The Oncogene \(^{\text{Nup98-HOXA9}}\) Induces Gene Transcription in Myeloid Cells*

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Most cases of AML are associated with chromosomal rearrangements that lead to the expression of chimeric fusion proteins (1). Genes encoding nuclear pore proteins (nucleoporins) are frequently rearranged in acute leukemia, particularly AML. Nup214 (CAN) was the first nucleoporin to be implicated in the pathogenesis of acute leukemia (2). However, the gene encoding another nucleoporin, Nup98, has recently emerged as a much more frequent target of chromosomal rearrangements in leukemia. At least 17 different chromosomal rearrangements involving nucleoporin genes have been described in leukemia, 15 of which involve Nup98 (3–12). Nup98 gene rearrangements are more frequent in patients that had been previously treated with topoisomerase II inhibitors and may also be more common in Asian populations (3–11, 13). Patients with nucleoporin gene rearrangements tend to be young, and their AML is usually refractory to therapy.

Nucleoporin gene rearrangements result in the expression of chimeric proteins that consist of a portion of the nucleoporin fused to a portion of a partner protein. Both Nup98 and Nup214 belong to a subset of nucleoporins containing FG repeats (14, 15). In all chromosomal rearrangements involving nucleoporins, the fusion product that is expressed in the leukemic cell contains these FG repeats (2–13). In 8 of the 15 Nup98 gene rearrangements, the fusion partner is a transcription factor of the homeodomain family (2–13). The prototype of these fusion proteins is Nup98-HOXA9, which results from t(7;11)(p15;p15) and consists of the N-terminal FG repeat region of Nup98 fused to the C terminus of HOXA9 that includes the DNA binding homeodomain (Fig. 1).

Nup98 is present both at the nuclear pore complex and within the nuclear interior (16–19). There is evidence that the FG repeat region of Nup98 interacts with RNA export proteins H20648 and H20649, and there is evidence that it plays a role in nuclear import (19–21). In addition, Nup98 interacts with the RNA export proteins RAE1 and TAP, and there is evidence that it functions in RNA export from the nucleus (3, 22–25). The function of Nup98 within the nucleus is not entirely clear. It has been reported that Nup98 is present on fibrillar structures inside the nucleus, suggesting a role in intranuclear trafficking (17). Other studies suggest that intranuclear Nup98 may be involved in the trafficking of RNA from transcription sites to the nuclear pore complex (16). Finally, there is evidence that the FG repeat region of Nup98 can activate transcription from a GAL4-responsive promoter when attached to a GAL4 DNA binding domain, suggesting a possible role for Nup98 in transcription (26).

HOXA9 is one of a large family of transcription factors characterized by the presence of a DNA binding homeodomain (27). Homeodomain transcription factors are involved in patterning the anteroposterior axis of the body during embryonic development (27) and play important and complex roles during hematopoiesis (28–31). There are two major groups of homeodomain
proteins. Class I includes the HOX genes that exist in 4 genomic clusters, designated A-D, on chromosomes 7, 17, 12, and 2, respectively, and are divided into 13 paralogous groups numbered 1–13 (30). Most of the homeodomain fusion partners of Nup98, including HOXA9, belong to the so-called Abd-B group of HOX genes that includes paralogous groups 9–13 (2–13). Class II includes genes such as PAX1, PBX1, and MEIS1, that are scattered throughout the genome (30). HOXA9 has been shown to bind DNA cooperatively with PBX1 and/or MEIS1 (32, 33). Although the normal functions of HOXA9 are not well understood, there is ample evidence that it is involved in the pathogenesis of AML, particularly in collaboration with MEIS1. HOXA9 overexpression can immortalize myeloid progenitors in vitro and inhibit some of their differentiation pathways (34). When mice are transplanted with bone marrow cells overexpressing HOXA9, they develop AML in an average of 128 days, a period that is shortened to 57 days by MEIS1 coexpression (35). Furthermore, HOXA9 and MEIS1 are frequently coexpressed in human AML (36).

The Nup98-HOXA9 fusion results in replacement of the transcriptional regulatory region of HOXA9 by the FG repeat region of Nup98 (Fig. 1). In contrast to wild-type Nup98, the Nup98-HOXA9 chimera is primarily intranuclear (26). Nup98-HOXA9 increases the proliferative capacity of bone marrow progenitors (35, 37). Transplanting mice with hematopoietic stem cells that express Nup98-HOXA9 induces a myeloproliferative disease with development of AML within an average of 230 days (35). Coexpression of Nup98-HOXA9 with MEIS1 shortens the period of AML development to an average of 142 days (35). Coexpression of Nup98-HOXA9 with the Bcr-Abl fusion oncogene reduces the period to AML even further, to 21 days (38). Thus, it is clear that Nup98-HOXA9 plays a causative role in the development of AML, although additional factors may be necessary for rapid induction of a full-blown AML phenotype. The mechanisms by which Nup98-HOXA9 and other Nup98 fusions contribute to the pathogenesis of AML are not well understood.

