Candidate Genes for Expansion and Transformation of Hematopoietic Stem Cells by NUP98-HOX Fusion Genes

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Background. Hox genes are implicated in hematopoietic stem cell (HSC) regulation as well as in leukemia development through translocation with the nucleoporin gene NUP98. Interestingly, an engineered NUP98-HOXA10 (NA10) fusion can induce a several hundred-fold expansion of HSCs in vitro and NA10 and the AML-associated fusion gene NUP98-HOXD13 (ND13) have a virtually indistinguishable ability to transform myeloid progenitor cells in vitro and to induce leukemia in collaboration with MEIS1 in vivo. Methodology/Principal Findings. These findings provided a potentially powerful approach to identify key pathways mediating Hox-induced expansion and transformation of HSCs by identifying gene expression changes commonly induced by ND13 and NA10 but not by a NUP98-Hox fusion with a non-DNA binding homedomain mutation (N51S). The gene expression repertoire of purified murine bone marrow Sca-1+Lin- cells transduced with retroviral vectors encoding for these genes was established using the Affymetrix GeneChip MOE430A. Approximately seventy genes were differentially expressed in ND13 and NA10 cells that were significantly changed by both compared to the ND13(N51S) mutant. Intriguingly, several of these potential Hox target genes have been implicated in HSC expansion and self-renewal, including the tyrosine kinase receptor Flt3, the prion protein, Prnp, hepatic leukemia factor, Hlf and Jagged-2, Jag2. Consistent with these results, FLT3, HLF and JAG2 expression correlated with HOX A cluster gene expression in human leukemia samples. Conclusions. In conclusion this study has identified several novel Hox downstream target genes and provides important new leads to key regulators of the expansion and transformation of hematopoietic stem cells by Hox.

INTRODUCTION

The 39 clustered Hox proteins are an evolutionary preserved family characterized by a 60 amino acid DNA-binding motif called the homeodomain. Hox genes of the A, B and C but not D clusters are transcribed during normal hematopoiesis with their expression being confined to the immature subpopulations. Elimination or enforced expression of certain clustered Hox genes in mouse models have demonstrated profound effects on hematopoietic differentiation and persistent expression of normal or mutated Hox proteins in hematopoietic progenitors can result in leukemia in both mice and humans [1,2]. Hox genes have also been linked to leukemia by virtue of their involvement in leukemia-specific translocations. Clustered Hox genes have repeatedly been identified in acute myeloid leukemia (AML) harboring translocations that generate novel fusion proteins containing the N terminal region of the nucleoporin gene, NUP98, and the C terminal Hox region including the homeodomain (HD). The involvement of homeobox genes as partners of NUP98 is of particular interest given the growing evidence linking Hox genes, particularly members of the 5'-located members of the HOXA cluster, such as HOXA9, to leukemia. To date at least seven clustered Hox genes have been found fused to NUP98 in human leukemia, interestingly only from the Abd-B clustered Hox [3–11]. Furthermore, two non-clustered homeodomain-containing genes PMX1 and PMX2 have also been identified as NUP98 fusion partners in de novo AML and t-AML respectively [12,13].

The mechanisms by which Hox proteins mediate their effects and how they perturb cellular functions are not well understood. They seem to be highly context dependent in their actions [14] and in normal hematopoiesis their expression is tightly regulated [15]. Expression analysis and gain- or loss-of-function studies have shown that Hox proteins play an important role in the regulation of early stages of hematopoiesis, including the self-renewal of hematopoietic stem cells and early progenitors [16]. DNA site-selection studies indicate that the homeodomain by itself has limited target sequence recognition. Additional binding specificity and stability is in part achieved through interaction with other homeodomain-containing proteins from the multimember PBX or MEIS1 families [17] and co-transduction of MEIS1 with Hox and NUP98-Hox genes also strongly accelerate the onset of leukemia in mice [18–20]. There is also a considerable redundancy between different NUP98-Hox fusions in effects when expressed in bone marrow cells in vitro and their ability to collaborate with MEIS1 in vivo. An engineered NUP98-HOXA10 (NA10) fusion and the AML-associated fusion gene NUP98-HOXD13 (ND13) have a virtually indistinguishable ability to transform myeloid progenitor cells in vitro and to induce leukemia in collaboration with MEIS1 in vivo [21]. Furthermore, it has been shown that a N51S mutation in the DNA binding homeodomain abolishes the DNA

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binding ability of several Hox genes [22] and, indeed, the AML-associated fusion gene NUP98-HOXD13 (ND13) fusion also loses its leukemic effect from this mutation [19].

The relatively long latency of Hox-induced AML in mouse models strongly indicates that additional genetic events are required for full leukemic progression [19,23,24]. Indeed, data supports that Hox-containing fusions, as for most transcription factor-containing fusion oncogenes, alter the growth and differentiation of early hematopoietic precursors leading to the establishment of a preleukemic population of cells that are then susceptible to the acquisition of cooperating mutations [25]. In concordance with such a model, we recently demonstrated that constitutive expression of ND13 or NA10 was sufficient to induce a preleukemic state in primary bone marrow (BM) cells after extended in vitro culture. Though these cells had short-term repopulating potential, they were for the most part incapable of inducing AML on their own but could readily be converted into AML-inducing cells when complemented with MEIS1 or other genes [26]. Intriguingly, in short term culture, NA10 can promote high level expansion of long term repopulating cells; moreover a NUP98-fusion restricted to the homeodomain of HOXA10 induces similar levels of expansion of HSC [27]. Together, these findings suggest that NUP98-Hox fusions genes impact on crucial genetic programs involved in stem cell self-renewal and proliferation that may also contribute to leukemic transformation.

In an effort to gain insight into the nature of the possible genetic programs impacted by Hox relevant to stem cell self-renewal and leukemogenesis we have used microarray technology to assess gene expression perturbations induced by NUP98-FOX fusions 24 hours post transduction into murine primary bone marrow Sca-1+Lin- cells enriched in HSC and progenitors cells. We compared two NUP98-FOX fusions (NA10 and ND13) with a non-DNA-binding and non-transforming ND13(N51S) homeodomain mutant. Surprisingly, a relatively small number of genes were significantly differentially expressed in cells harboring the ND13 or NA10 fusions compared to control cells and several potential target genes implicated in HSC expansion and self-renewal were identified with this approach.

