Tumor and Stem Cell Biology

Nm23-H1 Indirectly Promotes the Survival of Acute Myeloid Leukemia Blast Cells by Binding to More Mature Components of the Leukemic Clone

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Abstract

Nm23-H1 plays complex roles in the development of diverse cancers including breast carcinoma, high-grade lymphomas, and acute myeloid leukemia (AML). In the case of AML and lymphomas, serum Nm23-H1 protein is elevated with the highest levels correlating with poorest prognosis. A recent study identified that this association is most likely causal in AML and that Nm23-H1 acts as an AML cell survival factor. In this study, we report heterogeneity in the ability of AML samples to bind and respond to Nm23-H1, and we offer evidence that binding is essential for improved survival. Further, we show that the subset of AMLs that bind Nm23-H1 do not do so through the putative Nm23-H1 receptor MUC1. Although rNm23-H1 promoted the survival of the most primitive blasts within responding AMLs, it was not these cells that actually bound the protein. Instead, rNm23-H1 bound to more mature CD34+/CD34– and CD11b+ cells, revealing an indirect survival benefit of Nm23-H1 on primitive blasts. In support of this finding, the survival of purified blast cells was enhanced by medium conditioned by more mature cells from the clone that had been stimulated by rNm23-H1. Levels of interleukin 1-β (IL1β) and IL6 in rNm23-H1 conditioned medium mirrored the potency of the conditioned media to promote blast cell survival. Furthermore, Nm23-H1 expression was significantly associated with IL1β and IL6 expression in primary uncultured AML samples. These findings have implications for the role of Nm23-H1 in AML and its use as a prognostic marker. Additionally, they offer the first evidence of novel cross-talk between cell populations within the tumor clone. Cancer Res; 71(3); 1-10. ©2010 AACR.

Introduction

A murine Nm23 gene identified by Steeg and colleagues (1) was the first metastasis suppressor gene discovered. The authors showed that high levels of Nm23 mRNA correlated with low metastatic potential, whereas highly metastatic cells expressed Nm23 mRNA at a lower level (1). Subsequently, 10 human Nm23 genes have been identified, which all share functionally diverse isoforms of a nucleoside diphosphate kinase domain (2). The Nm23-H1 and -H2 genes have been extensively studied in human breast carcinomas, melanomas, and ovarian cancers, where the general trend is an inverse relationship between Nm23 expression and metastatic stage (3). This has been supported by transfection experiments in breast cancer MDA cell lines, where overexpression of Nm23-H1 and -H2 genes resulted in decreased metastatic potential (4-6).

In contrast, elevated Nm23-H1 expression is related to a more aggressive disease in neuroblastomas and many hematologic malignancies. In acute myeloid leukemia (AML) and high-grade lymphoma, Nm23-H1 and -H2 are often overexpressed and correlate with patient white cell count, and decreased overall patient survival (7-9). Nm23-H1 and -H2 are highly expressed in normal CD34+ hemopoietic progenitors, but are downregulated during normal hemopoietic maturation (10). Similarly, studies in cell lines have shown that Nm23-H1 expression decreases during induced differentiation (11). This suggests an important role for Nm23-H1 and -H2 in controlling hemopoietic differentiation and leukemic progression.

Extracellular Nm23-H1 has been reported to control differentiation in later stages of normal hemopoiesis and inhibit the induced differentiation of myeloid cell lines (12-15). These observations are significant because Nm23-H1 levels are elevated in the serum of AML patients, where levels can reach up to 200 ng/mL levels 25-50 times those found in normal individuals (16). Furthermore, patients with high levels of

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serum Nm23-H1 have significantly lower survival rates than those with low serum levels (16). Recently it has been shown that recombinant Nm23-H1 (rNm23-H1) supports the growth and survival of primary AML cells, indicating a role for extracellular Nm23-H1 in exacerbating the tumor burden (17). The authors showed that the prosurvival activity of Nm23-H1 was associated with activation of p38 mitogen activated protein kinase (MAPK) and STAT pathways, and the release of a number of supportive cytokines including granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 1β (IL1β), and IL6.

