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REVIEW

The case for extracellular Nm23-H1 as a driver of acute myeloid leukaemia (AML) progression

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Abstract Studies in the 1990s identified a link between extracellular Nm23 proteins and acute myeloid leukaemia (AML). Confidence in the importance of these observations was undermined by a lack of appreciation that extracellular Nm23 proteins were relevant to either normal or pathophysiology coupled with the lack of demonstrable activity of Nm23 proteins against human AML cell lines. However, independent studies have highlighted the importance of Nm23-H1 in AML and have identified an elaborate Nm23-H1-mediated cross talk between cells within the AML clone. In other studies, roles for Nm23-H1 have now also been implicated in the maintenance of the stem cell state of embryonic stem (ES) cells and induced pluripotent stem (IPS) cells. In this review, we have generated a unifying model of the action of Nm23-H1 in AML, including previously unpublished data from our laboratory, and provide arguments as to why we consider this role to be distinct from that in ES and IPS cells.

Keywords Nm23 · AML · MUC1* · ESCs · IPS cells

Acute myeloid leukaemia

The acute myeloid leukaemias (AMLs) are a heterogeneous group of aggressive malignancies characterised by the clonal accumulation of immature myeloid blast cells in the bone marrow and peripheral blood. Expansion of the differentiation-defective AML clone interferes with normal haemopoiesis resulting in anaemia, neutropenia and thrombocytopenia. Median age of presentation is in the late 1960s. In younger patients, the incidence is two to three per 100,000, which rises to ~15 per 100,000 in the seventh and eighth decades (Burnett et al. 2011; Lowenberg et al. 1999).

In patients younger than 60 years, 40 to 45 % of patients can be cured using current chemotherapy regimens reflecting a steady improvement over the past 30 years. Progress in older patients has shown a more modest improvement over the same period. Approximately 40 to 65 % of these patients achieve remission, but over 80 % will relapse within 2 to 3 years. Much of the improvement made in either age group can be attributed to a better understanding and deployment of supportive care necessary to overcome the life-threatening pancytopenia caused by chemotherapy (Burnett et al. 2011).

A notable exception is the case of acute promyelocytic leukaemia (APL) which represents approximately 10 % of all AML. If untreated APL demonstrates particularly rapid progression with significant deaths arising from disease-associated haemorrhages (Hillestad 1957; Warrell et al. 1993). APL is associated with specific chromosomal translocations that always involve the retinoic acid receptor α (RARA) gene on chromosome 17 (Piazza et al. 2001). The most common translocation is the t(15,17) translocation encoding the PML/RARA fusion (de The et al. 1990, 1991; Rowley et al. 1977) which is associated with >98 % of APL cases. These patients respond to all trans-retinoic acid (ATRA) as part of their therapy and, as a consequence, enjoy far better prognosis than all other AML subgroups with over 90 % of patients achieving complete remission and around 80 % of patients achieving cures. More recently, arsenic trioxide (ATO) has also been identified to target the PML/RARA fusion protein and to be efficacious in patients. Many trials are now testing the combination of ATRA and ATO in chemotherapy-free regimens in APL (reviewed in Park and Tallman 2011).

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The success of APL therapy indicates that understanding and targeting the abnormal biology of AML provide the possibility of improving treatment outcomes. However, targeting of other recurrent mutations in AML has been disappointing. There is therefore a growing need to look beyond the genetics of these diseases and understand other aspects of AML biology. One approach to gaining this understanding is the identification of biomarkers that correlate with AML prognosis.

Nm23-H1 and Nm23-H2 and AML

In seminal studies in the early 1990s, Okabe-Kado and colleagues sought to identify secretory factors that inhibit myeloid differentiation since failed differentiation is a central feature of AML. They used the murine M1 leukaemic cell line which can be induced to differentiate along the monocytic lineage in culture. Their studies identified that the supernatant from a differentiation-deficient M1 subclone conferred differentiation resistance upon parental M1 cells. In an impressive execution of what has since been termed proteomics, they identified that the differentiation inhibitory factor was an Nm23/nucleoside diphosphate kinase (NDPK) and that the differentiation inhibitory activity was independent of its kinase activity (Okabe-Kado 1992; Okabe-Kado et al. 1995). They went on to demonstrate that the human and mouse orthologues Nm23-H1, Nm23-M1, Nm23-H2 and Nm23-M2 also shared capacity to inhibit the differentiation of the HEL human erythroid leukaemia model, identifying that the phenomenon was neither species specific nor restricted to single lineage (Yokoyama et al. 1996).

