Enforced Expression of NUP98-HOXA9 in Human CD34+ Cells Enhances Stem Cell Proliferation

Ki Y. Chung,1 Giovanni Morrone,4 Jan Jacob Schuringa,3 Magdalena Plasilova,2 Jae-Hung Shieh,2 Yue Zhang,2 Pengbo Zhou,3 and Malcolm A.S. Moore2

1Department of Medicine and 2Moore Laboratory, Cell Biology Program, Memorial Sloan-Kettering Cancer Center; 3Department of Pathology and Laboratory Medicine, Weill Medical College and Graduate School of Medical Sciences of Cornell University, New York, New York; 4Department of Experimental and Clinical Medicine “Gaetano Salvatore,” University of Catanzaro Magna Graecia, Catanzaro, Italy; and 5Department of Hematology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

Abstract

The t(7;11)(p15;p15) translocation, observed in acute myelogenous leukemia and myelodysplastic syndrome, generates a chimeric gene where the 5’ portion of the sequence encoding the human nucleoporin NUP98 protein is fused to the 3’ region of HOXA9. Here, we show that retroviral-mediated enforced expression of the NUP98-HOXA9 fusion protein in cord blood–derived CD34+ cells confers a proliferative advantage in both cytokine-stimulated suspension cultures and stromal coculture. This advantage is reflected in the selective expansion of hematopoietic stem cells as measured in vitro by cobblestone area–forming cell assays and in vivo by competitive repopulation of nonobese diabetic/severe combined immunodeficient mice. NUP98-HOXA9 expression inhibited erythroid progenitor differentiation and delayed neutrophil maturation in transduced progenitors but strongly enhanced their serial replating efficiency. Analysis of the transcriptome of transduced cells revealed up-regulation of several homeobox genes and of CAAT/enhancer binding protein α. The latter gene, when coexpressed with NUP98-HOXA9, reversed the enhanced proliferation of transduced CD34+ cells. Unlike HOXA9, the NUP98-HOXA9 fusion was protected from ubiquitination mediated by Cullin-4A and subsequent proteasome-dependent degradation. The resulting protein stabilization may contribute to the leukemogenic activity of the fusion protein. (Cancer Res 2006; 66(24): 11781-91)

Introduction

NUP98 is a component of the nuclear pore complex (1). It is mapped to 11p15.5 and fused to several distinct partners as a consequence of leukemia-associated chromosomal translocations that juxtapose the NH2 terminus of NUP98 to the COOH terminus of the partner gene (2, 3). Nine of the 21 reported partner genes are of the homeobox (HOX) family (HOXA9, HOXA11, HOXA13, HOXC11, HOXC13, HOXD11, and HOXD13) or HOX related (PAX1 and PAX2; refs. 2, 3). NUP98 is present both at the nuclear pore complex and within the nuclear interior (4, 5). The NH2-terminal M9, GLEB, and FG domains of mammalian NUP98 bind to multiple RNA export factors, including Kap, β2B, Rael, and TAP, which facilitate mRNA export from the nucleus (6-8). FG repeats of NUP98 also bind and may facilitate interaction with the transcriptional coactivators cyclic AMP-responsive element binding protein–binding protein (CBP) and p300 (9). This latter interaction may provide a link between NUP98 protein mobility and active transcription.

Mounting evidence suggests that HOXA9 plays an important role in normal hematopoiesis. HOXA9, HOXA7, and Meis1 are expressed in early self-renewing CD34+ cells and downstream-regulate with differentiation (10, 11). In normal CD34+ cells, HOXA9 is preferentially expressed in a subfraction enriched for hematopoietic stem cells (HSC). HOXA9 has been shown to bind DNA cooperatively with either PBX1 or Meis1, two other members of the homeodomain family (12). Meis1 was originally described as a HOX cofactor that alters HOX DNA-binding specificity and affinity and increases HOX transcriptional activity (12). Targeted disruption of HOXA9 in mice leads to reduced numbers of progenitor cells and to a profound defect in HSC (13). Conversely, enforced expression of HOXA9 promoted proliferative expansion of HSC and progenitor cells and subsequently inhibited their differentiation (14). These data highlight the importance of precise control of HOXA9 protein levels during hematopoiesis. We have shown that the Cullin-4A (CUL-4A) ubiquitin ligase regulated HOXA9 protein levels by ubiquitination and degradation of the protein (15). Knockdown of CUL-4A by small interfering RNA in interleukin (IL)-3-dependent 32D myeloid cells enhanced their proliferation and blocked their differentiation in response to granulocyte colony-stimulating factor (G-CSF).

HOXA9 is one of the top 20 genes distinguishing acute myelogenous leukemia (AML) from acute lymphocytic leukemia and correlates with poor prognosis (16). HOXA9, HOXA7, and Meis1 genes are coexpressed strongly in all but the acute promyelocytic subset of AML (13, 17). HOXA9 behaves as an oncogene in leukemia following mutations that induce its persistent expression or that convert it into a persistent transcriptional activator. Leukemias associated with mixed lineage leukemia (MLL) gene translocations show uniform activation of HOXA9, which may be the common pathway that unifies diverse initiating events in many myeloid leukemias (18). Although no individual HOX gene is essential, Kumar et al. (19) proposed that the “HOX code,” minimally defined by the HOX5-A9 cluster, is central to MLL leukemogenesis.

HOXA9 overexpression can immortalize myeloid progenitors in vitro and inhibits some of their differentiation pathways (20, 21). When mice are transplanted with bone marrow cells overexpressing HOXA9, they develop AML after 8 to 12 months, a period that is shortened to 50 to 60 days by Meis1 coexpression (20). Pineault et al. (21) noted that Meis1 caused leukemic transformation of...
HOX9-immortalized progenitors, which did not have long-term repopulating capacity, supporting a two-step model of leukemogenesis.

Clinically, NUP98-HOXA9 is associated with AML and myelodysplastic syndrome (MDS), both de novo and therapy related, and with blast crisis of chronic myelogenous leukemia (CML; refs. 2, 3, 22, 23). Expression of NUP98-HOXA9 in murine bone marrow resulted in a myeloproliferative disease in transplanted mice, with neutrophil leukocytosis and extramedullary hematopoiesis, progressing to AML by 7 to 8 months (20, 23). Retroviral insertional mutagenesis identified several cofactors that collaborate with NUP98-HOXA9 in leukemia progression, the most frequent being Meis1. Collaboration between Meis1 and NUP98-HOXA9 reduced the latency of AML development to 4 to 5 months (20). Coexpression of NUP98-HOXA9 with the BCR-ABL fusion oncoprotein reduced the latency period to AML development even further to 21 days (24). Thus, it is clear that NUP98-HOXA9 plays a causative role in the development of AML, although additional genetic or epigenetic changes are necessary for progression to overt AML.