In this study we provide strong evidence that Nup98-HOXA9 acts as an aberrant transcription factor in myeloid cells and identify 102 target genes. Of these, 92 are up-regulated, whereas only 10 are down-regulated, indicating that Nup98-HOXA9 acts primarily as a transcriptional activator. In contrast, wild-type Nup98 has only 13 target genes, and Nup98 has none. Several of the genes induced by Nup98-HOXA9 are associated with increased cell proliferation and survival, suggesting possible pathways involved in leukemogenesis. Interestingly, the gene encoding a drug-metabolizing enzyme, CYP3A5, is markedly induced by Nup98-HOXA9 and may express some aspects of the epidemiology of Nup98-HOXA9-associated AML, particularly its association with etoposide treatment and possibly some pathways involved in leukemogenesis. 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in Fig. 6 represent the mean of 3
Avidities to correct for differences in transfection efficiency. The data shown
were run in triplicate on the same plate (except for the negative control). Data analysis was performed using the iCycler iQ Optical System Software Version 3.0a (Bio-Rad).

Transfection of K562 Cells and Luciferase Assay—K562 cells were transfected with a 10 μg of the pXP1/cyclooxygenase-1 (COX-1) con-
struct that expresses firefly luciferase driven by the promoter for hu-
man COX-1, kindly provided by Dr. Kenneth Wu (University of Texas, Houston, TX) (40) and 20 μg of either empty pcDNA3 vector, pcDNA3/Nup98, pcDNA3/HOXA9, or pcDNA3/Nup98–HOXA9 con-
structs. One μg of a plasmid expressing Renilla luciferase driven by the herpes simplex virus thymidine kinase promoter (pRL-TK-lucif-
erase)(Promega) was used as a control for the efficiency of transfection.

The cells were incubated with the DNA for 10 min at room temperature and subjected to electroporation at 250 V and 960 microfarads in a GenePulser (Bio-Rad). The cells were further incubated at room tem-
perature for 10 min and cultured in 10 ml of Iscove’s modified Dulbecco’s medium supplemented with 10% FBS. The cells were then analyzed
48 h post-transfection using the Dual-Luciferase® Reporter Assay Sys-
tem (Promega) on a TD-20/20 Luminometer (Turner BioSystems). Fire-
fly luciferase activities were normalized to the Renilla luciferase activities to correct for differences in transfection efficiency. The data shown in Fig. 6 represent the mean of 3–6 independent transfections.

RESULTS

Expressing Nup98–HOXA9 in K562 Myeloid Cells—To begin analyzing the effect of Nup98–HOXA9 on myeloid cells, HA-
tagged cDNA encoding Nup98–HOXA9 (26) was subcloned into a bicistronic retroviral vector, MSCV-IRES-GFP (39) (Fig. 1). In this vector the gene of interest and the GFP are separated by an internal ribosomal entry site (IRES) and are expressed from a single mRNA. Virus was produced by transient transfection of GP-293 cells with the MSCV constructs along with the pVSV-G plasmid (see “Experimental Procedures”). The myeloid cell line K562 (41) was transduced with either empty MSCV-
IRES-GFP retrovirus or retrovirus carrying Nup98–HOXA9 (see “Experimental Procedures”). After 48 h, the percentage of GFP-positive cells was generally in the 30–40% range (Fig. 2). GFP-positive cells were sorted using a Beckman Coulter Elite ESP cell sorter, and cell lysates were subjected to immunoblotting with anti-HA-tag antibodies to determine whether Nup98–
HOXA9 is expressed. K562 cells showed consistent high level expression of the transduced Nup98–HOXA9 gene (Fig. 2). Sim-
ilar analysis with other commonly used myeloid cell lines such as U937, HL60, and NB4 did not show significant expression of transduced Nup98–HOXA9 (data not shown). K562 cells were therefore used in all subsequent experiments.

Identification of Nup98–HOXA9 Target Genes in Myeloid Cells with Microarrays—Possible mechanisms by which Nup98–HOXA9 contributes to the pathogenesis of AML include inhibition of the nuclear import of transcription factors, inhibition of the nuclear export of RNAs, and/or aberrant transcription. To determine the global effect of Nup98–HOXA9 on gene expression in myeloid cells, K562 cells were transduced with either empty MSCV-IRES-GFP vector or with vector encoding Nup98–HOXA9 and sorted for GFP expression as described above. Cytoplasmic RNA was extracted from GFP-positive cells, and the two samples were submitted for comparative analysis using the Affymetrix HG-U133A array that contains ~21,000 probe sets. Cytoplasmic RNA was chosen because it would reveal a block of RNA export by Nup98–HOXA9 that could be missed if total RNA were used. Changes in expression level of 2-fold or more in the Nup98–HOXA9 sample compared with the empty vector control were considered significant. This experiment was repeated three independent times, and only those probes that showed reproducible significant change in all three experiments were considered to represent Nup98–
HOXA9 target genes. The results showed significant reproducible regula-
 tion by Nup98–HOXA9 of 102 genes represented by unique UniGene Cluster Identifiers (Table I).