MATERIALS AND METHODS

cDNA constructs and retroviral vectors

The NUP98-HOXA10 and NUP98-HOXD13 fusion gene as well as the N51S-ND13 dead homeodomain mutant gene constructs have been described elsewhere [19]. In short, the cDNAs were subcloned into the murine stem-cell virus (MSCV) 2.1 vector upstream of the internal ribosomal entry site (IRES) sequence linked to the gene encoding the enhanced green fluorescence protein (EGFP, Clontech). As a control, the MSCV vector carrying only the IRES-GFP cassette (GFP virus) was used. Production of high-titer, helper-free retrovirus was carried out by standard procedures [28]. Constructs have been validated by sequencing and correct expression and transmission were confirmed by western blot and Southern blot analysis.

Retroviral infection of primary bone marrow cells

Mice were bred and maintained at the British Columbia Cancer Research Centre animal facility. Donors of primary BM cells were older than 12-weeks (C57Bl/6Ly-Pep3b C3H/HeJ) F1 (PepC3) mice. Primary mouse BM cells were transduced as previously described [29]. Briefly, BM cells were harvested from mice treated 4 days previously with 150 mg/kg 5-fluorouracil (Faulding, Underdale, Australia) and prestimulated for 48 hours in Dulbecco modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 10 ng/mL human interleukin-6 (hIL-6), 6 ng/mL murine interleukin-3 (IL-3), and 100 ng/mL murine stem cell factor (mSCF) (StemCell Technologies, Vancouver, BC, Canada). Cells were infected by co-cultivation with irradiated (4600 cGy x-ray) GP+E86 viral producer cells with the addition of 5 μg/mL protamine sulfate (Sigma, Oakville, ON, Canada). Loosely adherent and nonadherent cells were harvested from the co-cultures after 2 days and were cultured for 24 hours in the same medium without protamine sulfate.

Cell sorting and harvest

The single cell suspensions collected were blocked for 10 min on ice with 5 μg/mL anti mouse CD16/CD32 (Fc Block, BD Pharmingen) in Phosphate Buffered Saline (STI)+2% Fetal Bovine Serum (PF). Cells were washed once with PF and then incubated on ice for 20 min with the primary mAb. Cells were then washed once, incubated with the secondary antibody if needed, washed again, and then analysed by flow cytometry using a FACS-Calibur™ flow cytometer and CELLQuest™ software (BD Pharmingen). GFP+ Sca-1+Lin- cells were sorted using a FACSVantage™ (BD Pharmingen). Purity>90% were confirmed by re-analysis of sorted cells. The forward versus side scatter profile was used to gate on viable cells and an unstained sample was used to determine appropriate gating for expression. Monoclonal antibodies (mAbs) were all purchased from PharMingen (San Diego, CA) (phycoerythin [PE]-labeled Gr-1, B220, Ter-119, CD4, CD5 and CD6).
sets. The array data is deposited at Gene Expression Omnibus (GEO), (http://www.ncbi.nlm.nih.gov/geo) and the MIAME (minimal information about a microarray experiment) guidelines was followed for data presentation.

Quantitative RT-PCR validation of murine bone marrow cells

Non-amplified RNA from the transfected murine primary bone marrow samples was used for validation with quantitative RT-PCR (qRT-PCR). RNA was isolated using Trizol® and the samples were then treated with DNase I (amplification grade, Invitrogen). Complementary DNA (cDNA) was generated by reverse transcription (RT) with the iScript cDNA Synthesis Kit (BioRad Inc., Hercules, CA). Gene transcripts were quantified by real-time PCR using the iCycler apparatus (BioRad Inc., Hercules, CA) and were detected with SYBR Green as fluorochrome (IQ™ SYBR Green Supermix, BioRad Inc.). Gene sequences for primer design were obtained from the NCBI Reference Sequences database (http://www.ncbi.nlm.nih.gov/RefSeq/). Primers were chosen using the Primer3 software (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi) and the specificity of all primer pairs was tested with electronic PCR using the mouse genome and the mouse transcript database (http://www.ncbi.nlm.nih.gov/sutils/e-pcr/). The relative expression changes were determined with the 2^ΔΔCt method [32] and the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene transcript was used to normalize the results. Primer sequences (5′ to 3′); 