In the light of their observations using monocytoid M1 cells, the group went on to demonstrate that Nm23-H2 and to a greater extent Nm23-H1 were highly expressed in myelomonocytic AML and that the highest expression was associated with worse prognosis (Yokoyama et al. 1996). Although particularly significant in monocytic AMLs, evaluation of Nm23 expression and other clinical parameters of patients with diverse forms of AML demonstrated that increased Nm23-H1 and Nm23-H2 messenger RNA (mRNA) levels were associated with resistance to initial chemotherapy and with reduced overall survival (Wakimoto et al. 1998; Yokoyama et al. 1998). There followed a unifying observation linking the laboratory studies and the measurement of Nm23-H1 and Nm23-H2 expression in AML when plasma levels of Nm23-H1 protein were found to correlate both with mRNA expression by AML cells and with prognosis (Niitsu et al. 2000). Although the study considered the importance of the overexpression of Nm23-H1 by AML cells *per se* in hindsight, these observations were the first to indicate that exogenous AML-derived Nm23-H1 protein may impact prognosis by exerting an autocrine activity against the tumour cells.

In 2009, almost a decade after their first observations, Okabe-Kado and colleagues demonstrated that the provision of extracellular recombinant Nm23 proteins (rNm23-H1 and rNm23-H2) promoted the survival of primary cultured AML cells in vitro (Okabe-Kado et al. 2009b). A total of 14 AMLs were studied, of which, 11 responded to rNm23-H1 and 3 were unresponsive.

Although the study focussed on Nm23-H1, the authors demonstrated that in two of the Nm23-H1-responsive AML samples, Nm23-H2 also promoted AML cell survival. Therefore, it was concluded that the pro-survival actions are not H1/H2 isotype specific. One drawback of the study was that responses were measured using MTT assays and were reported as dose-dependent survival and growth responses. However, the MTT assay does not strictly measure growth but rather is a direct measure of viable cells, and it is not possible to discern whether their data relates to growth or preferential survival.

Cytokine antibody arrays and ELISA assays identified that the response of AML cells to rNm23-H1 exposure was associated with induced cytokine expression. The patterns of expressed cytokines were inconsistent amongst three rNM23-H1-responsive AMLs which demonstrated increased survival/proliferation when compared against a single non-responsive AML. Nonetheless, it was noted that cytokines known to be active against AML cells such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-1β (Delwel et al. 1989) were elevated in the conditioned medium (CM) of the three rNm23-H1-responsive AMLs but not in the case of the unresponsive AML. The study also demonstrated that exposure of responsive AML cells to rNm23-H1 was associated with activation of P38 MAPK and STAT1, the 3 and 5 signalling pathways (Okabe-Kado et al. 2009b). A limited study in one AML that exhibit improved survival/proliferation in response to rNM23-H1 for which sufficient cells were available identified that inhibitors of these pathways diminished the rNm23-H1 response.

The action of Nm23-H1 against AML blasts is indirect

Although highly indicative of a potential mechanism, the above study was hampered by restricted numbers of cells and required secondary confirmation. In 2011, we published a study that confirmed the promotion of in vitro survival of primary AML cells by rNm23-H1 (Lilly et al. 2011). We used a flow cytometry approach to study binding of Nm23-H1 to AML cell populations and observed that not all AMLs bound rNm23-H1 (Fig. 1). In a sample set of 18 AMLs, 11 bound rNm23-H1 and enhanced survival was directly correlated with binding. In many cases, we could detect surface endogenous Nm23-H1 bound to AML cells taken directly from the patient,

indicating that binding of rNm23-H1 is not an in vitro artefact (Fig. 1). Our study used direct cell counting rather than MTT, and we observed that responses varied amongst rNm23-H1 binders. The majority displayed enhanced survival over controls, but 3 out of 11 also demonstrated a modest increase in cell number over 5 days.

Combined staining for the AML blast marker CD34 and the myeloid differentiation marker CD11b identified that Nm23-H1 did not bind the most immature blasts in AML mononuclear cell preparations. Instead, Nm23-H1 bound to cells displaying signs of differentiation as described by loss of CD34 expression and gain of CD11b ($CD34^{lo}/CD11b^{+}$). This observation suggested that rNm23-H1 indirectly enhanced survival, and in some cases growth, of AML blasts via acting on the more mature cells in the clone.

To test this, we separated AML samples into immature $CD34^{+}$ blast populations and more mature $CD34^{lo/-}$ fractions. The $CD34^{+}$ cells were stored overnight at 4°C. In parallel, the more mature $CD34^{lo/-}$ cells were exposed to rNm23-H1 and their supernatant harvested after 20 h and depleted of rNm23-H1 to generate a conditioned medium (CM) devoid of rNm23-H1. This CM was then used to culture the stored $CD34^{+}$ blasts. In all cases where isolated $CD34^{lo/-}$ bound rNm23-H1, their CM recapitulated the survival effect of rNm23-H1 (Lilly et al. 2011). A cytokine array analysis identified a number of cytokines that were enhanced in the CM of rNm23-H1-binding $CD34^{lo/-}$ cells when compared to non-binders. Unlike in the studies of Okabe-Kado et al. (2009b), we observed a small and inconsistent increase in GM-CSF secretion (unpublished). However, like the earlier study, we did observe an increase in IL-1β secretion. Increases in IL-6 and IL-1β levels best correlated with the degree of support that each CM exerted on the survival of their autologous $CD34^{+}$ cells identifying them as potential mediators of the indirect survival action. Furthermore, culture of $CD34^{+}$ cells from three AML patients with either rIL-1β or rIL-6 recapitulated the survival effects of the medium conditioned by Nm23-H1-stimulated $CD34^{lo/-}$ cells (Lilly et al. 2011). In contrast, direct culture of purified $CD34^{+}$ AML cells with rNm23-H1 failed to enhance their survival compared to untreated controls. Finally, we were able to show that in vitro, there is a positive association between Nm23-H1, IL-1β and IL-6 expressions in AML cells.