The mechanisms by which extracellular Nm23 proteins activate the survival pathways remains unclear. In one study in non-Hodgkin's lymphoma (NHL), more than 20% of cells were positive for Nm23-H1 cell surface expression in more than 25% of cases (18). The authors went on to show that NHL patients with high Nm23-H1 cell surface expression had significantly reduced survival rates. Weak Nm23-H1 surface expression has been reported on some myeloid and erythroid cell lines (10, 19) whereas to date no surface Nm23-H1 expression has been detected on normal peripheral blood cells (10). These data suggest that extracellular Nm23 proteins may provide an advantageous autocrine signal to malignant cells by binding cell surface receptors; however, Nm23-H1 cell surface binding on primary AML cells has not been investigated in detail.

Recent evidence suggests that Nm23-H1 can bind a cleaved form of Mucin1 called MUC1*, which is present on the surface of many cancer cell lines and on pluripotent stem cells (20, 21). The binding of Nm23-H1 to MUC1* was reported to result in dimerization of MUC1*, and subsequent activation of the MAPK pathway to increase proliferation of the breast cancer cell line T47D (20). Expression of MUC1* in the hematologic system and AML is poorly understood.

This study investigated the binding of rNm23-H1 to AML cells, and how binding relates to both their in vitro survival, and the expression of MUC1*. In particular, the effects of Nm23-H1 on more immature and mature components of the leukemic clone was studied. Importantly, we show that not all AMLs bind Nm23-H1. Surprisingly, in the binding cohort, Nm23-H1 only bound a more mature subpopulation within the clone, causing an indirect signal for the survival of the primitive blasts cells.

Materials and Methods

Recombinant Nm23-H1

The Nm23-H1 protein coding region was cloned into the pET-15b 6×His-tag expression vector, and transformed into Escherichia coli BL21 (DE3) cells. Bacterial cultures grown to an OD600 of 0.6 were induced to produce recombinant Nm23-H1 with 1 mmol/L isopropyl β-D-1-thiogalactopyranoside for 4 hours. Recombinant Nm23-H1 protein was purified from bacterial lysates using the Bugbuster NI-NTA His*Bind Purification kit (Novagen) according to manufacturer’s instructions. The purity of Nm23-H1 protein was assessed by coomassie stain and Western blotting of SDS-PAGE gels.

Cell culture

K562 and NB4 cells were obtained directly from the ATCC cell bank, which perform cell line characterization using short tandem repeat profiling, karyotyping, and by checking cell morphology. The cell lines were resuscitated, stored in multiple vials, and cultured for a maximum of 4 months. T47D cells were a kind gift from Dr. Moray Campbell (Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY), who acquired them directly from ATCC and were tested and treated as described earlier. Cells were maintained in a humidified atmosphere of 5% CO2 in RPMI 1640 medium with 2 mmol/L glutamine, supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS (Gibco, Invitrogen Ltd.).

Primary AML mononuclear cells were isolated using Ficoll Paque-Plus (Amersham) from presentation aspirates and peripheral blood samples provided after informed consent and ethical committee approval from ongoing phase I/II trials currently undertaken within the University of Birmingham Hospitals NHS Trust and from the Medical Research Council (MRC) AML 15 phase III trial. AML cells were cultured at 1×10⁶ cells/mL in RPMI 1640 medium with 2 mmol/L glutamine (Gibco), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 1% serum replacement ITS+ (Becton Dickinson). Where stated, cultures were supplemented with Polymyxin B (Sigma) at a previously optimized concentration of 1.25 µg/mL (22), to inhibit contaminating lipopolysaccharides (LPS) in the rNm23-H1 protein preparations.