We have evaluated the biological effects of NUP98-HOXA9 by retrovirally transducing the most physiologically relevant cells, human CD34+ HSC. Enforced expression of NUP98-HOXA9 conferred a proliferative advantage and enhanced HSC self-renewal, evident in stromal coculture and in the competitive repopulation of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. It also alters differentiation, inhibiting erythropoiesis relative to myelopoiesis, delaying neutrophil maturation, and inhibiting myeloid colony formation in responses to G-CSF or granulocyte macrophage colony-stimulating factor (GM-CSF). Several of these changes may be attributed to altered transcriptional activity, and we implicate down-modulation of CAAT/enhancer binding protein α (C/EBPα) as playing a significant role. We further show that the HOX protein component of the fusion is protected from CUL-4A–mediated ubiquitination, resulting in increased stability of HOX9 protein that may contribute to HSC transformation.

Materials and Methods

Retroviral vectors and constructs. A murine stem cell virus–based retroviral expression vector (MIGR1) was used for all experiments. The MIGR1 plasmid (kindly provided by Dr. Warren Pear, University of Pennsylvania, Philadelphia, PA) contains a multicloning site upstream of the ARES2 element and the coding sequence for the enhanced green fluorescent protein (EGFP) downstream of the latter (Fig. L4). The cDNA encoding human NUP98-HOXA9 was kindly provided by Dr. Nabeel Yaseen (Department of Pathology, Northwestern University, Chicago, IL) and cloned into the MIGR1 retrovirus. To generate stable, high-titer retroviral packaging cell lines, H29 cells were transiently transfected with 10 μg of vector DNA by calcium phosphate precipitation. After 72 hours, the H29 supernatant, containing vesicular stomatitis virus (VSV)-pseudotyped retrovirus, was used for cross-transduction of PG13 packaging cells in the presence of polybrene (8 μg/mL; Sigma, St. Louis, MO). High-titer retrovirus-producing PG13 subclones were selected by EGFP+ cell sorting, pRRL-C/EBPα-EYFP lentiviral vectors that encoded a 4-hydroxytamoxifen (4-OHT)-inducible C/EBPα-EYFP fusion were cloned by swapping the EcoRI fragment from C/EBPα-EYFP from MmN1-C/EBPα-EYFP as described previously (25) blunt into the SnuB1 site of pRRL-YFP. Lentiviral particles were produced in 2.5 × 10^6 293T human embryonic kidney cells that were transduced with 3 μg pCMV 0.891, 0.7 μg VSV-G, and 3 μg pRRL-C/EBPα-EYFP (pRRL-YFP was a kind gift from Dr. C. Baum, Department of Experimental Hematology, Hannover Medical School, Hannover, Germany).

Retroviral transduction protocol. Human cord blood–derived CD34+ cells isolated as detailed below were prestimulated for 48 hours in serum-free QBSF-60 medium (Quality Biological, Gaithersburg, MD) supplemented with c-Kit ligand (100 ng/mL; Kirin, Gunma, Japan), Flt3 ligand (100 ng/mL; Imclone Systems, New York, NY), and thrombopoietin (100 ng/mL; Kirin). Retrovirus-containing supernatants were harvested from PG13 clones incubated in QBSF for 8 to 12 hours, supplemented with 100 ng/mL of c-Kit ligand, Flt3 ligand, and thrombopoietin and 4 μg/mL polybrene, filtered through a 0.45-μm filter (Costar, Acton, MA), and applied immediately to the CD34+ cells on retroenamine-coated six-well plates (Takara, Otsu, Japan). Three consecutive transduction rounds, every 8 to 12 hours, were done.

Cell culture and stromal cell lines. Human umbilical cord blood was kindly provided by the Cord Blood Bank subdivision of the New York Blood Bank from healthy full-term pregnancies. Human CD34+ cells were selected from the Ficollic-separated mononuclear cord blood cells using the MiniMACS CD34 isolation kit (Miltenyi Biotech, Auburn, CA). The murine MS-5 stromal cell line was kindly provided by Dr. Itoh (Department of Biology, Niigata University, Niigata, Japan) and grown in α-MEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). The AGM-S2 stromal line was developed from the aorta-gonad-mesonephros region of a 10.5-day mouse embryo (26). H29 cells were cultured in DMEM (Life Technologies) supplemented with 10% FBS, penicillin-streptomycin, and 200 μmol/L glutamine. MS-5 stromal cocultures and long-term culture–initiating cell (LTC-IC) experiments were done in Gardner’s medium (α-MEM containing 12.5% FBS, 12.5% horse serum (HyClone), penicillin and streptomycin, 200 μmol/L glutamine, 57.2 μmol/L β2-mercaptoethanol (Fisher Scientific, Fair Lawn, NJ), and 1 μmol/L hydrocortisone (Sigma)).

Cytokine-stimulated suspension cultures were done in Iscove’s modified Dulbecco’s medium (Life Technologies) containing 20% FBS and 100 ng/mL of c-Kit ligand, Flt3 ligand, and thrombopoietin. Population doubling curves were calculated based on weekly total nucleated cell counts and replating of 1 × 10^6 cells.

Colony-forming cell, cobblestone area–forming cell, and LTC-IC and serial cobblestone area–forming cell assays. Colony assays were done in triplicate in 35-mm plates using 1.2% methylcellulose (Dow, Niagara Falls, NY) 30% FBS, 57.2 μmol/L β2-mercaptoethanol, 2 μmol/L glutamine, 0.5 μmol/L hemin (Sigma), 20 ng/mL of IL-3, IL-6, G-CSF, and c-Kit ligand, and 6 units/mL erythropoietin. IL-3 and IL-6 were from PropeTox (Rocky Hill, NJ), G-CSF was from Amgen (Thousand Oaks, CA), and erythropoietin was from Ortho Biotech (BridgeWater, NJ). In some assays, single cytokines (G-CSF, GM-CSF, and erythropoietin) were used. Colonies were scored 14 days after plating.

Cobblestone area–forming cell (C AFC) assays were done as described (27) by plating 1 × 10^6 cord blood CD34+ cells onto MS-5 monolayers in T12.5 tissue culture flasks (Becton Dickinson, Franklin Lanes, NJ) in triplicate. Weekly demidepopulations were done, with phenotypic analysis of nonadherent cells. Cobblestone areas were defined as groups of at least 10 phase-contrast dark cells tightly associated beneath the MS-5 monolayer and were scored over the course of 5 weeks.

Limiting dilution week 5 CAFC assays were done by plating sorted EGFP+ MIGR1-transduced and NUP98-HOXA9–transduced CD34+ cells in 24-well tissue culture plates containing MS-5 monolayers at five progressive dilutions with 12 replicate wells per dilution. Additional experiments were done using sorted EGFP+ CD34+/CD38– and CD34+CD38– to determine the CAFC frequency.

Secondary CAFC assays were done by trypsin harvesting week 5 adherent cells and replating all cultures, or sorted EGFP+ cells, on fresh MS-5 monolayers.