**For Flt3:**
- Flt3 forward primer ATCGGGGAGACTTTACAGCA
- Flt3 reverse primer AGGGTGTTAGATGCTGAG

**For Gapdh:**
- Gapdh forward primer ATCGGGGACATTTTACAGCA
- Gapdh reverse primer AGGGTGTTAGATGCTGAG

**For Ptprf:**
- Ptprf forward primer ATCGGGGACATTTTACAGCA
- Ptprf reverse primer AGGGTGTTAGATGCTGAG

**For Tgfbi:**
- Tgfbi forward primer ATCGGGGACATTTTACAGCA
- Tgfbi reverse primer AGGGTGTTAGATGCTGAG

**For Meis2:**
- Meis2 forward primer ATCGGGGACATTTTACAGCA
- Meis2 reverse primer AGGGTGTTAGATGCTGAG

**For Pbx2:**
- Pbx2 forward primer ATCGGGGACATTTTACAGCA
- Pbx2 reverse primer AGGGTGTTAGATGCTGAG

**For Evi1:**
- Evi1 forward primer ATCGGGGACATTTTACAGCA
- Evi1 reverse primer AGGGTGTTAGATGCTGAG

**For HoxA5:**
- HoxA5 forward primer ATCGGGGACATTTTACAGCA
- HoxA5 reverse primer AGGGTGTTAGATGCTGAG

**For Pbx1:**
- Pbx1 forward primer ATCGGGGACATTTTACAGCA
- Pbx1 reverse primer AGGGTGTTAGATGCTGAG

**For Meis3:**
- Meis3 forward primer ATCGGGGACATTTTACAGCA
- Meis3 reverse primer AGGGTGTTAGATGCTGAG

**For Pbx3:**
- Pbx3 forward primer ATCGGGGACATTTTACAGCA
- Pbx3 reverse primer AGGGTGTTAGATGCTGAG

**For HoxA7:**
- HoxA7 forward primer ATCGGGGACATTTTACAGCA
- HoxA7 reverse primer AGGGTGTTAGATGCTGAG

**For HoxA9:**
- HoxA9 forward primer ATCGGGGACATTTTACAGCA
- HoxA9 reverse primer AGGGTGTTAGATGCTGAG

**For MEF2C:**
- MEF2C forward primer ATCGGGGACATTTTACAGCA
- MEF2C reverse primer AGGGTGTTAGATGCTGAG

**For PRNP:**
- PRNP forward primer ATCGGGGACATTTTACAGCA
- PRNP reverse primer AGGGTGTTAGATGCTGAG

**For DDX4:**
- DDX4 forward primer ATCGGGGACATTTTACAGCA
- DDX4 reverse primer AGGGTGTTAGATGCTGAG

**For JAG2:**
- JAG2 forward primer ATCGGGGACATTTTACAGCA
- JAG2 reverse primer AGGGTGTTAGATGCTGAG

**For HOX7:**
- HOX7 forward primer ATCGGGGACATTTTACAGCA
- HOX7 reverse primer AGGGTGTTAGATGCTGAG

**For HOX9:**
- HOX9 forward primer ATCGGGGACATTTTACAGCA
- HOX9 reverse primer AGGGTGTTAGATGCTGAG

**For HOX10:**
- HOX10 forward primer ATCGGGGACATTTTACAGCA
- HOX10 reverse primer AGGGTGTTAGATGCTGAG

**For PBX1:**
- PBX1 forward primer ATCGGGGACATTTTACAGCA
- PBX1 reverse primer AGGGTGTTAGATGCTGAG

**For PBX2:**
- PBX2 forward primer ATCGGGGACATTTTACAGCA
- PBX2 reverse primer AGGGTGTTAGATGCTGAG

**For PBX3:**
- PBX3 forward primer ATCGGGGACATTTTACAGCA
- PBX3 reverse primer AGGGTGTTAGATGCTGAG

**For PBX4:**
- PBX4 forward primer ATCGGGGACATTTTACAGCA
- PBX4 reverse primer AGGGTGTTAGATGCTGAG

**For PBX5:**
- PBX5 forward primer ATCGGGGACATTTTACAGCA
- PBX5 reverse primer AGGGTGTTAGATGCTGAG

**For PBX6:**
- PBX6 forward primer ATCGGGGACATTTTACAGCA
- PBX6 reverse primer AGGGTGTTAGATGCTGAG

**For PBX7:**
- PBX7 forward primer ATCGGGGACATTTTACAGCA
- PBX7 reverse primer AGGGTGTTAGATGCTGAG

**For PBX8:**
- PBX8 forward primer ATCGGGGACATTTTACAGCA
- PBX8 reverse primer AGGGTGTTAGATGCTGAG

**For PBX9:**
- PBX9 forward primer ATCGGGGACATTTTACAGCA
- PBX9 reverse primer AGGGTGTTAGATGCTGAG

**For PBX10:**
- PBX10 forward primer ATCGGGGACATTTTACAGCA
- PBX10 reverse primer AGGGTGTTAGATGCTGAG

The following genes were analyzed (TaqMan assay ID number is indicated, Applied Biosystems); H0X7 (Hs00600484_m1), H0X9 (Hs00635956_m1), H0X10 (Hs00538103_m1), PBX1 (Hs00231228_m1), PBX2 (Hs00853502_s1), PBX3 (Hs00600415_m1), MEIS1 (Hs00180200_m1), MEF2C (Hs00231149_m1), JAG2 (Hs00171432_m1), PRNP (Hs00753591_m1), PAX3 (Hs00170334_m1), DXX4 (Hs00170334_m1), and HLF (Hs00171465_m1).

Statistical analysis

Microsoft Excel® in combination with the Excel plug-in software Analyse-IT® v1.73 was used for the statistical calculations. Pearson regression was used to compare gene array and qRT-PCR results. Spearman rank correlation was used to test possible associations. Non-parametric Kruskal-Wallis 1-way ANOVA was used to evaluate differences between groups.

RESULTS

Transduction of murine primary bone marrow

Adult murine bone marrow cells were transduced with vectors carrying ND13, NA10, ND13(N51S) or an empty GFP control vector were isolated on the basis of GFP expression by FACS 24 hours post-transduction. Viable transduced cells were further enriched for primitive hematopoietic cells by exclusion of cells expressing lineage markers (Gr-1, B220, Ter-119, CD4, CD5 and CD8) and selection for cells expressing the stem cell antigen-1 (Sca-1). This resulted in an overall recovery of 0.25–6% of all cells with a purity of 90–95% for GFP expression. The cells were kept on ice during sorting and immediately lysed in Trizol for RNA extraction. Three independent experiments were performed for each of the four different conditions included in the study.

Gene array analysis and validation

After extraction, RNA was amplified and analyzed using the Affymetrix GeneChip MOE430A array containing 23,000 probe sets. The Gene Chip robust multi-array analysis (GC-RMA) was...
used for initial normalization and the GFP control was used for per gene normalization. Pearson correlation coefficient between the experimental replicates ranged between $r = 0.92$–0.99 suggesting low inter-experimental variation and the 3'-to-5' ratios for Gapdh and Actin in all samples were less than 3.0 (ranging from 1.7–2.7), indicating that no serious bias was introduced by the RNA amplification procedure. Our main interest was to define the subset of genes that could explain the transforming and cell expansion potential of NUP98-HOX fusions in the Sca-1+, GFP+, Lin- cell population and that also could be direct NUP98-Hox binding target genes. First, all genes that were significantly differentially expressed between ND13, NA10 or the presumably non DNA-binding ND13(N51S) and the GFP control were identified. Genes were considered differentially expressed if they had a change in expression level compared to control of at least 50% and the extent of difference in expression was statistically significant ($p<0.05$) in a parametric Welsh-ANOVA t-test. More genes were activated than repressed by all NUP98-HOX fusions, 560 activated and 43 repressed genes with ND13, 414 activated and 20 repressed genes with NA10 and 204 activated and 82 repressed genes with ND13(N51S). (Figure 1). ND13 and NA10 had relatively more activation compared to the ND13(N51S) mutant and there was only a significant overlap on activated genes between ND13 and NA10 (Figure 1). Furthermore, of the 170 differentially expressed genes that were induced by both ND13 and NA10, 74 of these genes also differed significantly between ND13 and NA10 and the functionally inert ND13(N51S) mutant genes with at least a 50% difference in expression level (Table 1). These results suggest that ND13 and NA10 mainly act as transcriptional activators in undifferentiated BM cells and that this effect is dependent on an intact homeodomain. Moreover, the gene array results also indicate that a relatively small number of activated genes can be linked to NUP98-Hox induced effects on primitive hematopoietic cell function.