Nm23-H1 AML cell survival assay

Primary AML cell cultures were seeded at 2×10⁵ cells/well in 96-well plate in RPMI 1640 ITS+ medium supplemented with rNm23-H1 protein at a final concentration of 2 µg/mL, or elution buffer control diluted to the same extent. Cells were incubated for 5 days before analyzing survival by viable cell counts. The Nm23-H1 survival index was calculated as the number of viable cells treated with Nm23-H1 divided by the number of cells treated with elution buffer control.

Flow cytometric analysis of rNm23-H1 binding to AML cells

A modified version of the method developed by Okabe-Kado and colleagues (19) was used. AML cells (1×10⁶) were washed in 1 mL cold PBS. Cells were resuspended in 100 µL PBS containing 1% bovine serum albumin (BSA) and either 1% mouse anti-Nm23-H1 antibody (GeneTEX) or its isotype control, and incubated at 4°C for 20 minutes. Cells were washed with 1 mL cold PBS and resuspended in 100 µL PBS containing 1% BSA and 1% anti-mouse immunoglobulin G fluorescein isothiocyanate (FITC)-conjugated antibody (Jackson Immunoresearch), and incubated at 4°C for 20 minutes. To assess surface Nm23-H1 binding to subpopulations, cells were resuspended in 100 µL PBS containing 1% BSA and 1% anti-CD34 aliphycocyanin (APC) and CD11b phycoerythrin (PE) antibodies (Becton Dickinson) and incubated at 4°C for 20 minutes. The cells were washed in cold PBS and resuspended in 200 µL FACS fix [2% (v/v) FBS, 1% (v/v) formaldehyde in PBS].
Western blot analysis

Protein was extracted from cell pellets (1×10⁶ cells) by boiling in 1×SDS gel loading buffer [3.7 mL dH₂O, 15.625 mmol/L Tris-HCl pH6.8, 10% (v/v) glycerol, 0.5% (v/v) SDS, 0.125 mL 2-βME, bromophenol blue]. Lysates were resolved on 7.5% or 15% acrylamide SDS-PAGE gels, before transfer onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 1 hour in 5% (w/v) nonfat milk powder (Marvel) in Tris-buffered saline Tween-20 (TBS-T) and probed overnight in 5% (w/v) nonfat milk powder in TBS-T with anti-MUC1 antibodies, Ab-5 (anti-hamster, 1:1,000; Lab Vision), and VU4H5 (anti-mouse, 1:500; Santa Cruz). Membranes were washed 3 times with TBS-T and probed with horseradish peroxidase conjugated anti-hamster (Sigma) or anti-mouse (Sigma) secondary antibodies diluted 1:1,000 in 5% (w/v) nonfat milk powder in TBS-T. The membranes were washed 3 times before detection with Supersignal West Pico Chemiluminescent substrate (Pierce). Equal loading was checked using an anti-β-actin antibody (1:25,000; Sigma) for 45 minutes, and an anti-mouse secondary (1:25,000; Sigma) for 45 minutes and was developed as described earlier.

CD34/CD117 cell sorting

Mononuclear cells isolated from AML/RAEB2 patients were sorted into immature (CD34+ and/or CD117+) and more mature (CD34+ and/or CD117+) fractions using CD34 or CD117 MACs sorting kits (Miltenyi) according to manufacturer’s instructions. CD34+/CD117+ cells were stored at 4°C overnight in RPMI 1640 ITS+ medium, whereas the CD34+/CD117+ cells were seeded at 5×10⁵ cells/well in a 48-well plate in RPMI 1640 ITS+ medium supplemented with either 2 μg/mL rNm23-H1 or elution buffer control. Cells were cultured for 20 hours, after which the resulting conditioned medium (CM) was removed.

Nm23-H1 depletion from CD34+/CD117+ conditioned medium

Nickel resin (Sigma; 100 μL/mL of CM), was washed in dH₂O, followed by a wash with equilibration buffer (50 mmol/L sodium phosphate pH 8, 0.3 mol/L sodium chloride, 10 mmol/L imidazole), and then 1 mL of CD34+/CD117+ CM was added. The CM and resin were mixed at 4°C for 20 minutes to remove rNm23-H1. The CM/resin was centrifuged at 14,000 rpm for 15 seconds and CM was removed, followed by a longer centrifugation step of 2 minutes to remove any residual nickel resin from the CM. rNm23-H1 depletion from the CM was checked by Western blot.