Western blots and morphologic analysis. Sorted EGFP+ MIGR1-producing and NUP98-HOXA9-producing PG13 cells (3 × 10^7) were centrifuged, and immunoblotting was done as described previously (25). Antibodies (Upstate Technologies, Lake Placid, NY) to HOXA9 were used at a dilution of 1:1,000 and the secondary horsedarshish peroxidase–conjugated rabbit anti-mouse antibodies at 1:200.

Cell cytopsins were stained with the Max-Grünwald-Giemsa method for morphologic evaluation.

Reverse transcription-PCR and microarray analysis. Reverse transcription-PCR (RT-PCR) was done using total RNA isolated from 0.5 × 10^8 to 1.0 × 10^8 sorted cells using the RNeasy kit and One-Step RT-PCR kit
(Qiagen, Valencia, CA) following the manufacturer’s protocol. All RT-PCR primers were from Sigma and are available on request.

For microarray analysis, 4 μg of total RNAs were isolated from sorted EGFP+ MIGR1 and NUP98-HOXA9 cells using the RNeasy kit (Qiagen), labeled, and hybridized to Affymetrix (Santa Clara, CA) Human Genome U133A chips. Comparative gene expression profiles were determined between MIGR1-transduced and NUP98-HOXA9–transduced cells. A significant difference in gene expression was defined as a fold change of ≥ 1.87, with a detection P value of < 0.05 and a signal value of > 200.

**Cell culture, plasmids, and transfection.** HeLa cells stably expressing tetracycline-repressible rtTA (HtTA) were cultured in DMEM containing 10% FCS, 0.5 mg/mL G418, and antibiotics. The plasmids expressing hemagglutinin (HA)-tagged HOXA9, HA-tagged NUP98-HOXA9 (gift of J. van Deursen, St. Jude Children’s Research Hospital, Memphis, TN), and FLAG-tagged CUL-4A were transfected using Fugene 6 transfection reagent (Roche, Chicago, IL). pGreenLantern (1 μg) that expressed EGFP was also included in each transfection to monitor the transfection efficiency. The cells were harvested 48 hours after transfection and lysed in radio-immunoprecipitation assay buffer, immunoprecipitated with the anti-HA (12CA5) monoclonal antibody, and analyzed by Western blotting using antibodies against HA (HA1, Covance, Berkeley, CA), FLAG (M2; Sigma), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

**Mice.** NOD/SCID, NOD/SCID h2Mnull, and NOD/SCID γ2null mice (The Jackson Laboratory, Bar Harbor, ME) were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities under an institute-approved animal protocol in accordance with the Principles of Laboratory Animal Care. Female mice (10–12 weeks old) were sublethally irradiated with 3 cGy from a cesium γ-radiation source and inoculated i.v. or into the right femur with 1 x 10⁵ CD34+ cells transduced with either MIGR1-EGFP or MIGR1-NUP-HOXA9-EGFP. Animals were sacrificed at 5 to 7 weeks, and bone marrow, spleen, and liver were removed for fluorescence-activated cell sorting (FACS) analysis.

![Figure 1.](image-url)

**Figure 1.** Enforced expression of control MIGR1 and NUP98-HOXA9 in cord blood CD34+ cells. A, map of MIGR1 and NUP98-HOXA9 retroviruses and an immunoblot of whole-cell protein extracts from control (MIGR1) and NUP98-HOXA9 retroviral packaging cells with anti-HOXA9 antibody. B, transduction efficiencies determined by FACS analysis of EGFP expression using purified cord blood CD34+ cells prestimulated for 48 hours in QBSF-60 supplemented with c-Kit ligand, Flt3 ligand, and thrombopoietin (100 ng/mL) and then subjected to three transduction rounds as described in Materials and Methods. C, proliferative advantage of cord blood CD34+ cells transduced with NUP98-HOXA9 (NH) relative to MIGR1 control vector–transduced cells in nonsorted MS-5 stromal coculture. Left, advantage in total cell expansion; right, fold increase in percentage of EGFP+ cells over time in a comparison of MIGR1-EGFP+ control and NUP98-HOXA9-EGFP+ cells relative to nontransduced cells over time. D, EGFP+ cells were sorted from MIGR1 control or NUP98-HOXA9 CD34+ populations 24 hours after transduction and placed in MS-5 (left) or AGM-S2 (right) stromal coculture. Cultures were subjected to weekly demi-depopulation of suspension cells with FACS analysis for EGFP expression. Absolute expansion of EGFP+ cells in NUP98-HOXA9–transduced versus MIGR1 control cultures.
Flow cytometry. Phycocerthrin-conjugated antibodies were from PharMingen (San Diego, CA). Cells were incubated with the relevant antibodies at 4°C for 1 hour, washed once with PBS/2% FCS, and analyzed on a FACSCalibur (Becton Dickinson). The data were acquired and analyzed with the CellQuest (Becton Dickinson) software. All cell sorted were done using MoFlo (DakoCytomation, Denver, CO).

Results

Retroviral transduction of human cord blood CD34+ cells. Gene transfer efficiencies into CD34+ cells were determined by measuring the EGFP fluorescence 24 hours after the final round of transduction. In multiple independent experiments, mean transduction efficiencies of 43 ± 12% and 21 ± 10% were obtained from control (MIGR1) and NUP98-HOXA9 viral supernatants, respectively (Fig. 1B). Expression of the NUP98-HOXA9 fusion protein in the NUP98-HOXA9 retroviral packaging cell line was verified by Western blotting (Fig. 1A).

Selective in vitro proliferative advantage of NUP98-HOXA9–expressing cells. The proliferative potential of NUP98-HOXA9–transduced or MIGR1 control-transduced cells was evaluated in vitro in stromal coculture and cytokine-stimulated suspension culture. In nonsorted cocultures, NUP98-HOXA9–expressing cells showed a proliferative advantage over the course of 5 weeks as determined by the increase of EGFP® nonadherent progeny by week 5 and a progressive increase in the percentage of total cells expressing EGFP respectively the MIGR1 EGFP® population remained constant (Fig. 1C). We then evaluated EGFP® sorted cells to remove any possible bystander effect of nontransduced cells. The proliferative advantage of NUP98-HOXA9 expression was still shown by total suspension cell production through week 5 of coculture on both MS-5 and AGM-S2 stroma (Fig. 1D).

Cytokine-stimulated suspension cultures also confirmed the proliferative advantage of NUP98-HOXA9–expressing cells, with an 8- to 10-fold greater expansion of EGFP® cells relative to control (MIGR1) over 5 weeks (Fig. 2A).

Morphologically, nonadherent NUP98-HOXA9–expressing cells in culture were predominantly myeloblast/promyelocyte, lacking the terminal band and segmented neutrophil differentiation noted in control cultures (Fig. 2B). However, by immunophenotype, these cells were indistinguishable from control MIGR1 cells in their expression of myeloid markers, CD33, and CD14 (data not shown).