Interestingly, of the 102 genes regulated by Nup98–HOXA9, 92 genes were up-regulated, whereas only 10 were down-regu-
lated (Table I). Thus, it appears that the predominant effect of Nup98–HOXA9 in myeloid cells is to induce gene expression. Because these results were obtained using cytoplasmic RNA, they argue against a significant inhibition of either transcription factor import or mRNA export from the nucleus by Nup98–
HOXA9. Instead, they are more consistent with a transcriptional activation function.

Validation of Microarray Data by Quantitative PCR—To confirm the data obtained with the microarrays, quantitative real time PCR was performed. Cytoplasmic RNA samples from K562 cells expressing Nup98–HOXA9 or empty vector controls were prepared as described above. The expression of 22 of the most highly induced genes and 2 of the down-regulated genes identified by microarray analysis was quantitated by real time PCR. The results of quantitative real-time PCR confirmed the microarray results for all but one of the genes tested (Table I). A few of the induced genes were also confirmed by traditional semi-quantitative PCR followed by agarose gel electrophoresis (Fig. 3).

HOXA9 Has a Limited Set of Target Genes Compared with Nup98–HOXA9—HOXA9 is a transcription factor of the homeobox family and is known to cause AML when overexpressed in mouse bone marrow cells (35). The AML induced by HOXA9 in mice differs from that induced by Nup98–HOXA9 in that the latter is preceded by a long myeloproliferative phase (35). This suggests that the two proteins might not target the exact same set of genes in myeloid cells. It was, therefore, important to compare the target genes of Nup98–HOXA9 to those of wild-
type HOXA9. Because the target genes of HOXA9 in myeloid cells are not known, we undertook a microarray study similar to that performed with Nup98 and Nup98–HOXA9. The open reading frame of wild-type human HOXA9 with an N-terminal HA tag was subcloned into MSCV-IRES-GFP (Fig. 1), and the vector was introduced into K562 cells by retroviral transduc-
tion. GFP-positive cells were purified and tested for HOXA9 expression by immunoblotting (Fig. 4). Cytoplasmic RNA was isolated and subjected to microarray analysis in triplicate as described above. Changes in expression levels of 2-fold or more in the HOXA9 sample compared with the empty vector control were considered significant. Only those genes that showed a change of 2-fold or more in all 3 experiments were considered to be regulated by wild-type HOXA9. By these criteria 13 genes were identified as HOXA9 targets (Table II). Of these, seven are definite targets of Nup98–HOXA9 as well, and another five show weak regulation by Nup98–HOXA9 in the same direction as HOXA9 (Table II). The term weak regulation is used here to refer to a change in gene expression that is observed in a majority of the readings from relevant probe sets but does not meet the strict criteria of at least a 2-fold change in all 3

![Fig. 1. Retroviral constructs. LTR, long terminal repeat; HD, homeodomain.](image)
triplicates for the same probe set. Only one of the HOXA9 target genes shows no change in response to Nup98-HOXA9 (Table II). On the other hand, the effects of HOXA9 on the 102 Nup98-HOXA9 target genes were as follows. Seven were definite targets of HOXA9 (Table II), 22 showed weak change in the same direction as with Nup98-HOXA9, 72 showed no change, and only one showed weak change in the opposite direction from that effected by Nup98-HOXA9 (data not shown). Taken together these data show that Nup98-HOXA9 and wild-type HOXA9 are primarily transcriptional activators, that they have similar effects on their shared target genes, and that Nup98-HOXA9 is a stronger transcriptional activator with substantially more target genes than HOXA9.

Wild-type Nup98 Has No Significant Effect on Gene Expression in Myeloid Cells—As discussed previously, there is evidence showing that the FG repeat portion of Nup98 can act as a transcriptional activation domain (26), a notion supported by our microarray analysis of Nup98-HOXA9 (Table I). On the other hand, a significant portion of wild-type Nup98 is normally present inside the nucleus (16–18), and its movement there is linked to transcriptional activity (16), raising the possibility that Nup98 itself might function as a transcription factor.