Culture of CD34+/CD117+ blast cells in CM

The previously stored CD34+/CD117+ cells were resuspended at 1×10⁶ per mL in the CD34+/CD117+ CM, and seeded at 2×10⁵ cells/well in a 96-well plate. Cell survival was analyzed by viable cell counts after 2 days in culture.

Cytokine analysis of the CD34+/CD117+ cell CM

CM from the CD34+/CD117+ cells, stimulated with rNm23-H1 or elution buffer control was analyzed for cytokine release using a Bio-Plex Pro cytokine assay according to the manufacturer’s instructions (Bio-Rad).

Culture of CD34+ blast cells with IL1β and IL6

The previously stored CD34+ cells were seeded at 2×10⁵ per well in a 96-well plate in RPMI 1640 ITS+ medium supplemented with 2 μg/mL Nm23-H1, 2.5 ng/mL IL1β, and/or 5 ng/mL IL6. Cell survival was analyzed by viable cell counts after 2 days in culture.

Quantitative real-time PCR analysis

Mucin1 mRNA expression was analyzed by quantitative real-time PCR (QRT-PCR) in T47D, K562, NB4, and primary AML cells. Gene-specific primers (Sigma Genosys) and TaqMan probes (Eurogentec) were designed using Primer Express ABI PRISM software (Applied Biosystems). The sequences of the primers and probes were as follows:

**Mucin1:** forward primer 5’-TGCCATGTCTATCTCATTGCGCCAAGTTTGC-3’; reverse primer 5’-CAGCTGCGGCTTTTCTCCTC-3’; probe 5’-6-FAM-TCGCTGTCTCAGTGGCCG-TAMRA-3’.

QRT-PCR reactions (20 μL) were set up in triplicate containing: 1×qPCR master mix (Eurogentec), 20 to 40 ng cDNA, 18 pmol of each primer, and 2.5 pmol of probe. QRT-PCR was performed using standard cycle conditions on an ABI PRISM 7000 sequence detector (Applied Biosystems).

**IL1β** and **IL6** mRNA expression was investigated in high (uppermost 20% and low (lowermost 20%) Nm23-H1–expressing AMLs out of 100 AML samples. Preoptimized Quantitect IL1β and IL6 primers (Qiagen) were used in a standard SYBR green QRT-PCR assay (Quantace). QRT-PCR reactions (25 μL) were set up in duplicate containing: 1×Sensmix, 20 to 40 ng cDNA, 9 pmol each primer, 1×SYBR-Green solution, 4 mmol/L MgCl₂ and 0.5 units uracil n-glycosylase. The IL1β and Nm23-H1 relationship was investigated in more detail by SYBR green QRT-PCR in all the 100 AML samples.

**Results**

**Exogenous rNm23-H1 supports the survival of AML cells**

Recent data suggest that extracellular Nm23-H1 acts to support AML cell survival (17). To further examine the role of extracellular Nm23-H1 in AML, primary AML cells from 18 patients were cultured in serum-free conditions with and without 2 μg/mL rNm23-H1 and cell survival analyzed at day 5. rNm23-H1 enhanced overall AML cell survival compared with elution buffer control (P < 0.001; Fig. 1A). However, the responses were variable with potent survival effects on some samples and little effect on others. In 3 samples, the response resulted in a modest increase in total cell number over 5 days. In one of these samples, enough cells were available to perform rNm23-H1 dose titration experiments that showed that the effect of rNm23-H1 was significant and near maximal at 0.25 μg/mL (Fig. 1B). Flow cytometry confirmed that leukemic blasts, as defined by CD34/CD117 positivity, were protected by rNm23-H1 (Fig. 1C). These...
Nm23-H1 was near maximal at 250 ng/mL (Fig. 2A). In 8 of the 9 Nm23-H1–binding AMLs, the survival response to rNm23-H1 was greater than 1.5-fold with a mean survival index across all 9 of 1.78 ± 0.17 (Fig. 2B). However, rNm23-H1 had little effect on AMLs that did not bind Nm23-H1 at the cell surface (mean survival index 1.15 ± 0.08). The rNm23-H1–mediated increase in cell survival among binders compared with nonbinders was significant (P = 0.004).