Overall, there is considerable consistency between the studies of Okabe-Kado et al. and ourselves. Together, they strongly implicate Nm23-H1 as a driver of AML progression rather than a passive correlate of prognosis. Our data indicating that more mature cells within the AML clone bind and respond to Nm23-H1 is also consistent with another study by our colleagues in which they demonstrated that rNm23-H1 activates the secretion of cytokines including rIL-1β from normal human peripheral blood mononuclear cells (Okabe-Kado et al. 2009a). Where there is some discrepancy is where they observed Nm23-H1 to inhibit the survival of more mature myeloid cells whilst we have observed the reverse in AML samples (Lilly et al. 2011). Several factors could

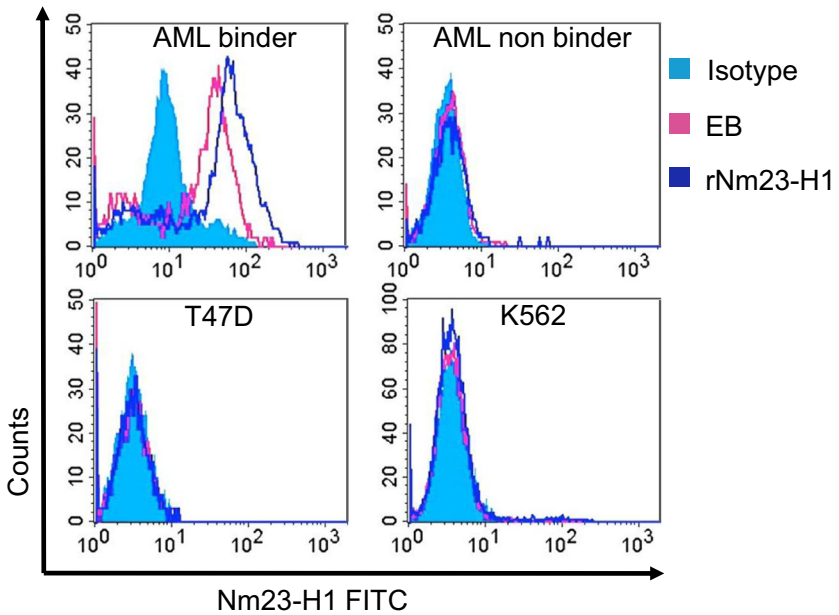

Fig. 1 Nm23-H1 cell surface binding in primary AMLs, T47D and K562 cells. Primary AML, K562 and T47D cells were cultured with 2 μg/ml Nm23-H1 or elution buffer control (EB). Cells were indirectly stained for cell surface Nm23-H1 using an anti-Nm23-H1 antibody and a FITC secondary antibody, and Nm23-H1 cell surface binding was measured by flow cytometry. The representative histograms shown are gated on the viable cell gate. Isotype control *solid light blue* histogram, EB-treated cells *pink line* histogram, rNm23-H1-treated cells *dark blue* histogram. AML samples were provided under ethical committee approval in compliance with the 1964 Declaration of Helsinki and its amendments (IRAS project code: 34495)

account for this discrepancy. Firstly, the more mature cells in AML are not the same as normal peripheral blood cells and are generally less differentiated. Secondly, technical differences exist between the protocols between our studies. Okabe-Kado and colleagues used foetal bovine serum in their experiments whereas we used serum-free conditions and higher rNm23-H1 concentrations in our studies.

KG1a cells are a $CD34^+$ AML cell line that provides a model for studying the behaviour of AML blasts. Consistent with primary AML samples where we have observed higher Nm23-H1 mRNA levels in the $CD34^+$ cells compared to $CD34^{lo/-}$ cells within the same AML sample (Fig. 2a), KG1a cells express significantly higher levels of Nm23-H1 mRNA than observed in the more differentiated HL60 cell line (Fig. 2a). The association between Nm23-H1 expression and differentiation state is further strengthened by our result showing that induction of neutrophil or monocytic differentiation of HL60 cells using ATRA or vitamin D_3, respectively, further decreases Nm23-H1 mRNA levels (Fig. 2a). KG1a also secretes endogenous Nm23-H1 protein into their media that is detectable by Western blotting (Fig. 2b).