To determine whether the HOXA9 moiety is required for gene activation by Nup98-HOXA9 and to explore the possibility that wild-type Nup98 might activate transcription, we transduced K562 cells with wild-type Nup98 using the retroviral system described above. Expression of Nup98 was confirmed by immunoblotting (Fig. 5), and cytoplasmic RNA from Nup98 and control transfectants was subjected to microarray analysis as described above. The experiment was repeated twice from independent transductions. There were no genes reproducibly up- or down-regulated by Nup98 in K562 cells. This result shows that the HOXA9 DNA binding domain is required for regulation of gene expression by Nup98-HOXA9 and further strengthens the notion that Nup98-HOXA9 acts as a transcriptional activator that binds to DNA through the HOXA9 homeodomain.

Nup98-HOXA9 Regulates Gene Expression at the Transcriptional Level—Although the results described so far suggest transcriptional regulation by Nup98-HOXA9, changes in mRNA levels could be due to post-transcriptional mechanisms. To show that Nup98-HOXA9 can up-regulate gene expression at the transcriptional level, we used a construct that expresses the firefly luciferase reporter gene driven by the promoter of cyclooxygenase-1 (COX-1; PTGS1) (40), one of the genes up-regulated by Nup98-HOXA9 (Table I). K562 cells were transiently transfected by electroporation with this construct in the presence of empty pcDNA3 vector or pcDNA3 expressing either Nup98-HOXA9, HOXA9, or Nup98. A construct expressing Renilla luciferase driven by the thymidine kinase promoter was included in each transfection as a control for transfection efficiency. After 48 h, the cells were lysed, and firefly luciferase activity was measured and normalized to Renilla luciferase activity. As shown in Fig. 6, Nup98-HOXA9 increased transcription from the COX-1 promoter, whereas wild-type HOXA9 and Nup98 had no significant effect. This result shows that Nup98-HOXA9 induces gene transcription in myeloid cells and that the HOXA9 homeodomain is required for this effect. The lack of induction by HOXA9 is consistent with our microarray data showing that COX-1 is not a target gene for HOXA9 (Table II).

**DISCUSSION**

As discussed previously, Nup98-HOXA9 is a nuclear protein consisting of the FG repeat region of Nup98 fused to the DNA binding homeodomain of HOXA9 (26). Both moieties are required for the transforming effect of Nup98-homeodomain fusions in vitro and in mouse models of AML (26, 35, 42). Based on the functions of the Nup98 FG repeats and the HOXA9 DNA binding homeodomain, at least two general mechanisms for Nup98-HOXA9 leukemogenesis can be envisioned; that is, 1) interference with nuclear transport, e.g., preventing nuclear...
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Table 1: Genes regulated by Nup98-HOXA9

| Transcription factors | -Fold change | Unigene ID |
|-----------------------|--------------|------------|
| Hairy enhancer-related | 4.23         | Hs.234434  |
| B6 (HOXB6)            | 5.67         | Hs.98428   |
| Homebox C6 (HOXC6)    | 4.62         | Hs.820     |
| e-Myc promoter-binding | 2.87         | Hs.145631  |
| protein (IRLB)        | 3.06         | Hs.57597   |
| Distal-less homeo box | 2.46         | Hs.419     |
| 2 (DLX2)              | 3.11         | Hs.12702   |
| Modulator recognition | 2.58         | Hs.155718  |
| factor 2 (MRF2)       | 2.54         | Hs.273663  |
| Goliath protein (GP)  | 3.92         | Hs.114360  |
| Firin                  | 2.47         | Hs.78995   |
| B-cell translocation  | 2.31         | Hs.77054   |
| gene 1 (BTG1)         | -2.15        | Hs.75424   |
| Inhibitor of DNA binding 1 (ID1) | -2          | Hs.75424   |

Calcium binding/regulation

| C-type lectin, superfamily member 2 (CLECSF2) | 2.15 | Hs.85201 |
| Annexin A3 (ANXA3)                           | 3.12 | Hs.1378  |
| Annexin A4 (ANXA4)                           | 2.76 | Hs.77840 |
| Calbindin 1, 28kD (CALB1)                    | 9.67 | Hs.65425 |
| Phospholamban (PLN)                          | 5.12 | Hs.85050 |
| S100 calcium-binding protein A10 (S100A10)   | 13.25| Hs.119301|

Proteolysis

| Carboxypeptidase D (CDP)                      | 2.19 | Hs.5057  |
| Ceroid-lipofuscinosis, neuronal 2, late infantile (CLN2) | 2.41 | Hs.20478 |
| Proteasome subunit, β type, 9 (large multifunctional protease 2) (PSMB9) | 6.97 | Hs.92580 |
| Ubiquitin-conjugating enzyme E2L 6 (UBE2L6)  | 2.05 | Hs.169895|
| Mouse zinc finger protein 103 homolog (ZFP103) | 2.94 | Hs.155968|
| Neuroepithelin (SERPINI1)                    | 6.59 | Hs.78589 |