Mucin1 and MUC1* expression in AML

The above data reveal that not all AMLs bind either endogenous Nm23-H1 or exogenously added rNm23-H1 and that the survival activity exerted by extracellular Nm23-H1 requires surface binding of the protein. Recently it has been reported that MUC1*, a truncated enzymatically cleaved form of Mucin1, is a receptor for Nm23-H1 (20). To investigate the possibility that MUC1* was mediating the prosurvival effects of Nm23-H1 on AML cells, MUC1* and Mucin1 expression was investigated in AML. In Western blotting experiments, neither Mucin1 nor MUC1* was detected in Nm23-H1 binding or nonbinding primary AML samples or in the AML cell line NB4. In contrast, MUC1* was readily observed in the chronic myeloid leukemia–derived line K562 and, as previously described (20), both Mucin1 and MUC1* were readily detected in the breast cancer cell line T47D (Fig. 3A). QRT-PCR analyses confirmed very low or absent expression of Mucin1 and MUC1* transcripts in both primary AMLs (n = 6) and NB4 cells when compared with T47D and K562 cells (Fig. 3B).

rNm23-H1 preferentially binds a population of more mature CD34+/CD11b+ cells within the AML clone

To identify the population of AML cells binding Nm23-H1, cell surface Nm23-H1 binding was studied by flow cytometry in combination with expression of myeloid markers CD34 and CD11b. Figure 4 reveals that both endogenous and recombinant Nm23-H1 preferentially bound populations of CD34+/CD11b+ cells rather than the more immature and more strongly CD34+ cells. The forward and side scatter plots show that these cells are larger and more granular than the AML blasts, indicative of a more mature phenotype. However, despite rNm23-H1 binding a discrete subpopulation of mature cells, the survival of both the immature and more mature cell populations was enhanced by the addition of rNm23-H1 in culture (Fig. 4).

Nm23-H1 supports the indirect survival of immature AML blast cells

The observation that Nm23-H1 was binding a population of CD34+/CD11b+ cells while enhancing the survival of more immature CD34+ cells indicated an indirect survival effect on the more primitive members of the malignant clone. To investigate this further, cells from 5 AML patients were sorted into immature CD34+ and more mature CD34lo fractions. In addition, an RAEB2 patient sample was sorted into more
immature and mature fractions using sorting for CD117+ immature cells. The more mature CD34−/CD0 (AML) and CD117−/CD0 (RAEB2) fractions were cultured in the presence or absence of 2 μg/mL rNm23-H1 for 20 hours, after which the resulting CM was harvested and depleted of rNm23-H1 using nickel resin (Fig. 5A). The CM from elution buffer control-treated cells was mock depleted of rNm23-H1 in the same way as the CM from the rNm23-H1−treated cells. Subsequently, the immature CD34+ or CD117+ cells, which had been stored at 4°C overnight, were cultured in these CM and cell survival analyzed after 2 days in culture. In 5 of 6 cases, the survival of immature CD34+/CD117+ blast cells was enhanced by CM from rNm23-H1−stimulated CD34−/CD117+ cells, but not by CM from elution buffer−treated CD34+/CD117+ cells (Fig. 5C). Notably, in the sixth case, where no survival advantage was observed, we failed to detect binding of rNm23-H1 to the CD34low− cells. Further, incubation of purified immature CD34+/CD17+ AML blasts with rNm23-H1 alone did not provide a survival advantage (data not shown). A possible explanation for these findings would be a survival effect triggered by the contamination of our rNm23-H1 with LPS derived from the bacteria in which the protein was generated. However, this was not the case because the addition of the LPS inhibitor polymyxin B to CD34−/CD117− cell culture had no significant effect on rNm23-H1−mediated indirect CD34+/CD117− cell survival (Supplementary Fig. 1).