We used the KG1a model to study the response of AML blast cells to rNm23-H1 CM, IL-1β and IL-6. NF-κB is constitutively active in AML stem cells, although it is uncertain whether this is due to direct molecular disruptions in the pathway or due to the paracrine/autocrine secretion of cytokines such as IL-1β (Guzman et al. 2001). Given that Okabe-Kado and colleagues and ourselves have observed IL-1β secretion in response to Nm23-H1, we tested whether rNm23-H1 CM and IL-1β induced NF-κB activation. As shown in Fig. 3a, rNm23-H1 CM activated NF-κB in KG1a cells as measured by a decrease in cytosolic IκBα and an increase in nuclear NF-κB P65. Consistent with this, rIL-1β also generated a similar NF-κB response (Fig. 3b, c). Importantly, we also observed rIL-1β-induced expression of GM-CSF in KG1a cells as determined by quantitative RT-PCR (Fig. 3d). This potentially explains the discrepancy between the detection of GM-CSF in the supernatant of rNm23-H1-treated cells in the studies of (Okabe-Kado et al. 2009b) and our own studies (Lilly et al. 2011). Our experiments suggest that $CD34^+$ AML cells are the source of GM-CSF that is secreted indirectly secondary to rNm23-H1-stimulated IL-1β release from the Nm23-H1 from more mature $CD34^{lo}$/$CD11b^+$ cells. Since Okabe-Kado et al. did not separate these populations, they would have been unable to observe this Nm23-H1-dependent cell–cell communication.

In their study, Okabe-Kado et al. did not mention whether they measured or observed rNm23-H1-induced secretion of IL-6 (Okabe-Kado et al. 2009b), the main cytokines they discussed were IL-1β and GM-CSF, and these were highlighted because of known activity against AML cells. The role of IL-6 in AML is poorly understood; however, evidence suggests that high IL-6 levels are responsible for constitutive activation of the STAT3 pathway, which has been observed in around 20–45 % of AML samples (Benekli et al. 2002; Schuringa et al. 2000). Thus, it was of interest that Okabe-Kado et al. (2009b) observed STAT activation in those AMLs exhibiting increased survival/proliferation in response to rNm23-H1. Importantly, IL-6 stimulation of KG1a cells resulted in a rapid Tyr 705 phosphorylation of STAT3 after 10 min indicating that IL-6 activates the STAT3 pathway in primitive AML cells (Fig. 3e, f).

Are Nm23 proteins secreted from cells?

The elevated serum levels of Nm23-H1 in haematological malignancies, together with the identification of Nm23 proteins in tissue culture supernatants from a range of cell lines from different tumours, suggests that Nm23-H1 can be secreted (Anzinger et al. 2001; Mahanta et al. 2008; Okabe-Kado 1992). Nm23-H1 has no secretion signal peptide, and the mechanism of its release from cells is unknown (Okabe-Kado et al. 2012). However, there is a growing list of proteins which do not contain secretion signal peptides and are secreted by unconventional mechanisms (Gardella et al. 2002; Mambula et al. 2007).

The Nm23 proteins can associate with membrane phospholipids which may mediate their secretion. Mitochondrial-specific Nm23-H4 can bind the phospholipid cardiolipin and intercalate the inner and outer mitochondrial membranes (Tokarska-Schlattner et al. 2008). A cardiolipin-like binding domain can be found in Nm23-H1, which may mediate the nucleotide-dependent binding of Nm23-H1 to plasma membrane phospholipids (Lacombe et al. 2009; Mitchell and Hatch 2009). One type of unconventional secretion mechanism involves the targeting of proteins to the membrane by phospholipids which then mediates the translocation of the protein across the membrane (Nickel 2010). Other unconventional secretion mechanisms involve protein containing vesicles, such as lysosomes and exosomes, fusing with the plasma membrane, thus releasing their contents out of the cell (Nickel 2010). Future studies into these unconventional secretion mechanisms may resolve the mystery of how Nm23 proteins are released from cells (Fig. 2).

MUC1* as a receptor for Nm23 proteins

MUC1 is a type I membrane glycoprotein of the mucin family that has an extracellular domain consisting of hundreds of tandem repeats, a single transmembrane domain and a C-terminal cytoplasmic tail. The large extracellular domain can be cleaved in situ by extracellular proteases leaving a truncated transmembrane protein that has been termed MUC1* (Mahanta et al. 2008). A series of studies by the Bamdad group have