Extracellular matrix

| Epidermal growth factor containing fibrillin-like extracellular matrix protein 1 (EFEMP1) | 3.65 | Hs.76224 |
| Collagen, type XV, α1 (COL15A1)              | 2.59 | Hs.83164 |
| Collagen, type IV, α2 (COLAA2)               | 2.47 | Hs.75617 |
| Latent transforming growth factor β-binding protein 1 (LTBP1) | 2.86 | Hs.241257|
| Reselin (RRLN)                               | 2.47 | Hs.12246 |
| Fibronectin 1 (FN1)                          | -4.38| Hs.287820|

Erythroid/megakaryocytic markers

| Glycoprotein A (GYP A)                        | 2.76 | Hs.108894|
| Glycoprotein B (GYP B)                        | 2.99 | Hs.438658|
| Glycoprotein E (GYPE)                        | 3.09 | Hs.395535|
| Hemoglobin, β (HBB)                           | 2.83 | Hs.155376|
| Hemoglobin, δ (HBD)                          | 3.27 | Hs.36977 |
| Glycoprotein lb (platelet), β polypeptide (GP1BB) | 2.41 | Hs.283743|
| Erythroblast membrane-associated protein (ERMAP) | 2.20 | Hs.183923|
| Rhesus blood group, CcEe antigens (RHCE)     | 2.87 | Hs.278994|

Drug transport/metabolism

| ATP-binding cassette, sub-family A (ABC1), member 8 (ABCA8) | 4.38 | Hs.38995 |
| Cytochrome P450, subfamily IIA, polypeptide 5 (CYP2A5)     | 3.13 | Hs.72913 |
| Cytochrome P450, subfamily IIIA, polypeptide 5 (CYP3A5)    | 3.23 | Hs.104117|

Cytoskeleton/membrane traffic

| CDC42-binding protein kinase a (DMPK-like) (CDC42BPA)       | 2.05 | Hs.18586 |
| Microtubule-associated protein, RP/EB family, member 2 (MAPRE2) | 3.26 | Hs.78335 |
| Microtubule-associated protein, RP/EB family, member 3 (MAPRE3) | 3.60 | Hs.172740|
| Tropomodulin (TMOD)                                        | 2.19 | Hs.170453|
| Tropomyosin 1 α (TM1)                                       | 2.76 | Hs.77899 |
| Rab acceptor 1 (prenylated) (RABAC1)                       | 2.78 | Hs.11417 |
| Rabconnectin-3 (RC3)                                       | 3.18 | Hs.13264 |

Lipid metabolism

| Prostaglandin-endoperoxide synthase 1 (cyclooxygenase-1) (PTGS1) | 6.12 | Hs.88474 |
| SA hypertension-associated homolog (rat) (SAH)                 | 3.37 | Hs.181345|
| Sialyltransferase 9 (GM3 synthase) (STIA9)                      | 3.84 | Hs.225939|
| Phytanoyl-CoA hydroxylase (Refsum disease) (PHYH)              | 2.84 | Hs.172888|
| COI-58 protein (COI-58)                                       | 3.41 | Hs.198985|
| 3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1 (soluble) (HMGC1) | -4.07| Hs.77910 |
| Steroyl-CoA desaturase (delta-9-desaturase) (SCD)              | -3.81| Hs.119597|

Signaling

| Phosphodiesterase 3B, cGMP-inhibited (PDE3B)                  | 2.25 | Hs.337616 |
| Membrane-interacting protein of RGS16 (MIR16)                | 3.71 | Hs.107014 |
| Membrane-spanning 4 domains, subfamily A, member 4 (MS4A4A)  | 2.72 | Hs.329560 |
| Putative transmembrane protein (NMA)                         | 2.77 | Hs.78776 |
| G protein-coupled receptor 56 (GPR56)                        | 15.23| 54.47     |
| Adaptor protein containing pH domain, PTB domain and leucine zipper motif (APPL) | 2.31 | Hs.274183 |
| N-Acylphosphatidyl ethanolamine amidohydrolase (acid ceramidase) (ASAH) | 2.45 | Hs.75811 |
| BMX non-receptor tyrosine kinase (BMX)                        | 4.62 | Hs.27372 |

Each fold change number represents the mean of three independent microarray experiments. -Fold change was also estimated by real-time quantitative PCR (RT PCR) for a subset of the genes as indicated. Gene names are followed (in parentheses) by other common names and/or abbreviations.
Nup98-HOXA9 Induces Gene Transcription in Myeloid Cells

import of transcription factors critical for myeloid differentiation, or interference with the export of RNAs needed for myeloid differentiation, and 2) aberrant transcription. Consistent with this notion, Nup98-HOXA9 is capable of binding to a homeobox DNA recognition site (26). Because homeodomain proteins play important roles in both normal hematopoiesis and in leukemogenesis (28–31), an aberrant homeodomain-containing protein such as Nup98-HOXA9 may cause leukemia by interfering with the transcriptional programs of hematopoietic differentiation and proliferation.