rNm23-H1−induced release of IL1β and IL6 from CD34+/CD117− cells promotes the survival of immature CD34+/CD117− blast cells

The above observations strongly indicate that rNm23-H1 promoted the survival of AML blasts via binding to more

Figure 2. Nm23-H1 supports the survival of AMLs that bind Nm23-H1 at the cell surface. AML cells were plated out at 1 × 10⁶ cells/mL in RPMI 1840 ITS+ medium, with rNm23-H1 or elution buffer only. A, after 5 days in culture, Nm23-H1 cell surface binding was measured using an anti-Nm23-H1 antibody and flow cytometry. Representative histograms of an Nm23-H1 nonbinding and binding AML (patient 4 and 2, respectively, Supplementary Table 1) are shown. Isotype control, solid gray histograms; elution buffer−treated cells, gray line histograms; rNm23-H1−treated cells, black line histograms. B, the survival index was calculated by dividing the number of viable rNm23-H1−treated cells by the number of viable elution buffer−treated cells after 5 days in culture. The difference in survival index in response to rNm23-H1 was compared between binding (n = 9) and nonbinding AMLs (n = 7; patients 1–16, Supplementary Table 1); P = 0.004 (Mann−Whitney U test).

Figure 3. Mucin1 and MUC1* expression in AML. A, Mucin1 and MUC1* protein expression in T47D, K562, NB4, and primary AML cells by Western blot analysis. Membranes were probed for full-length Mucin1 (VU4H5), MUC1* (Ab5), and β-actin as a loading control. The ability of the AMLs to bind Nm23-H1 is indicated by +−−−; ND, not done. B, Mucin1 mRNA expression in T47D, NB4, and primary AML cells relative to expression in K562 cells was measured by QRT-PCR. Mean AML Mucin1 expression was calculated from 6 independent AML samples (patients 2–4, 6, 18, and 19, Supplementary Table 1) as 6.5% ± 2.1 relative to expression in K562 cells.
mature cells in the clone, resulting in the release of secondary survival signals that then act on the immature AML blasts. To further investigate the possible nature of the secondary survival signals we used a Luminex assay to simultaneously measure the levels of 28 cytokines (Supplementary Table 1).

Figure 5. Nm23-H1 supports the indirect survival of immature AML blast cells. A, to test the CM of CD34+/CD117− cells following exposure to Nm23-H1 it was necessary to efficiently deplete the protein from the CM. A representative Western blot of CD34+/CD117− CM pre- and post- rNm23-H1 depletion is shown (patient 21, Supplementary Table 1). However, this was done on all experiments. B, AML cells were sorted for CD34+/CD117− and CD34+/CD117+ cells using CD34 or CD117 MACs sorting kits. CD34+/CD117−–purified AML cells were treated with either elution buffer control or 2 μg/mL rNm23-H1 for 20 hours. Viable cell numbers were counted following 2-day treatments of the more immature CD34+/CD117− AML cells with CM from elution buffer–treated CD34+/CD117− cells (CM EB) or rNm23-H1–treated CD34+/CD117+ cells (CM rNm23-H1). White circles are n = 5 AML samples (patients 1, 21–24, Supplementary Table 1), and the black triangle is an RAEB2 sample (patient 20, Supplementary Table 1); P = 0.028 (Wilcoxon signed rank test).