To confirm the fidelity of the microarray data a subset of 15 genes was selected for validation using quantitative RT-PCR (Table 2). Quantitative real-time RT-PCR was used to measure relative abundances. The housekeeping gene GAPDH was used as an endogenous control to normalize the data. The fold change was calculated between the GFP control and the NA10, ND13 and ND13(N51S) samples respectively. The majority of the individual fold changes determined from the gene array was verified by qRT-PCR (Table 2). Overall the analysis revealed a good correlation between the gene array data and the qRT-PCR results (Pearson correlation, $r = 0.83$), with a tendency that the PCR results showed greater changes than what the array suggested. Taken together the validation analysis provides confidence in our approach to identify differentially expressed genes with a high likelihood of exhibiting true expression level changes.

**Functional classification and analysis of differentially expressed genes**

The 74 genes that were both induced by ND13 and NA10 and whose expression was dependent on an intact homeodomain were classified into gene ontology categories according to involvement in different biological processes. The genes were separated into 12 main categories (Figure 2). A relatively high number of differentially expressed genes were classified as being involved in development and differentiation (16 genes), cell cycle, cell growth and/or cell proliferation (13 genes) or signal transduction (19 genes). At least 16 genes were classified as being involved in transcriptional regulation (Table 1). Thus, processes potentially important for cell self-renewal, expansion and transformation. Several of the genes induced by ND13 and NA10 were Hox or Hox cofactors (Hoxa5, Hoxa7, Hoxa9 and Pbx3, Table 1). No significant change of gene expression could be seen for Pbx1, Pbx2 or Meis1, also verified with Q-RT-PCR (Table 2), but all three genes were expressed in the Sca1+, Lin- BM cell population in concordance with published results [33]. Meis2 and Meis3 could not be detected either by gene array or Q-RT-PCR (data not shown). These findings are basically in line what have been reported in other studies [34–36]. Besides the Hox or Hox co-factors several other putative target genes of ND13 and NA10 are involved in cell development and proliferation. This included induction of the DEAD-box protein gene Ddx4, the hepatic leukemia factor (Hlf), the MADS box transcription enhancer factor 2C (Mef2c), the prion protein gene (Prnp) and Jagged-2 (Jag2). The Drosophila ortholog of Ddx4, VASA, has a central role in germ cell development and is conserved in invertebrates and vertebrates. Ddx4 is a member of the DEAD box family of ATP-dependent RNA helicases, the same gene family as Ddx10 belongs to, which has been found in translocations with NUP98 [37]. The prion protein has been shown to be present on human CD34+ bone marrow (BM) stem cells [38] and was recently shown to be expressed on long-term hematopoietic stem cells and to be important in hematopoietic stem cell self-renewal [39]. The HLF gene has been found in translocations together with E2A in human acute lymphoblastic leukemia [40] and enforced HLF expression has been reported to enhance both HSC engraftment and to inhibit apoptosis [41]. The MEF2 family of regulatory proteins are
Table 1. Genes changed by NA10 and ND13 but not by the ND13(N51S) mutant compared to the GFP control.