Our microarray data show that of the 102 cytoplasmic mRNAs whose amount is significantly altered by Nup98-HOXA9, 92 are increased, and only 10 are decreased (Table I). These results argue against significant inhibition of nuclear import of transcription factors or of mRNA export to the cytoplasm. Instead, the results are most consistent with a transcriptional activation function for Nup98-HOXA9. Consistent with this notion, Nup98-HOXA9 activated transcription from the COX-1 promoter in myeloid cells (Fig. 6). The fact that wild-type Nup98 had no significant effect on gene expression or transcription in myeloid cells further indicates that the effect of Nup98-HOXA9 in myeloid cells is mediated by binding to DNA through the HOXA9 homeodomain. However, these results were obtained in the absence of differentiating stimuli, and it is, therefore, still possible that Nup98-HOXA9 may exert a significant inhibitory effect on the genes induced during myeloid differentiation. The K562 myeloid cell line used for these studies is derived from a patient with chronic myelogenous leukemia and tends to differentiate along the erythroid and megakaryocytic lineages, although monocytic differentiation has also been reported (43). It would be of interest to study the effect of Nup98-HOXA9 on gene expression under differentiating conditions.

### Nup98-HOXA9 and Malignant Transformation

Several of the genes induced by Nup98-HOXA9 are associated with increased cell proliferation, malignant transformation, increased tumorigenic potential, and inhibition of apoptosis. The following are some of the more prominent examples.

**COX-1**—One of two isoforms of cyclooxygenase which catalyzes a step in prostaglandin synthesis (44). Increased COX-2 levels are associated with colorectal and mammary tumors among others, and COX inhibitors decrease the incidence of colorectal and other tumors (44–46). It is possible that COX-1 plays a role in myeloid tumorigenesis similar to the role that COX-2 plays in the induction of adenocarcinoma.

**Granulin**—In cell lines granulin acts as a mitogenic growth factor (47, 48), and its antisense cDNA inhibits tumorigenicity (49, 50). It is preferentially overexpressed in malignant tumors of the brain, stomach, ovary, and kidney (51–55) and is involved in the wound response as a growth factor (56).

### Table I—continued

| Unigene ID | Fold change | Array | RT-PCR |
|------------|-------------|-------|--------|
| Hs.70434   | 2.54        | 5.23  |        |
| Hs.286124  | 2.58        |       |        |
| Hs.150402  | 2.53        |       |        |
| Hs.12956   | 2.05        |       |        |
| Hs.298323  | 2.86        |       |        |
| Hs.180577  | 2.24        |       |        |
| Hs.35       | −2.34       |       |        |
| Hs.95327   | −2.89       |       |        |
| Hs.99960   | 4.76        | 2.25  |        |
| Hs.106070  | 2.31        |       |        |
| Hs.4854    | 2.25        |       |        |
| Hs.75586   | −2.30       |       |        |
| Hs.180566  | 3.12        | 1.83  |        |
| Hs.184542  | 2.75        |       |        |
| Hs.14125   | 2.20        |       |        |
| Hs.110386  | 2.61        |       |        |
| Hs.50985   | 2.84        |       |        |
| Hs.301763  | 2.60        |       |        |
| Hs.324784  | 2.47        |       |        |
| Hs.50550   | 26.33       | 39.04 |        |
| Hs.22915   | 3.26        |       |        |
| Hs.179526  | 2.31        |       |        |
| Hs.79103   | 12.12       | −1.18 |        |
| Hs.71869   | 2.59        |       |        |
| Hs.10235   | 4.14        | 1.15  |        |
| Hs.15671   | 2.51        |       |        |
| Hs.7594    | −2.56       |       |        |
| Hs.317432  | −2.54       |       |        |
| Hs.286049  | −2.30       | −2.44 |        |
| Hs.20981   | 3.50        |       |        |
| Hs.71968   | 2.35        |       |        |
| Hs.301658  | 4.76        | 3.3   |        |
| Hs.7314    | 2.25        |       |        |
| Hs.61273   | 2.36        |       |        |
| Hs.419780  | 2.41        |       |        |
| Hs.118174  | 2.55        |       |        |
| Hs.35660   | 2.36        |       |        |
| Hs.118978  | 2.82        |       |        |
transforming growth factor-β and bone morphogenetic proteins are associated with reduced proliferation and differentiation of myeloid cells (60–62), the induction of NMA by Nup98-HOXA9 may contribute to leukemogenesis by enhancing proliferation and inhibiting differentiation.