These assays used CM taken from CD34+/CD117− cells from 3 AML samples and 1 RAEB2 sample. The CD34+ or CD117+ cells from these samples had shown a range of survival responses to rNm23-H1 CM (Fig. 6A). Exposure of CD34+/CD117− to rNm23-H1 resulted in multiple and variable changes in cytokine release across the 4 samples (Supplementary Table 1). However, rNm23-H1 consistently stimulated CD34+ or CD117+ cells to release IL1β and IL6 at levels that mirrored the potency of the CM to promote the survival of CD34+ or CD117+ blast cells from the same samples (Fig. 6A–C).

The above observations suggest an association between the Nm23-H1–induced release of IL1β and IL6 from CD34+/CD117− cells and the promoted survival of immature blast cells. To investigate this association further, CD34+ cells from 3 AML patients were cultured with rIL1β and/or rIL6 (Fig. 6D and E). In all 3 cases tested, the direct addition of rIL1β and/or rIL6 recapitulated the survival effects of the medium conditioned by Nm23-H1–stimulated CD34+/CD117− cells.

The relationship between Nm23-H1 expression and IL1β and IL6 mRNA expression in vivo

As rNm23-H1 stimulated the release of IL1β and IL6 in vitro, we wished to seek evidence that endogenous expression of Nm23-H1 by AML cells was correlated with expression of IL1β and IL6 in vivo. Nm23-H1 levels were measured in 100 primary AML samples using QRT-PCR, and IL1β and IL6 expression measured in the 20 samples that displayed the highest and lowest expression of Nm23-H1. Median IL1β and IL6 expression in the uppermost 20% of Nm23-H1–expressing AMLs was 12-fold and 5-fold higher, respectively, compared with the lowest 20% of Nm23-H1–expressing AMLs (Fig. 7A and B; P < 0.001 and P = 0.004, respectively). A highly significant correlation between IL1β and Nm23-H1 mRNA expression...
(r = 0.473, P < 0.001; Fig. 7B) was also observed across all 100 samples studied.

Discussion

In this study, we show a strong association between the ability of the AML cells to bind rNm23-H1 at the cell surface and cell survival in vitro. Cell surface Nm23-H1 has been previously observed on NHL cells (18) and some myeloid cell lines (10, 19). However, to our knowledge, this is the first time that binding of Nm23-H1 to AML cells from an exogenous soluble pool of protein, commensurate with that found in AML patients, has been shown. Importantly, not all AML samples bound either endogenous Nm23-H1 or exogenously added rNm23-H1. This has notable implications for the biology of the disease. It has been previously documented that expression of Nm23-H1 transcripts, and more so levels of Nm23-H1 protein in serum, provide strong indicators of prognosis with higher values associating with poorer overall survival (7, 16). The data of Okabe-Kado and colleagues (17) and our observations here strongly indicate that Nm23-H1 can act as a tumor-derived survival factor in AML, and therefore that these associations with prognosis are causal. However, the influence of this mechanism on patient outcome is likely to have been underestimated because earlier studies were not able to delineate between Nm23-H1 binding AMLs in which the mechanism is likely to be active, from nonbinders in which Nm23-H1 levels are less likely to be of importance. Our data also imply that some AMLs express an Nm23-H1 receptor whereas others do not.

A previous study in T47D breast cancer cells identified MUC1* as a potential Nm23-H1 receptor (20). Mahanta and colleagues used extracellular MUC1* amino acids as bait to “fish” for possible dimerizing ligands in the T47D CM.
Nm23-H1 dimerizing ligand was found and was subsequently identified as Nm23-H1 by immunoprecipitation (20). Furthermore, the authors showed the capacity of MUC1* to dimerize and activate the MAPK pathway to increase proliferation of the T47D cells (20). A second study described that Nm23-H1 promoted growth of MUC1*-expressing pluripotent stem cells, an activity that was recapitulated by antibody induced dimerization of MUC1* (21). Although these studies indicate that MUC1* can act as a receptor for Nm23-H1 we have shown here that cell surface binding to AML cells is independent of MUC1*, and therefore an alternative receptor must be present on these cells. Future studies should investigate the relationship between Nm23-H1 binding and responses to AML therapies and aim to determine the nature of the Nm23-H1 receptor in AML, which may provide a novel target for adjunctive therapies.