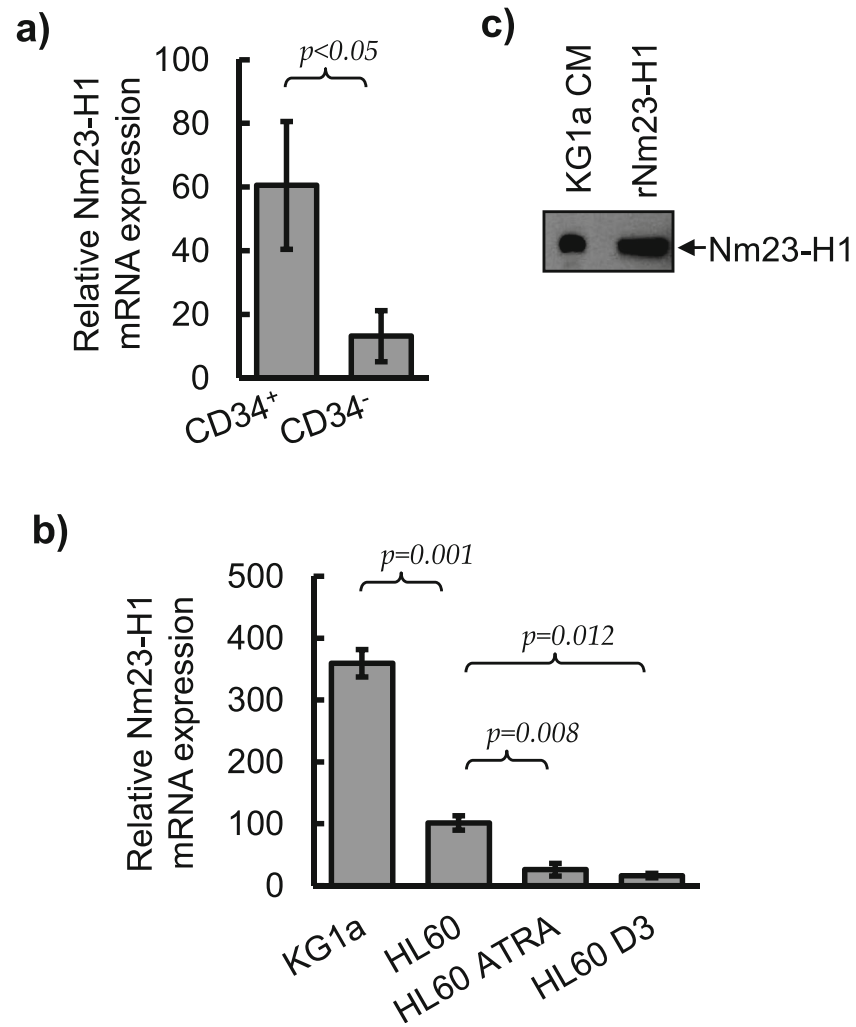

Fig. 2 Nm23-H1 expression in immature leukaemic blast cells and its expression during haematopoietic maturation. **a** Nm23-H1 expression in $CD34^+$ and $CD34^-$ AML cells. AML samples were sorted into $CD34^+$ and $CD34^-$ fractions. Nm23-H1 mRNA expression in the samples relative to expression in HL60 cells was measured by QRT-PCR. The data is presented as the mean ±SEM (n=5, Wilcoxon signed rank test). **b** Nm23-H1 gene expression in AML cell lines. HL60 cells were induced to differentiate for 5 days with 100 nM vitamin D_3 or ATRA. Nm23-H1 mRNA expression was measured by QRT-PCR in untreated KG1a cells and HL60 cells, as well as in the HL60 cells induced to differentiate. Results are expressed relative to expression in uninduced HL60 cells. Data is presented as the mean±SEM (n=3, t test). **c** Nm23-H1 expression in KG1a-conditioned medium was determined by Western blotting. rNm23-H1 (15 ng) was used as a positive control

demonstrated that MUC1* acts as a biologically active receptor for Nm23-H1 and Nm23-H2 that has important roles both in cancer and stem cell self-renewal (Fessler et al. 2009; Hikita et al. 2008; Mahanta et al. 2008; Smagghe et al. 2013). In their first study, they used immobilised MUC1* to probe for ligands,first in cell lysates and then in supernatants of T47D breast cancer cells. Both approaches captured Nm23-H1, as shown by sequencing of excised bands and by western blots, respectively. Although not verified by westerns, sequencing approaches also identified Nm23-H2 binding to MUC1*, indicating that Nm23-H2 is likely to act as a ligand for MUC1*. However, Nm23-H2 was not investigated in subsequent studies. A gold particle aggregation (GPA)-based assay and surface plasmon resonance (SPR) assays confirmed binding of purified bovine Nm23 (bNm23) by MUC1*. Importantly, bNm23 protein enhanced T47D cell proliferation in the low nanomolar (0.1–3 nM) range, a response which was absent in MUC1* knockdown T47D cells (Mahanta et al. 2008).