**BMX Non-receptor Tyrosine Kinase (Btk)**—A non-receptor tyrosine kinase of the Btk family, BMX is thought to be involved in hematopoietic cell growth and differentiation (63). It is expressed in the granulo-monocytic lineage including progenitors and mature cells as well as in acute and chronic myeloid leukemias (64, 65). BMX mediates Src-induced cell transformation through signal transducer and activator of transcription (STAT) 3 activation (66). It promotes cell proliferation and tumorigenicity and inhibits apoptosis (67–69). It induces vascular endothelial growth factor expression and angiogenesis (68, 70), which are known to be increased in AML (71).

**Serum and Glucocorticoid-Inducible Kinase**—Serum and glucocorticoid-inducible kinase is a serine-threonine kinase transcriptionally induced by serum and glucocorticoids (72, 73) that mediates cell growth and survival (74–82). It is overexpressed in hepatocellular carcinoma and tumorigenic cell lines (83, 84) and is induced by granulocyte-macrophage colony-stimulating factor in human granulocytes (85).

**Phosphodiesterase 3B**—Phosphodiesterase 3B is a cyclic AMP phosphodiesterase that mediates the antiapoptotic functions of insulin-like growth factor 1 (86). It is activated by protein kinase B (Akt) in response to insulin-like growth factor 1 in the myeloid cell line FDCP2, resulting in increased thymidine incorporation (87). Its inhibition augments apoptosis in chronic lymphocytic leukemia cells (88).

**HOXB6**—A HOX transcription factor expressed during early myelopoiesis and suppressed in later stages of myelopoiesis (89–91). It also functions in erythropoiesis (92, 93). Overexpression of HOXB6 in myeloid cell lines inhibits their myeloid differentiation (89). HOXB6 is expressed in many cases of AML, particularly those with worse prognosis and those that lack the common chromosomal rearrangements (89, 94).

**HOXC6**—HOXC6 is expressed in a large fraction of AML cases but not in mature neutrophils or monocytes (95). It is also expressed in lymphoid cells during maturation and in a variety of lymphoid neoplasm (96–98). HOXC6 is consistently overexpresed in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) (96, 97). Its expression is not observed in normal lymphoid cells or in cases lacking common chromosomal rearrangements (98).

**TABLE II Genes regulated by HOXA9**

Each-fold change number represents the mean of three independent microarray experiments. Only genes regulated 2-fold or more by HOXA9 in all three experiments are included. Of these seven are regulated 2-fold or more by Nup98-HOXA9 as well, as indicated by the numbers under Nup98-HOXA9. The remainder show either weak induction (WI), weak repression (WR), or no change (NC) in response to Nup98-HOXA9 as indicated. WT, wild-type.

| Gene name       | -Fold change | Unigene ID |
|-----------------|--------------|------------|
| CD10           | 19.04        | HS.1298    |
| Calbindin 1.28 kD (CALB1) | 4.62        | HS.65452   |
| HEY1           | 4.01         | HS.234434  |
| Secretogranin III (SCG3) | 3.04        | HS.22215   |
| SAH            | 2.58         | HS.181345  |
| ASC            | 3.19         | HS.71869   |
| PSMB9          | 2.81         | HS.9280    |
| EBV-induced gene 2 (EBI2) | 4.00        | HS.784     |
| Ankyrin 3 (ANK3) | 2.47        | HS.75882   |
| BMX            | 2.42         | HS.27372   |
| KIAA0342 gene product | 2.65        | HS.16950   |
| Hypothetical protein | 2.94        | HS.277431  |
| FLJ23356       | –2.21        | HS.276770  |
pressed in T or B acute lymphoblastic leukemia associated with mixed-lineage leukemia gene rearrangements (99).

**MEF2C—**MEF2C is a transcription factor of the MEF2 (myocyte enhancer factor) family involved in central nervous system and muscle development (100, 101). Also important for angiogenesis and vascular endothelial growth factor expression (102). Its activity is induced by lipopolysaccharide in monococytes through p38-mediated phosphorylation, with a resulting increase in c-jun gene transcription (103). MEF2C can activate c-jun transcription in response to G protein-coupled receptor stimulation (104). Epidermal growth factor leads to MEF2C activation via ERK5 (B MK1) in the early response to serum stimulation (105, 106). MEF2C mediates inhibition of apoptosis by p38 during neuronal differentiation (107). MEF2C inhibition by caspases in neurons results in apoptosis (108). Thus, MEF2C participates in both anti-apoptotic and mitogenic pathways.

**HEY1—HEY1** is one of a family of basic helix-loop-helix putative transcription factors involved in mouse embryogenesis (109–111). It is a target of the Notch signaling pathway and is up-regulated by constitutively active forms of Notch (112, 113). The Notch pathway is involved in hematopoiesis, where it can increase stem cell expansion and survival and either inhibit or promote myeloid differentiation (114, 115).