Defective myeloid and erythroid colony formation by NUP98-HOXA9–expressing CD34+ cells. Clonogenic assays were used to evaluate lineage commitment at the progenitor cell level following NUP98-HOXA9–transduction. Sorted NUP98-HOXA9–expressing and MIGR1 control transduced CD34+ cells cloned in the presence of IL-3, IL-6, G-CSF, c-Kit ligand, and erythropoietin (6 units) showed comparable numbers of myeloid granulocyte-macrophage colony-forming unit (CFU-GM), whereas blast-forming unit-erythroid (BFU-E) was significantly (P < 0.01) fewer in NUP98-HOXA9 cultures (Fig. 2C). A comparable reduction of BFU-E relative to CFU-GM was noted when both NUP98-HOXA9–transduced CD34+, CD38dm, and CD34+, CD38fr cultures were cultured separately (Fig. 2D). Significantly fewer BFU-E were present in NUP98-HOXA9 cultures relative to control when erythropoietin was used at both high and low concentrations, either alone or with c-Kit ligand (Fig. 2C). In contrast to results seen with a cocktail of cytokines, when cultures were stimulated by GM-CSF alone or by G-CSF at both high and low concentrations, significantly fewer myeloid colonies developed (Fig. 2C). In G-CSF–stimulated cultures, analysis of the colony to cluster ratio (with >40 cells as the minimum definition of a colony) revealed >2-fold more clusters in NUP98-HOXA9 cultures relative to control. Replating of primary colonies from cultures stimulated with a combination of cytokines yield secondary myeloid colonies but no BFU-E, with NUP98-HOXA9–expressing primary colonies generating significantly more secondary colonies than MIGR1 control colonies (P < 0.01; Fig. 3A). Tertiary passage was obtained only with NUP98-HOXA9 cells. This suggests that the expression of NUP98-HOXA9 resulted in acquisition of some measure of self-renewal by immature myeloid progenitors.

Weekly evaluation of progenitor [colony-forming cell (CFC)] production in stromal coculture showed that there was a consistently greater generation of CFC in the NUP98-HOXA9 cultures than in MIGR1 control, with a 4- to 5-fold greater colony-forming unit granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), CFU-GM, and BFU-E output at week 5 (Fig. 3B).

Enhanced CAFC formation by NUP98-HOXA9–expressing cells. NUP98-HOXA9–transduced CD34+ cells were evaluated for HSC function using weeks 5 to 6 CAFC assay on MS-5stromal cells (Fig. 3C). NUP98-HOXA9–expressing cells began to form cobblestone areas by 1 week, and these progressively increased in number until approaching confluence by 3 to 4 weeks (Fig. 3C and D). Limiting dilution assays (Fig. 3C and D) were used to evaluate weeks 5 to 6 CAFC frequency, and in three separate experiments, there were consistently greater numbers (4- to 8-fold more) of these candidate HSC in NUP98-HOXA9 cultures compared with control. CAFC comprised 2% to 4% of NUP98-HOXA9–transduced CD34+ cells compared with 0.3% to 0.5% for control cells. This reflects an expansion of the primitive stem/progenitor cell pool. This was further confirmed by the replating capacity of week 5 NUP98-HOXA9 CAFCs in fresh MS-5 cocultures, showing generation of secondary (Fig. 3C) and tertiary (data not shown) week 5 CAFCs (Fig. 3D). Week 5 cobblestone areas were harvested, and cells were sorted by EGFP expression, characterized morphologically and immunophenotypically, and plated in semisolid medium for progenitor evaluation. The isolated EGFP® cells seemed to be predominately myelomonocytic blasts (Fig. 3D), and their immunophenotype showed 10% CD34+ cells, 17% CD14+, and >90% CD33+ (data not shown). Colony assays with sorted EGFP® week 5 cobblestone area–derived cells highlighted the following: (a) the presence of a much higher number of progenitors in the NUP98-HOXA9 cobblestone areas than in those derived by MIGR1–transduced cells and (b) a significant proportion of CFCs were BFU-Es and CFU-GEMMs in the NUP98-HOXA9 cobblestone areas but not in the MIGR1 cobblestone areas. These data imply that enforced expression of the fusion protein, particularly in the context of the hematopoietic microenvironment, maintains the transduced cells in a more immature state.

Experiments where sorted HSC-enriched CD34+/CD38− and progenitor-enriched CD34+/CD38+ NUP98-HOXA9–transduced populations were assayed in MS-5 cocultures revealed that only the former subset was able to generate weeks 5 to 6 CAFCs (data not shown), suggesting that the proliferative advantage and enhanced self-renewal of NUP98-HOXA9-expressing cells reflect an effect at the level of primitive HSCs.

Engraftment and expansion of NUP98-HOXA9–expressing cells in NOD/SCID, NOD/SCID β2mull, and NOD/SCID γ2mull mice. To determine whether the in vitro proliferative advantage of enforced NUP98-HOXA9 expression in CD34+ cells persisted in vivo, equivalent numbers of nonsorted NUP98-HOXA9–transduced and MIGR1-transduced cells were transplanted by the i.v. route into...
Figure 2. Selective expansion of NUP98-HOXA9-EGFP-expressing cells in liquid cytokine-stimulated cultures in nonsorted competitive repopulation experiments. A, fold expansion of EGFP+ cells over 5 weeks in cultures of MIGR1 control-transduced or NUP98-HOXA9–transduced cells. B, morphology of suspension cells at 3 weeks in MIGR1 control-transduced (left) and NUP98-HOXA9–transduced (middle) cultures. Note the significant (P = 0.001) reduction in mature granulocytes (bands and segmented forms) and predominance of immature myeloid lineage cells (myeloblasts and promyelocytes) in the NUP98-HOXA9 suspension cultures relative to control cultures (right). C, comparison of myeloid colony formation (CFU-GM) by EGFP+ MIGR1 (Control) and NUP98-HOXA9–transduced (NH9) CD34+ cells. Note no significant difference when stimulated by a combination of cytokines (IL-3, IL-6, G-CSF, c-Kit ligand, and erythropoietin) but significantly (P < 0.05–0.01) fewer colonies developed from NUP98-HOXA9–transduced cells stimulated by single myeloid growth factors (G-CSF and GM-CSF). Erythroid burst formation was significantly less (P < 0.05–0.01) with NUP98-HOXA9–transduced cells than with MIGR1 control cells irrespective of the cytokine combinations used, including a combination of cytokines (IL-3, IL-6, G-CSF, c-Kit ligand, and erythropoietin), erythropoietin alone at a high and low concentration, and erythropoietin with 20 ng/mL of c-Kit ligand. D, CD34+ cells transduced with NUP98-HOXA9 (NH) or control vector (MIGR1) were separated into EGFP+ CD34+ populations and further divided into CD38+ and CD38–/lo fractions that were plated for colony formation in the presence of IL-3, IL-6, G-CSF, c-Kit ligand, and erythropoietin. Erythroid, myeloid, and multilineage progenitors were distributed in all fractions, but the ratio of myeloid to erythroid colonies was significantly different when either the CD38+ or CD38–/lo fractions of MIGR1 (erythroid predominant) were compared with NUP98-HOXA9 (erythroid depleted relative to myeloid).