A follow-up study using immunohistology and a MUC1*-truncated extracellular domain selective rabbit polyclonal antibody (raised by the investigators) identified exclusive expression of MUC1* and not MUC1 by undifferentiated human embryonic stem cells (hESCs) (Hikita et al. 2008), a situation that was reversed in newly differentiated hESC progeny. Furthermore, antibody-mediated dimerization of the MUC1* receptor on hESCs stimulated cell growth. Staining for MUC1* and Nm23-H1 showed co-localisation further suggesting that Nm23-H1 may be an endogenous ligand of MUC1* in hESCs. In addition, exogenous rNm23-H1 promoted hESC growth in the absence of feeder cells and produced pluripotent colonies that resisted spontaneous differentiation. The study also used a mutant rNm23-H1 (rNm23-S120G) that preferentially forms dimers rather than the preferred hexameric state of the wt protein (Chiadmi et al. 1993; Dumas et al. 1992). In a limited number of repetitions, equimolar rNm23-S120G appeared more efficient than wt rNm23-H1 in supporting undifferentiated hESC colony formation, although both appeared to be functional.

A third study further investigated the role of Nm23-H1 in supporting the undifferentiated proliferation of hESCs and

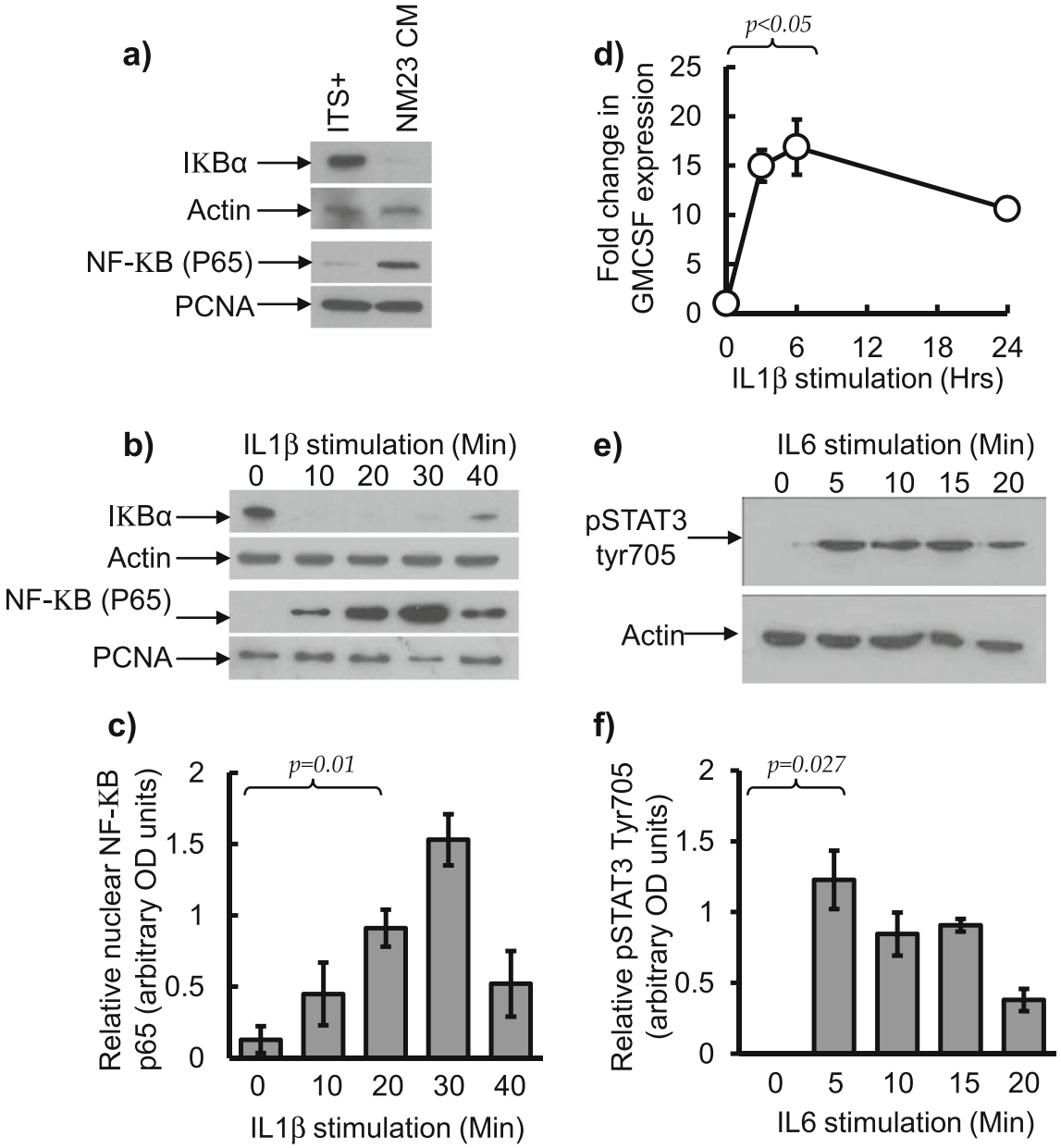

Fig. 3 The response of KG1a AML blast cells to Nm23 CM, IL-1β and IL-6. **a** Conditioned media from Nm23-H1-stimulated primary CD34$^-$ AML cells activates the NF-κB pathway in KG1a cells. KG1a cells were stimulated with either CM from Nm23-H1-stimulated CD34$^-$ primary AML cells (Nm23 CM) or ITS+media as a control for 20 min. Cytosolic IκBα and nuclear NF-κB (P65) levels were determined by Western blotting. **b** IL-1β activates the NF-κB pathway in KG1a cells. KG1a cells were stimulated with 2.5 ng/ml IL-1β for up to 40 min. Cytosolic IκBα and nuclear NF-κB (P65) levels were determined by Western blotting. **c** The nuclear NF-κB P65 band density was determined relative to PCNA band density using Image J software. **d** IL-1β increases GM-CSF gene expression in KG1a cells. KG1a cells were stimulated with 2.5 ng/ml IL-1β for up to 24 h. Fold change in GM-CSF mRNA expression levels was determined by QRT-PCR (SYBR green) relative to