| Gene name and description | Accession # | ND13 fold change | ANOVA p-value | NA10 fold change | ANOVA p-value | Biological process |
|---------------------------|-------------|------------------|---------------|------------------|---------------|--------------------|
| Crisp1, cysteine-rich secretory protein 1 | NM_009638 | 18.3 | 6.04E-06 | 30.8 | 7.92E-06 | Unknown |
| Nr4a1, nuclear receptor subfamily 4, group A, member 1 | NM_010444 | 8.1 | 2.90E-02 | 1.8 | 5.70E-03 | Transcription |
| IgG-6, immunoglobulin heavy chain 6 (heavy chain of IgM) | BB226392 | 5.0 | 2.90E-03 | 3.2 | 3.81E-05 | Signal transduction/Immune response/Cell proliferation |
| Ptprf, protein tyrosine phosphatase, receptor type, F | BF235516 | 4.5 | 3.69E-03 | 1.8 | 1.10E-03 | Signal transduction |
| Pdcd1lg2, programmed cell death 1 ligand 2 | NM_021396 | 4.1 | 3.79E-05 | 2.0 | 2.42E-03 | Cell proliferation |
| Pbx3, pre B-cell leukemia transcription factor 3 | NM_016768 | 3.9 | 6.23E-06 | 4.8 | 1.19E-05 | Transcription/Development |
| Hlf, hepatic leukemia factor | NM_172563 | 3.4 | 3.52E-02 | 6.8 | 4.03E-04 | Transcription/Cell proliferation |
| Ahr, aryl-hydrocarbon receptor | NM_013464 | 3.4 | 2.54E-03 | 3.1 | 2.35E-05 | Transcription/Signal transduction |
| Hlx1, H2.0-like homeo box 1 | BM240030 | 3.3 | 9.17E-04 | 2.3 | 1.21E-03 | Transcription/Cell proliferation |
| Ier3, immediate early response 3 | U96693 | 2.8 | 5.50E-03 | 2.0 | 8.22E-04 | Unknown |
| Cables1, Cdk5 and Abl enzyme substrate 1 | AF328140 | 2.8 | 2.87E-03 | 1.8 | 2.59E-02 | Cell cycle/Development |
| H2-DMa, histocompatibility 2, class II, locus DMa | NM_010386 | 2.8 | 7.61E-03 | 2.2 | 1.74E-02 | Immune response/Transport |
| Ahrr, aryl-hydrocarbon receptor repressor | BM240030 | 2.8 | 9.31E-04 | 1.5 | 8.00E-03 | Cell cycle/Transcription/Metabolism/Cell adhesion |
| Fads3, fatty acid desaturase 3 | BB041811 | 2.9 | 2.99E-03 | 2.9 | 3.04E-03 | Signal transduction/Transport |
| Tmem71, transmembrane protein 71 | AV173260 | 3.0 | 4.03E-05 | 2.5 | 4.65E-05 | Unknown |
| Pkp2, plakophilin 2 | AA516617 | 3.0 | 7.13E-06 | 1.7 | 9.35E-06 | Cell adhesion/Immune response |
| Pira1, paired-Ig-like receptor A1 | NM_011093 | 3.0 | 1.56E-02 | 2.3 | 2.13E-02 | Cell cycle/Immune response |
| Tmem51, transmembrane protein 51 | BC003277 | 2.4 | 5.61E-03 | 2.1 | 3.87E-03 | Cell growth/Signal transduction |
| RCIEN NDA, RAB4A, member RAS oncogene family | NM_009003 | 2.4 | 1.14E-04 | 1.7 | 2.10E-03 | Unknown |
| RCIEN NDA, RAB4A, member RAS oncogene family | BC023470 | 2.4 | 1.07E-02 | 1.9 | 7.20E-03 | Metabolism |
| Tspan6, tetraspanin 6 | NM_009644 | 2.7 | 9.31E-04 | 2.2 | 2.48E-03 | Signal transduction/Transport/Cell adhesion |
| Pscdbp, pleckstrin homology, Sec7 and coiled-coil domains, binding protein | BC007144 | 2.5 | 3.65E-03 | 2.4 | 4.42E-03 | Cell cycle/Cell proliferation/Signal transduction |
| F2rl2, coagulation factor II (thrombin) receptor-like 2 | BC007144 | 2.4 | 3.04E-03 | 1.6 | 4.34E-02 | Cell adhesion |
| F2rl2, coagulation factor II (thrombin) receptor-like 2 | NM_010170 | 2.4 | 8.34E-04 | 2.2 | 9.01E-04 | Coagulation/Signal transduction |
| Cish, cytokine inducible SH2-containing protein 1 | BC009174 | 2.4 | 6.78E-04 | 1.6 | 3.19E-02 | Cell growth/Signal transduction |
| Tsc22d1, TSC22 domain family, member 1 | BB357514 | 2.4 | 2.60E-03 | 2.0 | 7.37E-03 | Transcription |
| Wdfy2, WD repeat and FYVE domain containing 2 | BB794924 | 2.4 | 6.33E-03 | 1.7 | 6.51E-03 | Unknown |
| Hoxa5, homeo box A5 | NM_011116 | 2.3 | 8.47E-03 | 2.6 | 4.39E-05 | Metabolism |
| Dnase1, deoxyribonuclease 1-like 1 | AK009174 | 2.3 | 1.84E-02 | 1.9 | 2.87E-02 | Metabolism |
| Myl2, myosin light chain 2, precursor | NM_021611 | 2.3 | 2.35E-02 | 2.9 | 1.82E-03 | Unknown |
| Cyp4f16, cytochrome P450, family 4, subfamily f, polypeptide 16 | BC019423 | 2.3 | 4.46E-04 | 2.2 | 8.76E-04 | Unknown |
| Hoxa5, homeo box A5 | BC011063 | 2.3 | 7.95E-04 | 3.9 | 3.02E-05 | Transcription/Development |
| Crisp3, cysteine-rich secretory protein 3 | NM_009639 | 2.2 | 1.20E-04 | 2.1 | 3.50E-02 | Unknown |
| Procr, protein C receptor, endothelial | NM_011171 | 2.2 | 8.99E-03 | 2.5 | 5.25E-05 | Coagulation |
| Plek, pleckstrin | AF181829 | 2.2 | 5.33E-03 | 1.8 | 1.44E-02 | Signal transduction |
| Mtf, microphthalmia-associated transcription factor | BB763517 | 2.2 | 9.81E-03 | 1.9 | 2.58E-03 | Development/Transcription |
involved in myogenesis and the *Mef2c* gene is important for normal morphogenesis [42]. Jagged-2 (*Jag2*) is a ligand that activates NOTCH1 and related receptors that are critical for various cell fate decisions. Furthermore, the tyrosine kinase receptor *Flt3* was found to be induced by ND13 and NA10 in Sca1+Lin- primary BM cells. We and others have previously shown that *Flt3* expression is induced by Meis1 in a context with either high expression of NUP98-Hox fusions [43] or HOXA9 [44] and the present finding further strengthen the conclusion that the *Flt3* is a direct target gene of Hox and Hox co-factors in primary HSCs or progenitor cells.

**Correlation of gene expression in human AML**

To discern what target genes identified in the microarray analysis might be involved in leukemic transformation and to investigate if they are associated with Hox and Hox co-factor expression, 34 de