subletally irradiated NOD/SCID $\beta_{2}M^{null}$ and $\gamma^{2ull}$ mice (nine NUP98-HOXA9 and six MIGR1). In addition, four NOD/SCID mice were engrafted locoregionally by intrafemoral injection of transduced cells. Engraftment was measured 5 to 7 weeks after transplantation by FACs analysis of human CD45 and EGFP expression in bone marrow mononuclear cells (Fig. 4). In all three models, human hematopoietic engraftment was obtained in bone marrow (2–42%). A selective in vivo proliferative advantage of NUP98-HOXA9–expressing cells relative to the nontransduced cells coinjected was revealed by a mean 3.3-fold increase in the percentage of human mature CD45 cells recovered at 5 to 6 weeks that expressed EGFP relative to the EGFP percentage in the input population (9–15%; Fig. 4A). This was significantly greater (P < 0.028) than the average expansion of EGFP cells seen in control mice (1.6-fold). The proliferative advantage of NUP98-HOXA9–expressing cells was also accompanied by extramedullary engraftment in the spleen and liver. These mononuclear EGFP+ cells were predominantly myelomonocytic blasts (Fig. 4C). Additional colony assays from engrafted CD34+–immunopurified mononuclear cells from bone marrow of the engrafted mice revealed a predominance of CFU-GM and CFU-GM colonies (data not shown). The greatest degree of human engraftment (31–34%) was observed in NOD/SCID $\gamma^{2ull}$ mouse marrow (Fig. 4D), with 20% to 28% of the human cells expressing the myelomonocytic marker CD14, 3% to 5% expressing the erythroid marker glycoporphin A, and 6% to 9% expressing lymphoid markers CD7, CD8, or CD4. The expression of EGFP in CD8 T cells (43% in control and 19–29% in the NUP98-HOXA9 engrafted mice) indicated that expression of the fusion protein was not incompatible with T lymphocyte differentiation, although this pathway of differentiation may be quantitatively impaired (Fig. 4D).

Validation of in vitro phenotype and discovery of potential downstream targets by microarray analysis. Comparative microarray analysis was undertaken on RNA isolated from freshly sorted CD34+ NUP98-HOXA9–expressing cells and MIGR1 control cells (Table 1). Consistent with several other published studies (3, 19, 28), expression of NUP98-HOXA9 up-regulated the expression of many HOX genes, including endogenous HOXA5, HOXA6, HOXA7, HOXA9, and HOXB5, important myeloid transcription factors, such as Meis1 and AML1, as well as the transcripts encoding the CD44 surface membrane protein, the Pim-1 serine/threonine kinase, and
hepatic leukemia factor (HLF). The level of mRNA for C/EBPα was decreased in NUP98-HOXA9− cells. The up-regulation of endogenous HOX genes and Pim-1 as well as the down-regulation of C/EBPα were validated by RT-PCR in transduced CD34+ cells in independent experiments (Fig. 5A). HOXA9, HOXA7, HOXA6, HOXA5, HOXB5, and Meis1 have all been implicated in leukemogenesis (19–23). Up-regulation of HOXA5 may also play a role in the suppression of erythropoiesis as reflected in the down-regulation of several globin genes (Table 1). Enforced expression of HOXA5 in human CD34+ cells has been shown to preferentially support myeloid differentiation, with a reduced frequency of erythroid progenitors (BFU-E; refs. 29, 30). The down-regulation of C/EBPα is consistent with the observed enhanced HSC proliferation because it has been reported that ectopic expression of HLF enhances HSC engraftment and inhibits apoptosis (37).

Validation of the role of C/EBPα down-modulation in NUP98-HOXA9 transformation. The down-regulation of C/EBPα by NUP98-HOXA9− expressing CD34+ cells seems to be consistent with the observed primitive myelomonocytic phenotype of their progeny and their delayed neutrophil maturation. C/EBPα plays an important role in myeloid differentiation as shown in C/EBPα−/− mice that display an accumulation of myeloblasts with myeloid maturation arrest (38) and as recently reported in human CD34−

Figure 3. Stem cell and progenitor cell expansion in NUP98-HOXA9−transduced cord blood CD34+ cell cultures. A, colony assays of week 0 sorted MIGR1 and NUP98-HOXA9−transduced (NH9) CD34+ cells in the presence of IL-3, IL-6, G-CSF, c-Kit ligand, and erythropoietin. The primary day 14 colony contents of each 35-mm Petri dish (140–180 CFC) were dissociated and passaged into secondary 1 ml cultures (in triplicate) and scored for secondary colonies after a further 14 days. Process repeated for a tertiary passage. Columns, mean of three experiments; bars, SE. B, left, total weekly progenitor cell production in MS-5 stromal cocultures of sorted EGFP+ cells from MIGR1-control-transduced or NUP98-HOXA9−transduced (NH9) CD34+ populations. Note the 3- to 4-fold greater number of progenitors recovered at week 5 in the NUP98-HOXA9 cultures. Right, morphology of EGFP+ CFC generated at 5 weeks of stromal coculture. Note that, in addition to the 4.5-fold greater numbers of CFC in NUP98-HOXA9 cultures, multilineage (CFU-GEMM) and erythroid (BFU-E) progenitors were still produced, in contrast to the exclusive GFU-GM production in MIGR1 control cultures. C, cobblestone area formation (arrows) in MS-5 stromal cocultures and in 1 and 2 weeks following addition of 2,000 sorted NUP98-HOXA9 (NH9) or MIGR1 control CD34+ cells (left). Limiting dilution week 5 CAFC assay of NUP98-HOXA9 (NH9) or MIGR1 control CD34+ cells on MS-5 stroma (0–800 input CD34+ cells per well) in control or transduced MS-5 cocultures (right). D, CAFC assay. Number of cobblestone areas formed on MS-5 stroma between 1 and 3 weeks following coculture with 2,000 sorted EGFP+ CD34+ cells transduced with NUP98-HOXA9 (NH9) or MIGR1 control. Columns, mean; bars, SE. Data on week 5 cobblestone areas obtained by limiting dilution assay. Left, representative experiment. One third of week 5 cobblestone areas derived from NUP98-HOXA9−transduced CD34+ cells were able to generate secondary week 5 cobblestone areas following repassage onto fresh MS-5, whereas no control cobblestone area had this capacity (right).
cells expressing a dominant-negative C/EBPα (39). The clinical relevance is highlighted by reports of dominant-negative C/EBPα mutations in AML patients leading to a block in myeloid differentiation (40). We have recently reported that down-modulation of C/EBPα by STAT5 in CD34+ cells was a prerequisite for STAT5-induced effects on enhancement of HSC self-renewal and inhibition of myeloid differentiation (25). A 4-OHT-inducible C/EBPα-ER protein was coexpressed with an activated STAT5 mutant in CD34+ cells using a lentiviral/retroviral approach, and reexpression of C/EBPα restored myeloid differentiation and inhibited HSC proliferation. We have used a comparable approach to evaluate the relevance of C/EBPα down-modulation in the observed NUP98-HOXA9 phenotype. CD34+ cells were transduced with NUP98-HOXA9-EGFP, with C/EBPα-ER-YFP, or with both vectors, plated on MS-5 stroma, and cultured for 5 weeks in the presence or absence of 4-OHT. The cultures were evaluated by weekly FACS analysis for cells expressing YFP, EGFP, or both. Measurement of the relative expansion of the transduced cells reveals that NUP98-HOXA9–expressing cells expanded to a greater degree than control nontransduced cells by weeks 3 to 5 in the presence or absence of 4-OHT (Fig. 5). Double-transduced cells expressing NUP98-HOXA9 and C/EBPα-ER, or cells expressing C/EBPα-ER alone, in the absence of 4-OHT, exhibited a relative expansion comparable with untransduced CD34+ cells. However, in the presence of 4-OHT, there was a very significant suppression of proliferation relative to control or NUP98-HOXA9 cultures within 1 to 2 weeks (Fig. 5).