Unexpectedly, our results show that Nm23-H1 preferentially binds the more mature cells of the AML clone that are CD34lo/CD11b+. However, the survival of the more mature (CD34+/CD11b+) and immature (CD34+/CD11b−) cells was enhanced. These data indicate that Nm23-H1 has indirect survival effects on the CD34+/CD11b− cells, by inducing supportive cytokine release from the more mature CD34+/CD11b+ cells.

Of the 28 cytokines measured in our assay, the release of IL1β and IL6 from the more mature cells best correlated with the survival of the immature cells. Furthermore, the direct addition of IL1β and/or IL6 recapitulated the survival effects of the CM to promote CD34+ blast cell survival. Unexpectedly, either IL1β or IL6 was sufficient in all cases tested, with each of the AML samples demonstrating different survival responses to the 2 cytokines.

The promoted release of IL1β and IL6 by Nm23-H1 was also observed by Okabe-Kado and colleagues (17). However, their experiments used unsorted cells, and therefore did not show that these cytokines form part of a feedback mechanism within the AML clone. Furthermore, we studied Nm23-H1, IL1β, and IL6 mRNA expression in freshly isolated AML mononuclear cell preparations and observed that elevated Nm23-H1 expression correlated with higher expression of IL1β and IL6. Thus, our data are consistent with the model that Nm23-H1 regulates IL1β and IL6 expression within AML cells in vivo. Once again our data are likely to underestimate the link between Nm23-H1 and IL1β and IL6 expression in AML because the RNA samples used were from the MRC-AML12 bank in which Nm23-H1 binding capacity had not been measured.

Our observations are consistent with other studies identifying the importance of IL1β in AML. Exogenous rIL1β has been shown to enhance the proliferation of AML cells, and a strong proliferative response in vitro to rIL1β predicts a reduced rate of complete remission for AML patients (23, 24). Evidence also suggests that IL1β modulates autocrine signaling by stimulating the release of growth factors such as GM-CSF from AML blast cells (25–27). More recently rIL1β has been shown to enhance AML cell survival by activating multiple survival pathways, which include the p38 MAPK, phosphoinositide 3-kinase, and NF-xB pathways (28).

Although IL6 is known to be a key growth factor in multiple myeloma (29), the role of IL6 in AML is poorly understood. There is evidence that the addition of exogenous rIL6 synergizes with other cytokines, including GM-CSF, IL3, and IL4, to enhance growth and survival of AML cells (30, 31). Elevated autocrine/paracrine secretion of IL6 in AML has been shown to constitutively activate Stat3 signaling (32), a feature associated with the upregulation of antiapoptotic proteins, and cyclins that regulate G1 to S-phase cell cycle transition (33, 34).

During normal hemopoiesis Nm23-H1 is highly expressed in immature CD34+ hemopoietic cells, and its expression decreases during maturation (10). Thus, it is likely that the
elevated serum Nm23-H1 levels found in AML patients derive predominantly from the more immature components of the clone. Collectively our findings indicate that the released Nm23-H1 is sensed by the more mature malignant cells, improving their survival, and stimulating a reciprocal cyto- 
kine-mediated survival signal directed by the immature cells. This novel feedback mechanism reveals that the action of Nm23-H1 in promoting AML cell survival and clonal expan- 
sion is more complex than expected. Following the demon-
stration that Nm23-H1 inhibits myeloid cell differentiation (14), it is attractive to hypothesize that this mechanism may reflect the subversion by AML cells of a previously unidentified process of "quorum sensing" during normal hemopoiesis that 
regulates the balance between transiently amplifying and 
maturing cell populations.

Disclosure of Potential Conflicts of Interest

C.M. Bunce is a director of Stemtrax Ltd., a company set up to exploit the actions of Nm23-H1 on normal hemopoietic stem cells. There are no other conflicts of interest.

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