| Gene name and description | Accession # | ND13 fold change | ANOVA p-value | NA10 fold change | ANOVA p-value | Biological process |
|---------------------------|-------------|------------------|---------------|------------------|---------------|--------------------|
| Metn1l, meteorin, gial cell differentiation regulator-like | BC024445 | 2.1 | 2.00E-02 | 2.2 | 1.79E-02 | Unknown |
| Prnp, prion protein | BE630020 | 2.1 | 2.13E-03 | 2.3 | 8.29E-03 | Metabolism |
| Sord, sorbitol dehydrogenase | AV253518 | 2.1 | 1.49E-02 | 2.3 | 5.31E-04 | Unknown |
| Rpgp1, retinitis pigmentosa GTPase regulator interacting protein 1 | AK015037 | 2.1 | 1.98E-04 | 1.6 | 3.35E-03 | Development |
| Gsn, gelsolin | NM_010354 | 2.1 | 8.22E-03 | 2.1 | 2.22E-02 | Transport |
| Hoxa7, homeo box A7 | NM_010455 | 2.0 | 2.45E-02 | 1.7 | 2.38E-02 | Transcription/Development |
| Mef2c, myocyte enhancer factor 2C | AI595932 | 2.0 | 2.58E-02 | 1.7 | 6.06E-03 | Transcription/Development |
| Eltd1, EGF, latrophilin seven transmembrane domain containing 1 | BC017134 | 2.0 | 2.94E-04 | 1.9 | 4.89E-04 | Signal transduction |
| Fli3, FMS-like tyrosine kinase 3 | NM_010229 | 2.0 | 2.44E-02 | 2.0 | 2.32E-02 | Signal transduction/Development |
| Ncoa1, nuclear receptor coactivator 1 | NM_010881 | 1.9 | 2.24E-02 | 1.5 | 6.81E-03 | Transcription/Signal transduction |
| Ddx4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 | AK014844 | 1.9 | 2.04E-03 | 1.3 | 1.92E-06 | Development |
| Calcr, calcitonin receptor-like | AF209905 | 1.9 | 1.37E-02 | 1.8 | 1.97E-02 | Cell proliferation/Signal transduction/Development |
| Slt1sl4a, ST8 alpha-N-acetyl-neuraminide alpha-2-8-sialyltransferase 4 | NM_009183 | 1.9 | 1.87E-03 | 2.0 | 1.77E-03 | Metabolism |
| Glul, glutamate-ammonia ligase | AI391218 | 1.9 | 1.09E-02 | 1.7 | 1.64E-02 | Metabolism |
| Lrp10, low-density lipoprotein receptor-related protein 10 | BC011058 | 1.9 | 3.27E-03 | 1.8 | 5.96E-03 | Metabolism/Transport |
| Hoxa9, homeo box A9 | NM_010456 | 1.8 | 1.06E-02 | 2.5 | 1.33E-03 | Transcription/Development |
| Malat1, metastasis associated lung adenocarcinoma transcript 1 | AW012617 | 1.8 | 3.31E-02 | 1.9 | 1.27E-03 | Unknown |
| Ptk2b, PTK2 protein tyrosine kinase 2 beta | AV026976 | 1.8 | 8.99E-03 | 1.7 | 1.34E-02 | Signal transduction |
| Igfbp7, insulin-like growth factor binding protein 7 | AI481026 | 1.8 | 1.25E-02 | 1.7 | 2.41E-02 | Cell growth/Metabolism |
| Mxd4, Max dimerization protein 4 | BG68949 | 1.8 | 1.23E-02 | 1.8 | 7.84E-03 | Transcription |
| Sesn1, sestrin 1 | BG076140 | 1.8 | 1.60E-03 | 1.8 | 1.42E-03 | Cell cycle |
| Itm2c, integral membrane protein 2C | NM_022417 | 1.8 | 3.67E-03 | 1.5 | 2.97E-02 | Unknown |
| Riken cdna 4930504e06 gene | BB010153 | 1.7 | 2.71E-02 | 1.8 | 2.82E-03 | Unknown |
| Man1a, mannosidase 1, alpha | NM_008548 | 1.7 | 9.38E-03 | 1.6 | 1.53E-02 | Metabolism |
| Aldoc, aldolase 3, C isofrom | BC008184 | 1.7 | 2.36E-02 | 1.8 | 2.93E-02 | Metabolism |
| Cin3, ceroid lipofuscinosis, neuronal 3, juvenile | NM_009907 | 1.7 | 4.55E-03 | 1.7 | 5.18E-03 | Unknown |
| Arbr1, arrestin, beta 1 | AK004614 | 1.7 | 6.77E-03 | 1.8 | 7.22E-03 | Signal transduction |
| Cast, calpastatin | AB026997 | 1.7 | 8.19E-03 | 1.8 | 2.99E-03 | Metabolism |
| Nupr1, nuclear protein 1 | NM_019738 | 1.7 | 4.92E-03 | 2.0 | 8.18E-03 | Unknown |
| Jag2, jugged 2 | AV264681 | 1.7 | 2.21E-02 | 1.6 | 1.15E-02 | Signal transduction/Development/Cell proliferation |
| Bckdha, branched chain ketoacid dehydrogenase E1, alpha polypeptide | NM_005733 | 1.6 | 3.32E-02 | 1.8 | 1.30E-02 | Metabolism/Transcription |
| Prkcn, protein kinase C, nu | BF160591 | 1.6 | 1.17E-02 | 1.7 | 3.48E-03 | Signal transduction |
| Pnp, purine-nucleoside phosphorylase | AK008143 | 1.5 | 3.02E-03 | 1.5 | 2.90E-02 | Metabolism |
| Tcf4, transcription factor 4 | AI639846 | 1.5 | 2.96E-02 | 1.5 | 1.42E-02 | Transcription/Development |

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Nova AML samples collected at diagnosis from adult patients were analyzed. Complete karyotype and FLT3-ITD status was known for all subjects and represented FAB subclass M0-M5 morphologically. Six patients had favorable, 19 had intermediate and 9 had unfavorable cytogenetics (Table 3). Quantitative real-time RT-PCR with TaqMan Low density array (TLDA) was used to measure gene expression, allowing analysis of all the selected genes in several samples at the same time lowering assay variation and increasing reproducibility. We tested a subset of Hox and Hox cofactor genes, HOXA5, HOXA7, HOXA9, HOXA10, PBX1, PBX2, PBX3, and MEIS1, and possible Hox target genes FLT3, HLF, JAG2, MEF2C, DDX4, and PRNP. The gene expressions in leukemia samples were normalized with the housekeeping gene GUS as an endogenous control and the expression levels were calculated relative to pooled normal bone marrow (Table 3).

Two of the selected genes could not be detected in the majority of the samples in either normal or leukemia bone marrow (HOXA10 and DDX4) and were therefore excluded from further analysis. Analysis of the selected Hox A cluster and Hox cofactor genes revealed a high degree of co-expression between these in leukemia samples, where correlation coefficient between HOXA5, HOXA7, HOXA9 and MEIS1 ranged between 0.83 and 0.92 (Spearman rank correlation, p < 0.0001 in all cases, e.g. in Figure 3A). PBX3 gene expression also correlated with these (r = 0.67–0.76, p < 0.0001) but PBX2 and PBX1 did not. Furthermore, patients with intermediate or unfavorable cytogenetics had significantly higher expression of MEIS1, PBX3, HOXA5, HOXA7 and HOXA9 compared to patients with favorable cytogenetics (non-parametric Kruskal-Wallis ANOVA, e.g. HOXA7 p = 0.0013 and p = 0.018 respectively) but there was no significant difference between patients with intermediate and unfavorable cytogenetics. These findings suggest a tight co-regulation of these factors and define a subset of homeodomain transcription factors linked to leukemic development and are in concordance with other reports [45,46].