Enhanced stability of the NUP98-HOXA9 fusion protein compared with wild-type HOXA9 may be mediated by decreased sensitivity to CUL-4A–dependent ubiquitination. The CUL-4A ubiquitination machinery has recently been shown to mediate ubiquitin-dependent proteolysis of HOXA9 (15). To examine whether the stability of the NUP98-HOXA9 chimera was also subjected to regulation by CUL-4A, HECa cells were transiently transfected with both HA-HOXA9 and HA-NUP98-HOXA9 together with increasing doses of CUL-4A. As shown in Fig. 5C, HA-HOXA9 protein was readily degraded in a CUL-4A dose-dependent manner as described previously (15). In striking contrast, the steady-state levels of HA-NUP98-HOXA9 protein were not affected significantly by the increased expression of CUL-4A. Pulse-chase analysis confirmed the difference in protein half-lives (Fig. 5D). These results indicate that the NUP98-HOXA9 chimera is resistant

Figure 4. A, comparison of the hematopoietic differentiation and expansion of EGFP+ cells following injection of 10^5 unsorted NUP98/HOXA9-transduced or MIGR1-transduced cells i.v. into irradiated NOD/SCID il2Mnull mice (Δ), i.v. into NOD/SCID γcnull mice (○), or into the right femur of NOD/SCID mice (□). Transduction efficiency ranged from 9% to 20%. Data expressed as fold change in percentage of EGFP+ cells in engrafted bone marrow harvested at weeks 5 to 7 (2–100%) to the EGFP% in the input CD34+ cell population (9–20%). NUP98-HOXA9 mean increase = 3.31-fold; MIGR1 fold increase = 1.63-fold; P = 0.028. B, evaluation of bone marrow harvested at week 7 after i.v. inoculation of NOD/SCID il2Mnull mice with nonsorted, transduced CD34+ cells. Prominent engraftment of human CD45+ cells identified by FACS analysis, with EGFP+ NUP98-HOXA9 cells representing ~50% of the engrafted human CD45+ cells. C, cytospin of FACS-sorted NUP98-HOXA9–expressing cells showing a primitive myelomonocytic blast morphology similar to isolated week 5 CAFC cells. D, human hematopoietic engraftment in bone marrow of NOD/SCID γcnull mice 5 weeks after i.v. injection of 10^6 NUP98/HOXA9–transduced (9.6% EGFP+) or MIGR1 control-transduced (14.1% EGFP+) CD34+ cells. Human CD45+ cells comprised 31% to 34% of the bone marrow with an additional 3% to 5% human glycophorin A+, CD45+ erythroid cells detected. EGFP+ cells comprised 30% of the NUP98 CD45 population (a 2.1-fold increase in % EGFP+ cells). Left, EGFP+ NUP98-HOXA9 cells comprised 29% and 52% of the CD45 population (a 3- and 5.4-fold increase in % EGFP+ cells). Lymphoid differentiation was obtained, with 2.5% to 5.5% CD7+, CD45+ cells and 1.5% to 5% CD8+, CD45+ T cells detected in the marrow, with 43% of the control expressing MIGR1-EGFP and 19% and 29% of the experimental mice expressing NUP98-HOXA9-EGFP (right).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Unigene accession</th>
<th>Gene symbol</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast growth factor 18</td>
<td>Hs.49585</td>
<td>FGF18</td>
<td>21.11</td>
</tr>
<tr>
<td>Urotensin 2</td>
<td>Hs.162900</td>
<td>UTS2</td>
<td>13.00</td>
</tr>
<tr>
<td>FBJ murine osteosarcoma viral oncogene homologue B</td>
<td>Hs.75678</td>
<td>FOSB</td>
<td>12.13</td>
</tr>
<tr>
<td>Homeobox A9</td>
<td>Hs.127428</td>
<td>HOXA9</td>
<td>6.50</td>
</tr>
<tr>
<td>Homeobox A5</td>
<td>Hs.37034</td>
<td>HOX5</td>
<td>4.92</td>
</tr>
<tr>
<td>CD44-Human CD44 antigen precursor</td>
<td>Hs.285173</td>
<td>CD44</td>
<td>4.92</td>
</tr>
<tr>
<td>CD69 (p60, early T-cell activation antigen)</td>
<td>Hs.82401</td>
<td>CD69</td>
<td>4.59</td>
</tr>
<tr>
<td>H2B histone family, member A</td>
<td>Hs.352109</td>
<td>H2BFA</td>
<td>4.00</td>
</tr>
<tr>
<td>Piccolo (presynaptic cytomatrix protein)</td>
<td>Hs.12356</td>
<td>PCKL</td>
<td>4.00</td>
</tr>
<tr>
<td>Phospholipase A2, group IVA</td>
<td>Hs.211587</td>
<td>PL2G4A</td>
<td>3.73</td>
</tr>
<tr>
<td>Cell division cycle 2-like 5</td>
<td>Hs.59498</td>
<td>CDC2L3</td>
<td>3.73</td>
</tr>
<tr>
<td>Fibroblast growth factor 18</td>
<td>Hs.49585</td>
<td>FGF18</td>
<td>21.11</td>
</tr>
<tr>
<td>Urotensin 2</td>
<td>Hs.162900</td>
<td>UTS2</td>
<td>13.00</td>
</tr>
<tr>
<td>FBJ murine osteosarcoma viral oncogene homologue B</td>
<td>Hs.75678</td>
<td>FOSB</td>
<td>12.13</td>
</tr>
<tr>
<td>Homeobox A9</td>