**MALT1**—**MALT1** is a B cell oncogene involved in at least two chromosomal rearrangements in MALT lymphoma; one results in an API2-MALT1 fusion transcript (116–118), and the other results in juxtaposition of MALT1 to the IgH locus, resulting in overexpression of MALT1 (119, 120). In addition, amplification of the MALT1 gene associated with MALT1 overexpression is seen in some B cell lymphomas and B cell lymphoma cell lines (120). MALT1 belongs to a caspase-like family of proteins called paracaspases and cooperates with Bcl10 to activate NFκB, whereas API2-MALT1 can activate NFκB on its own (121–123). NFκB activation or gene rearrangement is seen in a large number of human malignancies including leukemias and lymphomas (124).

**Nup98-HOXA9 and Cell Cycle Regulation**

It is intriguing that Nup98-HOXA9 induces two cyclin-dependent kinase inhibitors, p18(Ink4c) and p57(KIP2), and down-regulates cyclin D2 (Table II). This may seem paradoxical in view of the ability of Nup98-HOXA9 to induce leukemic transformation. However, the relationship between differentiation, proliferation, malignant transformation, and the cell cycle is not always straightforward. For example, cyclin D2 can be induced by signals that inhibit macrophage proliferation (125) and is repressed in pancreatic neoplasms and pancreatic carcinoma cell lines (126). Furthermore, some tumors, such as hepatoblastoma, pancreatic carcinoma, and colorectal carcinoma, show a higher level of p57(KIP2) than their corresponding normal tissues (127–129). In addition, p18(Ink4c) is increased in AML and in CD34+ progenitors but not in normal differentiated myeloid cells (130). Finally, it has been shown that AML1-ETO, the most common leukemogenic fusion protein in AML, inhibits growth and induces apoptosis while also inhibiting differentiation when expressed in a myeloid cell line (131). It remains to be seen whether changes in gene expression that appear to favor decreased proliferation are part of the leukemogenic process induced by Nup98-HOXA9 or whether they represent negative feedback mechanisms that attempt to counteract the growth stimulating changes outlined above.

**Nup98-HOXA9 and Drug Metabolism**

Of the genes implicated in drug metabolism that are induced by Nup98-HOXA9, CYP3A5, which belongs to the cytochrome P450 family, is of particular interest. The CYP3A subfamily is the major drug-metabolizing family in humans, and CYP3A5 is the major extrahaemopoeitic CYP3A enzyme seen among others in leukocytes (132–134). Nup98 gene rearrangements are associated with therapy-related AML, particularly after treatment by epipodophyllotoxins such as etoposide, which induce DNA strand breaks by inhibiting topoisomerase II. These drugs are metabolized by CYP3A4 and CYP3A5 to catechol derivatives through O-demethylation (135), a pathway that can result in the formation of DNA damaging metabolites (136, 137). Indeed, a mutation in the promoter region of CYP3A4 is associated with a lower incidence of therapy-related leukemia after epipodophyllotoxin treatment than wild-type CYP3A4 (138). These findings led Felix et al. (138) to suggest that wild-type CYP3A4 activity may result in more conversion of epipodophyllotoxins to genotoxic metabolites that in turn increase the incidence of therapy-related leukemia. It is of interest in this regard to note that Nup98 gene rearrangements may be more common in Asian populations (3). This could be explained by the fact that the wild-type variant of CYP3A5 is present only in about 10% of Caucasians but is at least three times as common in Asian populations (139–142). A possible model for therapy-related leukemia with a Nup98-HOXA9 gene rearrangement that emerges from these considerations is that the epipodophyllotoxin-induced DNA strand breaks result in a Nup98-HOXA9 fusion that induces the CYP3A5 enzyme, which then generates further genotoxic metabolites, resulting in further mutations and a full-blown leukemogenic phenotype.

**Nup98-HOXA9 Versus Wild-type HOXA9**

Our microarray analysis shows that Nup98-HOXA9 has about eight times as many target genes as wild-type HOXA9. Most HOXA9 target genes are regulated by Nup98-HOXA9, but the reverse is not true. Thus, there are a substantial number of genes that are regulated only by Nup98-HOXA9. As previously discussed, both Nup98-HOXA9 and wild-type HOXA9 can induce AML in mice, but only Nup98-HOXA9 induces myeloproliferation before the development of AML. Thus, genes that are regulated by both proteins, such as BMX and HEY1, may be important for the development of AML, whereas genes uniquely regulated by Nup98-HOXA9 may play a role in inducing myeloproliferation.

In summary, our data provide strong evidence that Nup98-HOXA9 acts as an aberrant transcription factor in myeloid cells, with a wider and stronger transcriptional effect than wild-type HOXA9. We have identified 102 Nup98-HOXA9 target genes that shed light on the etiology and mechanisms of Nup98-HOXA9-induced AML. Future studies will be aimed at identifying the cognate DNA-binding site for Nup98-HOXA9, identifying its direct target genes, and determining its effect on gene expression during myeloid differentiation.

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