Among the selected possible direct target genes, FLT3 gene expression showed the closest association with HOXA5, HOXA7, HOXA9 and MEIS1 (Spearman, r-value between 0.45–0.57, p-value 0.016–0.0020) but no significant correlation was seen with PBX3 expression (r = 0.22, p = 0.20). Furthermore, FLT3 expression showed a weak but significant correlation with bone marrow blast counts (r = 0.36, p = 0.049). Moreover, FLT3 expression was significantly higher in patients with intermediate or unfavorable cytogenetics (Kruskal-Wallis, p = 0.047 and

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**Table 2. Validation of microarray results with quantitative RT-PCR on unamplified RNA.**

| Gene transcript | qRT-PCR NA10 | Array NA10 | qRT-PCR ND13 | Array ND13 | qRT-PCR ND13 N515 | Array ND13 N515 |
|-----------------|--------------|------------|--------------|------------|-------------------|----------------|
| Pbx1            | 1.7          | 1.1        | 2.6          | 1.3        | 1.0               | 0.1            |
| Pbx2            | 0.9          | 1.3        | 1.1          | 1.1        | 0.6               | 1.0            |
| Pbx3            | 5.4          | 5.0        | 4.2          | 4.9        | 0.4               | 0.8            |
| HoxA5           | 7.5          | 3.2        | 7.7          | 2.1        | 1.7               | 0.9            |
| HoxA7           | 1.7          | 2.1        | 3.0          | 2.8        | 0.5               | 0.2            |
| HoxA9           | 2.4          | 1.6        | 2.9          | 1.2        | 0.8               | 1.0            |
| HoxB4           | 2.9          | 2.5        | 1.5          | 1.4        | 0.8               | 1.1            |
| Meis1           | 1.6          | 1.1        | 1.7          | 0.9        | 1.4               | 1.2            |
| Flt3            | 5.8          | 2.2        | 10.1         | 2.3        | 2.1               | 1.1            |
| Evl             | 28.1         | 4.2        | 8.6          | 2.9        | 3.8               | 1.4            |
| Anxa1           | 3.6          | 2.0        | 4.9          | 2.2        | 1.0               | 1.3            |
| Tgfbi           | 2.2          | 1.8        | 5.6          | 2.2        | 1.7               | 1.1            |
| c-Jun           | 1.1          | 1.5        | 5.3          | 3.4        | 1.1               | 3.4            |
| Csf2rb          | 4.9          | 2.4        | 5.6          | 4.9        | 1.6               | 2.2            |
| Ptprf           | 24.5         | 10.8       | 100.5        | 27.2       | 2.7               | 2.4            |

Fold changes are calculated against an empty MIG control and Gapdh as endogenous control gene.

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**Figure 2. Annotation of differentially expressed genes.** Genes that were differentially expressed by both ND13 and NA10 but not the mutant ND13(N51S) mutant were classified according to involvement in different biological processes. Some genes are classified in more than one category resulting in the total number of genes indicated in the figures being greater than the total number of differentially expressed genes.

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| Patient | Cytogenetics | FLT3 | FAB | Marrow blasts |
|---------|-------------|-----|-----|--------------|
| 1       | favorable   | ITD+| AML | M3           |
| 2       | favorable   | Normal | AML | M4           |
| 3       | favorable   | Normal | AML | M3 unknown   |
| 4       | favorable   | ITD+ | AML | M2           |
| 5       | favorable   | ITD+ | AML | M3           |
| 6       | favorable   | Normal | AML | M2 unknown   |
| 7       | intermediate| Normal | AML | M1 unknown   |
| 8       | intermediate| Normal | AML | M1           |
| 9       | intermediate| Normal | AML | M2           |
| 10      | intermediate| Normal | AML | M3 unknown   |
| 11      | intermediate| Normal | AML | M1           |
| 12      | intermediate| Normal | AML | M2           |
| 13      | intermediate| Normal | AML | M3           |
| 14      | intermediate| Normal | AML | M2           |
| 15      | intermediate| Normal | AML | M1           |
| 16      | intermediate| Normal | AML | M2           |
| 17      | intermediate| Normal | AML | M1           |
| 18      | intermediate| Normal | AML | M2           |
| 19      | intermediate| Normal | AML | M3           |
| 20      | intermediate| Normal | AML | M2           |
| 21      | intermediate| Normal | AML | M1           |
| 22      | intermediate| Normal | AML | M1           |
| 23      | intermediate| Normal | AML | M2           |
| 24      | intermediate| Normal | AML | M1           |
| 25      | intermediate| Normal | AML | M3           |
| 26      | intermediate| Normal | AML | M2           |
| 27      | intermediate| Normal | AML | M1           |
| 28      | intermediate| Normal | AML | M3           |
| 29      | intermediate| Normal | AML | M2           |
| 30      | intermediate| Normal | AML | M1           |
| 31      | intermediate| Normal | AML | M2           |
| 32      | intermediate| Normal | AML | M1           |
| 33      | intermediate| Normal | AML | M1           |
expression correlated ($r = 0.47$, $p = 0.0052$, Figure 3B) and there was also a significant correlation between $JAG2$ and $FLT3$ expression ($r = 0.39$, $p = 0.024$). Altogether, these findings suggest that $HOXA7$ and $HOXA9$ could be directly involved in $JAG2$ and $HLF$ gene regulation and possibly, unlike $FLT3$, independent of MEIS1.

The expression of the $MEF2C$ and $PRNP$ genes showed a tight co-association ($r = 0.70$, $p < 0.0001$) and both showed significantly higher expression in patients with unfavorable vs. favorable cytogenetics (Kruskal-Wallis ANOVA, $p = 0.017$ and $p = 0.026$ respectively). However, their expression did not correlate with $HOXA5$, $HOXA7$, $HOXA9$ or MEIS1 and only weakly with $PBX3$ expression in human leukemia (Spearman, $r = 0.37$, $p = 0.033$ and $r = 0.44$, $p = 0.078$ respectively). This suggests that their induction may be restricted to NUP98-Hox fusions and that they are not regulated by the native HOXA5, HOXA7, HOXA9 or MEIS1 in human bone marrow cells.

### DISCUSSION

The goal of this study was to identify gene expression changes that may underlie the potent growth promoting effects of Hox on primitive hematopoietic cells. Our strategy included use of two NUP98-Hox fusions with strong overlapping functional effects versus a functionally “dead” mutant form coupled with analysis of early induced gene expression changes in a HSC/progenitor cell enriched fractions. Key results included the identification of a limited number of induced genes mainly involved in cell development, cell proliferation and signal transduction, consistent with the potent effects of these fusions on promoting primitive hematopoietic cell expansion and differentiation block in vitro and their ability to collaborate in leukemic transformation [19,21,27]. Among the identified genes are several intriguing candidates as HSC regulators and/or possible leukemogenic targets e.g. the tyrosine receptor Flt3, the prion protein Prnp, the transcription factor Hif and the Notch ligand Jag2. Moreover, induction of $HOXA5$, $HOXA7$, $HOXA9$ and $PBX3$ by ND13 and NA10 was observed in HSC/progenitor cells that may define a minimal Hox transforming profile that seems to be shared with other NUP98-Hox and MLL fusions. Furthermore, $FLT3$, HLF and Jag2 expression correlated with Hox genes in human AML that both confirms the fidelity of the microarray analysis and suggests the importance of these genes for Hox genes ability to trigger HSC expansion and to serve as a first step in leukemic transformation.