<td>Hs.127428</td>
<td>HOXA9</td>
<td>6.50</td>
</tr>
<tr>
<td>Homeobox A5</td>
<td>Hs.37034</td>
<td>HOX5</td>
<td>4.92</td>
</tr>
<tr>
<td>CD44-Human CD44 antigen precursor</td>
<td>Hs.285173</td>
<td>CD44</td>
<td>4.92</td>
</tr>
<tr>
<td>CD69 (p60, early T-cell activation antigen)</td>
<td>Hs.82401</td>
<td>CD69</td>
<td>4.59</td>
</tr>
<tr>
<td>H2B histone family, member A</td>
<td>Hs.352109</td>
<td>H2BFA</td>
<td>4.00</td>
</tr>
<tr>
<td>Piccolo (presynaptic cytomatrix protein)</td>
<td>Hs.12356</td>
<td>PCKL</td>
<td>4.00</td>
</tr>
<tr>
<td>Phospholipase A2, group IVA</td>
<td>Hs.211587</td>
<td>PL2G4A</td>
<td>3.73</td>
</tr>
<tr>
<td>Cell division cycle 2-like 5</td>
<td>Hs.59498</td>
<td>CDC2L3</td>
<td>3.73</td>
</tr>
<tr>
<td>Fibroblast growth factor 18</td>
<td>Hs.49585</td>
<td>FGF18</td>
<td>21.11</td>
</tr>
<tr>
<td>Urotensin 2</td>
<td>Hs.162900</td>
<td>UTS2</td>
<td>13.00</td>
</tr>
<tr>
<td>FBJ murine osteosarcoma viral oncogene homologue B</td>
<td>Hs.75678</td>
<td>FOSB</td>
<td>12.13</td>
</tr>
<tr>
<td>Homeobox A9</td>
<td>Hs.127428</td>
<td>HOXA9</td>
<td>6.50</td>
</tr>
<tr>
<td>Homeobox A5</td>
<td>Hs.37034</td>
<td>HOX5</td>
<td>4.92</td>
</tr>
<tr>
<td>CD44-Human CD44 antigen precursor</td>
<td>Hs.285173</td>
<td>CD44</td>
<td>4.92</td>
</tr>
<tr>
<td>CD69 (p60, early T-cell activation antigen)</td>
<td>Hs.82401</td>
<td>CD69</td>
<td>4.59</td>
</tr>
<tr>
<td>H2B histone family, member A</td>
<td>Hs.352109</td>
<td>H2BFA</td>
<td>4.00</td>
</tr>
<tr>
<td>Piccolo (presynaptic cytomatrix protein)</td>
<td>Hs.12356</td>
<td>PCKL</td>
<td>4.00</td>
</tr>
<tr>
<td>Phospholipase A2, group IVA</td>
<td>Hs.211587</td>
<td>PL2G4A</td>
<td>3.73</td>
</tr>
<tr>
<td>Cell division cycle 2-like 5</td>
<td>Hs.59498</td>
<td>CDC2L3</td>
<td>3.73</td>
</tr>
<tr>
<td>Fibroblast growth factor 18</td>
<td>Hs.49585</td>
<td>FGF18</td>
<td>21.11</td>
</tr>
<tr>
<td>Urotensin 2</td>
<td>Hs.162900</td>
<td>UTS2</td>
<td>13.00</td>
</tr>
<tr>
<td>FBJ murine osteosarcoma viral oncogene homologue B</td>
<td>Hs.75678</td>
<td>FOSB</td>
<td>12.13</td>
</tr>
<tr>
<td>Homeobox A9</td>
<td>Hs.127428</td>
<td>HOXA9</td>
<td>6.50</td>
</tr>
<tr>
<td>Homeobox A5</td>
<td>Hs.37034</td>
<td>HOX5</td>
<td>4.92</td>
</tr>
<tr>
<td>CD44-Human CD44 antigen precursor</td>
<td>Hs.285173</td>
<td>CD44</td>
<td>4.92</td>
</tr>
<tr>
<td>CD69 (p60, early T-cell activation antigen)</td>
<td>Hs.82401</td>
<td>CD69</td>
<td>4.59</td>
</tr>
<tr>
<td>H2B histone family, member A</td>
<td>Hs.352109</td>
<td>H2BFA</td>
<td>4.00</td>
</tr>
<tr>
<td>Piccolo (presynaptic cytomatrix protein)</td>
<td>Hs.12356</td>
<td>PCKL</td>
<td>4.00</td>
</tr>
<tr>
<td>Phospholipase A2, group IVA</td>
<td>Hs.211587</td>
<td>PL2G4A</td>
<td>3.73</td>
</tr>
<tr>
<td>Cell division cycle 2-like 5</td>
<td>Hs.59498</td>
<td>CDC2L3</td>
<td>3.73</td>
</tr>
<tr>
<td>Fibroblast growth factor 18</td>
<td>Hs.49585</td>
<td>FGF18</td>
<td>21.11</td>
</tr>
<tr>
<td>Urotensin 2</td>
<td>Hs.162900</td>
<td>UTS2</td>
<td>13.00</td>
</tr>
<tr>
<td>FBJ murine osteosarcoma viral oncogene homologue B</td>
<td>Hs.75678</td>
<td>FOSB</td>
<td>12.13</td>
</tr>
<tr>
<td>Homeobox A9</td>
<td>Hs.127428</td>
<td>HOXA9</td>
<td>6.50</td>
</tr>
<tr>
<td>Homeobox A5</td>
<td>Hs.37034</td>
<td>HOX5</td>
<td>4.92</td>
</tr>
<tr>
<td>CD44-Human CD44 antigen precursor</td>
<td>Hs.285173</td>
<td>CD44</td>
<td>4.92</td>
</tr>
<tr>
<td>CD69 (p60, early T-cell activation antigen)</td>
<td>Hs.82401</td>
<td>CD69</td>
<td>4.59</td>
</tr>
<tr>
<td>H2B histone family, member A</td>
<td>Hs.352109</td>
<td>H2BFA</td>
<td>4.00</td>
</tr>
<tr>
<td>Piccolo (presynaptic cytomatrix protein)</td>
<td>Hs.12356</td>
<td>PCKL</td>
<td>4.00</td>
</tr>
<tr>
<td>Phospholipase A2, group IVA</td>
<td>Hs.211587</td>
<td>PL2G4A</td>
<td>3.73</td>
</tr>
<tr>
<td>Cell division cycle 2-like 5</td>
<td>Hs.59498</td>
<td>CDC2L3</td>
<td>3.73</td>
</tr>
<tr>
<td>Fibroblast growth factor 18</td>
<td>Hs.49585</td>
<td>FGF18</td>
<td>21.11</td>
</tr>
<tr>
<td>Urotensin 2</td>
<td>Hs.162900</td>
<td>UTS2</td>
<td>13.00</td>
</tr>
</tbody>
</table>
to CUL-4A-mediated proteolysis. The increased stability of NUP98-HOXA9 fusion protein may therefore further contribute to the generation of the phenotype observed.