expression levels in untreated cells. **e** IL-6 activates the STAT3 pathway in KG1a cells. KG1a cells were stimulated with 5 ng/ml IL-6 for up to 40 min. Total intracellular pSTAT3 tyr705 and β-actin control levels were determined by Western blotting. **f** The pSTAT3 band density was determined relative to β-actin band density using Image J software. In all cases, the data is presented as the mean±SEM (n=3, t test)

extended the study to include induced pluripotent stem (IPS) cells (Smagghe et al. 2013). In this study, the case was made that dimeric rNm23-S120G is a far superior ligand for MUC1* and, substantially, more biologically active against HEScs and IPS cells than hexameric wt rNm23-H1. Indeed, it is argued that hexameric wt rNm23-H1 is not a relevant MUC1* ligand (Smagghe et al. 2013). This assertion appears to be at odds with the earlier study where wt rNm23-H1 appeared able to support undifferentiated hESC colony formation (Hikita et al. 2008). It has not been reported that T47D cells express Nm23-S120G or another variant of Nm23-H1 or Nm23-H2 that preferentially forms dimers. However, if the

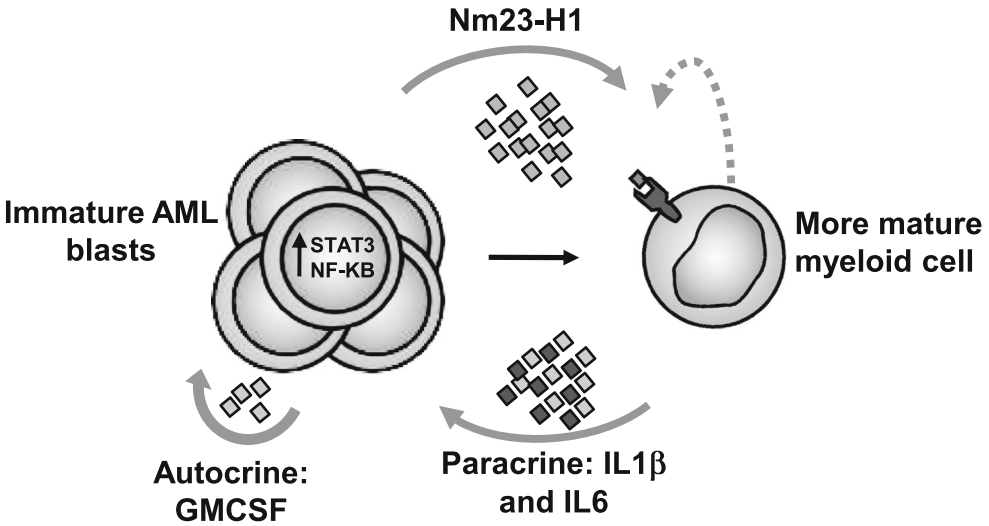

Fig. 4 Summary of the role of Nm23-H1 in AML. An increase in the number of immature AML blast cells highly expressing Nm23-H1 leads to elevated levels of extracellular Nm23-H1. Nm23-H1 binds to more mature myeloid cells, inducing the release of cytokines including IL-1β and IL-6. These cytokines reciprocally enhance the survival of the immature AML blast cells by activating survival pathways such as STAT3 and NF-κB, as well as by increasing the expression of other cytokines such as GM-CSF. Ultimately, this cross-talk mechanism leads to a 'vicious cycle' enhancing the expansion of the malignant clone

assertion is that only dimeric Nm23 forms are ligands for MUC1*, then the pulldown experiments that first directed the groups' interest to Nm23 would suggest that this is the case. Similarly, in the T47D-based paper of Mahanta et al., data is shown using the same GPA and SPR technologies to argue that wt Nm23 (albeit bovine) is a ligand for MUC1* (Mahanta et al. 2008). It is therefore also interesting to consider whether bovine Nm23 preferentially forms dimers.