Importantly, several of the suggested target genes reported herein overlap with those recently published by the study of Chung et al., in which human CD34+ cord blood was used to investigate the effects induced by NUP98-HOXA9. These included the $HOXA5$, $HOXA7$, $HOXA9$ and $PBX3$ genes but perhaps more intriguing also $HLF$. Elevated $HLF$ expression can both enhance HSC engraftment and inhibit apoptosis [41]. The homeodomain dependent induction of $HIF$ expression by ND13 and NA10 may explain the proliferative advantage induced by these genes on primary HSC and progenitor cells in vitro and the high level expansion of long term repopulating cells induced NA10 [19,27]. The finding that $HLF$ also correlated with $HOXA7$ and $HOXA9$ in human leukemia suggests that this gene also might be important for Hox induced cell transformation and development of leukemia.

The finding of homeodomain dependent induction of Jag2 by ND13 and NA10 in Scal+, Lin− BM cells, and that Jag2 expression correlated with $HOXA7$ and $HOXA9$ expression in AML samples is also very interesting since Jag2 was recently found to be overexpressed in CD34+/CD38− isolated leukemic stem cells from AML patients [47]. Moreover, Serrate, the Drosophila homologue of Jag2, has been identified as a component of Hox-dependent pathways [48] and in C. elegans the Hox

![Figure 3. Correlation between HOXA7 and HOXA9 (A) and JAG2 and HLF (B) gene expression in human AML analyzed with TaqMan Low Density Array (TLDA). Spearman rank correlation analysis was done on the log ratio values obtained from the TLDA assay and calculated with the $2^{-ΔΔCT}$ method (n = 34).](image-url)
protein LIN-39 and its Pbx-like cofactor CEH-20 are required for the expression of the genes encoding the LIN-2/Notch receptor and its ligand LAG-2/Delta/Serrate [49]. Thus, the Notch signaling pathway might be part of the effects induced by Hox and Hox co-factors.

Calvo et al. have reported that NUP98-HOXA9 enforce strong transcription of endogenous Hoxa9 and Hoxa7, which further strengthen that different NUP98-Hox fusions have common target genes. HOXA9 is frequently induced in human AML with poor prognosis [50] and Hoxa9 can induce leukemia in murine BM transplantation model in collaboration with Meis1 [51] similar to what we have shown for ND13 and NA10 [19,21]. Furthermore, mixed-lineage-leukemia (MLL) fusion genes, induce a characteristic pattern of Hox A cluster genes, including Hoxa7 and Hoxa9 in myeloid cells [52]. Both these genes are required for efficient in vivo myeloid immortalization by MLL-ENL and in a bone marrow transplantation model Hox9 is essential for MLL-dependent leukemogenesis in vivo [52]. Furthermore, deregulation of FLT3 or FLT3 mutations are frequently found in AML [53]. Meis1 was recently shown to directly induce Flt3 expression in murine BM cells together with either Hoxa9 or ND13 or NA10 [43,44] and we have also shown that high Flt3 expression is sufficient to induce AML transformation in mice using preleukemic BM cells expressing either ND13 or NA10 [43]. Interestingly, Flt3 has also been reported to be expressed together with Pbx3, HoxA7, HoxA9 and Meis1 in a gene expression profile induced by MLL-ENL [54], thus similar to what we observed for ND13 and NA10 in this study. In addition, our results reveal an association between HOXA5, HOXA7, HOXA9, MEIS1 and FLT3 expression in human AML. In conclusion these findings indicate that induction of Flt3, Hoxa7, Hoxa9 and Pbx3 and possibly other Hox genes (i.e. Hoxa5) in part underlie the transforming effects of ND13 and NA10 and perhaps support a common mechanism for leukemogenesis triggered by both NUP98 and MLL fusion genes.

Finally, the gene array results also indicated that the NUP98-Hox genes act principally as strong transcriptional activators. Importantly, the set of genes that showed overlap between ND13 and NA10 were all induced genes suggesting that gene transcription activation rather then repression is the key to their functional effects on primitive hematopoietic cells. Interestingly, in the study by Ghannam et al. where HOXA9 or NUP98-HOXA9 were expressed in myeloid cell lines, the majority of the genes showed predominant induced expression [53]. Furthermore, NUP98-HOXA9 affected about eight times more genes than HOXA9, with a substantial number of them regulated by the fusion but not by the native HOXA9 protein, intriguingly including the Mef2c gene. The finding that the Mef2c gene was induced also by both ND13 and NA10, but did not correlate with Hox or Hox co-factor expression in human AML samples indicate that this gene, plus the prion protein, Prnp, could be novel direct targets of these NUP98-Hox fusions and not normally effected by native HOXA5, HOXA7, HOXA9 or MEIS1 genes. However, other Hox genes not included in our analysis could of course still be involved in their regulation. In conclusion our findings support that NUP98-Hox fusion proteins are aberrant transcriptional activators whose activity depends on the DNA binding homeodomain but also has stronger and wider transcriptional effects than the native Hox protein. Findings by Chung et al. provide evidence that this effect, at least in part, is mediated by decreased susceptibility to CUL-4A-dependent ubiquitination of NUP98-Hox fusions increasing their protein half-lives [56].

In summary this study identify gene expression changes that involve different biological processes important for HSC self-renewal and proliferation and point to several interesting genes suggesting that NUP98-Hox fusions target multiple mechanisms that could potentially explain how they both transform and induce expansion of HSCs, which in turn may lead to leukemia. The next step in our investigation will be to elucidate their relative role in these processes in animal models for HSC expansion and for transforming activity. These results will also be of great help in ongoing efforts for genome wide analysis of Hox binding sites and for investigating direct binding of Hox and Hox co-factors to regulator sequences.

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Author Contributions

Conceived and designed the experiments: RH NP LP.Performed the experiments: NP LP CW. Analyzed the data: LP. Contributed reagents/materials/analysis tools: RH NP LP. Wrote the paper: RH NP LP.
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