Discussion

The establishment of a retroviral transduction model to enforce expression of the NUP98-HOXA9 gene in human CD34+ cells has permitted evaluation of its action in primary, nonleukemic human HSC and progenitor cells. We show that NUP98-HOXA9 expression confers a selective growth advantage on CD34+ cells as measured by in vitro expansion in stromal and cytokine-stimulated culture systems. Evidence that the fusion gene acts to enhance HSC proliferation was provided in two in vitro HSC assay systems. In the LTC-IC assay, we showed an increased number of progenitors generated by week 5 of culture. In the week 5 CAF assay, we showed a 4- to 8-fold expansion of CAF and ~33% of these could be passaged for an additional 5 to 10 weeks whereas none of the control CAF could. The transduced CAFC resided in the CD34+, CD38−/lo fraction, traditionally enriched for HSC, and not in the HSC-depleted, progenitor-enriched CD34+, CD38+ fraction. In the NOD/SCID assay for week 5+ human hematopoietic engraftment, traditionally considered as a HSC assay, NUP98-HOXA9–transduced cells were significantly more effective in competing against coinjected nontransduced cells than were MIGR1 control cells. The data showing increased secondary and tertiary myeloid colony formation by NUP98-HOXA9-transduced primary colonies relative to control colonies indicate that more committed progenitor cells can acquire some measure of self-renewal. The differential microarray analysis also supports our contention that NUP98-HOXA9 enhances HSC proliferation because there is up-regulation of genes, such as HOXA9 and HLF, implicated in HSC self-renewal (3, 37) and identified as up-regulated in leukemic stem cells (41). In addition to proliferative defects, the fusion gene alters differentiation. Erythroid differentiation is suppressed, terminal neutrophil maturation is inhibited, and clonogenic response to G-CSF or GM-CSF is blunted. In vivo, NUP98-HOXA9–transduced cells can differentiate to CD8+ T cells but possibly with reduced efficiency.

Table 1. Differential gene expression in cord blood CD34+ cells expressing NUP98-HOXA9 relative to CD34+ cells transduced with the MIGR1 control vector (Cont’d)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Unigene accession</th>
<th>Gene symbol</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wingless-type MMTV integration site family, member 5B</td>
<td>Hs.306051</td>
<td>WNT5B</td>
<td>-2.64</td>
</tr>
<tr>
<td>Hemoglobin, α1</td>
<td>Hs.272572</td>
<td>HBA1</td>
<td>-2.83</td>
</tr>
<tr>
<td>Hemoglobin, α2</td>
<td>Hs.347939</td>
<td>HBA2</td>
<td>-3.03</td>
</tr>
<tr>
<td>Hemoglobin, ε</td>
<td>Hs.117848</td>
<td>HBE1</td>
<td>-3.25</td>
</tr>
<tr>
<td>Hemoglobin, γA</td>
<td>Hs.266959</td>
<td>HBG1</td>
<td>-4.00</td>
</tr>
<tr>
<td>Hemoglobin, δ</td>
<td>Hs.36977</td>
<td>HBD</td>
<td>-4.59</td>
</tr>
<tr>
<td>Hemoglobin, β</td>
<td>Hs.155376</td>
<td>HBB</td>
<td>-4.92</td>
</tr>
<tr>
<td>Platelet factor 4</td>
<td>Hs.81564</td>
<td>PF4</td>
<td>-8.00</td>
</tr>
</tbody>
</table>

NOTE: Cord blood CD34+ cells were prestimulated for 48 hours in QBSF-60 medium with c-Kit ligand, Fl3 ligand, and thrombopoietin (100 ng/ml of each) followed by three transduction rounds in the next 48 hours on retronectin. GFP+ cells were sorted and total RNA was isolated and used to hybridize gene arrays. Data shown are the comparison of GFP+ NUP98-HOXA9 and GFP+ MIGR1 cells. A change in gene expression was only considered significant when the fold change was >1.87, with a statistical P value of <0.05 and a signal value of >200.
C/EBPα alone, or coexpressed with NUP98-HOXA9, profoundly suppressed CD34+ cell proliferation, possibly reflecting an impairment in HSC proliferation and enhancement of myeloid differentiation comparable with our observations in the STAT5 system (25).

The transforming potential of NUP98-HOXA9 may in part be related to its enhanced stability, with 3-fold longer half-life of the fusion HOXA9 protein relative to wild-type HOXA9, due to the resistance of the fusion HOXA9 protein to CUL-4A-mediated ubiquitination and degradation. Enhanced NUP98-HOXA9 stability would allow for more robust expression of endogenous HOX genes. The ubiquitination site on the first helix of HOXA9 is present in the fusion protein, but it is possible that some form of steric hindrance mediated by the NUP98 component, dimerization of NUP-HOXA9 or its binding to wild-type NUP98, blocks interaction with CUL-4A. An alternative mechanism may involve a CBP/p300-mediated acetylation of lysine residues required for ubiquitination, p53 and its structural and functional homologue p73 are protected from ubiquitination by such an acetylation process (44, 45). Competition between ubiquitination and acetylation of overlapping lysine residues constitutes a novel mechanism regulating protein stability (46).

A second hypothesis for the role of NUP98-HOXA9 in leukemogenesis is based on disruption of NUP98 function in the nuclear import of transcription factors or in the export of mRNAs. This in turn could disrupt pathways critical to both HSC proliferation and differentiation. We have obtained preliminary data showing impaired assembly of the nuclear pore complex with formation of distinct NUP98 colocalization bodies in NUP98-HOXA9–transduced CD34+ cells (47). NUP98 maps to 11p15.5 and in AML and MDS, among all regions studied, this region showed the highest frequency of loss of heterozygosity (LOH; 47%; ref. 48). We have also noted a high frequency of LOH specifically at the NUP98 locus in AML (47).

It has been proposed that one class of leukemogenic mutant confers a proliferative or survival advantage, whereas a second class primarily interferes with differentiation and subsequent apoptosis (49). Our studies point to multiple roles for the NUP98-HOXA9 fusion protein in the dysregulation of several critical pathways that influence HSC and progenitor self-renewal, proliferation, differentiation, and interaction with bone marrow stroma. However, additional mutations would seem to be needed.
for progression to acute leukemia. Transduction of normal CD34+ cells with NUP98-HOXA9 and additional leukemogenic genes (e.g., Flt3 internal tandem duplication; ref. 35) provides a model for testing this concept in a human system.

Acknowledgments

Received 3/29/2006; revised 9/5/2006; accepted 10/20/2006.

References


Enforced Expression of \textit{NUP98-HOXA9} in Human CD34\textsuperscript{+} Cells Enhances Stem Cell Proliferation

Ki Y. Chung, Giovanni Morrone, Jan Jacob Schuringa, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/24/11781

Cited articles
This article cites 48 articles, 28 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/24/11781.full.html#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/66/24/11781.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.