The importance of MUC1* as receptor for embryonic stem cells (ESCs) was further demonstrated by enhanced proliferation and suppressed differentiation when ESCs were grown on surfaces coated with a rabbit polyclonal antibody to MUC1* (Smagghe et al. 2013). It was also stated that proliferation was vastly improved when exogenous dimeric Nm23-H1 was also added to these cultures although this data was not shown. These observations would suggest that cross-linking of MUC1* by immobilised antibody delivers a suboptimal signal that is 'topped up' by dimeric Nm23-H1. This may be a threshold effect or may indicate that optimal MUC1* signalling requires internalisation of liganded receptor. Alternatively, dimeric Nm23-H1 may have an additional yet unidentified receptor.

It is interesting to consider the roles of dimeric Nm23-H1 versus hexameric Nm23-H1. In the cell, the only catalytically active form of Nm23-H1 is hexameric, and its NDPK activity centres around the catalytic histidine 118 (H118) residue (Gilles et al. 1991). Mutational studies have indicated that Nm23 NDPK activity is not required for its extracellular pro-survival or differentiation inhibitory functions (Okabe-Kado et al. 1995, 2009b; Willems et al. 2002). Therefore, at least some of the extracellular functions of Nm23-H1 are independent of its NDPK activity, but likely reflect its interaction with cell surface receptors. In the case of MUC1*, it seems that dimeric Nm23-H1 is the form that most efficiently cross-links MUC1* extracellular domains resulting in downstream signal transduction in a mechanism similar to class 1 growth factor receptors (Mahanta et al. 2008). However, to date, a detailed structural analysis of the Nm23-H1-MUC1* complex has not been undertaken.

The receptor for Nm23 proteins on AML cells remains unknown

The presence and relative abundance of MUC1 and MUC1* in cell extracts can be determined using differential staining of western blots by commercially available antibodies that recognise epitopes in the tandem repeats of the extracellular domain and, thus, detect only MUC1 and antibodies that recognise the C-terminus that recognise both MUC1 and MUC1*. We used this approach to interrogate expression of both proteins in primary AMLs responsive and non-responsive to wt rNm23-H1 using T47D as a positive control (Lilly et al. 2011). Consistent with the study of Mahanta et al. (2008), we observed very strong binding of antibody directed at the MUC1/MUC1* C-terminus in T47D cells and only weak binding of antibody to the MUC1 extracellular tandem repeat epitopes, indicating strong and predominant expression of MUC1* over MUC1 (Lilly et al. 2011; Mahanta et al. 2008). We also detected MUC1* expression in the bcr-abl-positive chronic myeloid leukaemia (CML) blast crisis cell

line K562 as also previously described by Mahanta et al. (2008). The signal for MUC1* in K562 cells was much lower than in T47D cells, and we were unable to detect MUC1 expression by this approach in K562 cells. In contrast, interrogation of three wt rNm23-H1-binding primary AML samples and five non-binding samples failed to detect either MUC1 or MUC1* in any sample. Two further AML samples, for which Nm23 binding was not determined, also failed to express detectable MUC1 or MUC1*. We also failed to detect either MUC1 or MUC1* in NB4 (AML M3) cell extracts. Consistent with the analyses by western blotting, we observed abundant expression of MUC1 mRNA by T47D cells and lesser, but strong, expression by K562 cells. However, MUC1 mRNA expression was very low/absent in both binding and non-binding primary AML cells (Lilly et al. 2011).

Figure 1 shows representative flow cytometric analyses of cell surface endogenous and rNm23-H1 binding to examples of a non-binding and a binding primary AML, T47D cells and K562 cells. As shown, the binding of endogenous Nm23-H1 was clearly detectable on the binding AML prior to exposure to rNm23-H1 and this signal increased post-exposure. In contrast, the non-binding AML sample displayed no Nm23 staining either before or after incubation with rNm23-H1. Similarly, neither MUC1* expressing T47D nor K562 cells bound either endogenous Nm23-H1 or exogenously applied rNm23-H1. These observations are therefore consistent with the conclusions of the Bamdad group that MUC1* is not a receptor for hexameric wt Nm23-H1. These data also indicate that the receptor responsible for the pro-survival effects of Nm23-H1 against AML cells is yet unknown.

Concluding remarks and future directions

We have discussed herein a small field within the wider scope of Nm23 (NME, NDPK) biology that nonetheless has big implications for developmental biology, cancer and regenerative medicine.

The data regarding wt Nm23-H1 in AML share considerable crossover between two independent laboratories and suggest that the area is worthy of wider investigation. Equally, our studies and those of the Bamdad group appear in good agreement that MUC1* is not a receptor for wt Nm23-H1 in its favoured hexameric form, yet it has not been independently corroborated that dimeric Nm23-H1 is a ligand for MUC1*. Neither has the assertion that MUC1* is a universal receptor on all stem cells (Hikita et al. 2008; Smagghe et al. 2013) been either substantiated or corroborated and this too needs further testing.

What is clear is that the biology in these two scenarios is different. Bamdad and colleagues describe Nm23-H1 activity against stem cells. The Okabe-Kado and Honma group and ourselves have described an activity of Nm23-H1 in AML that acts against differentiated non-self-renewing cells that solicits a response that reciprocally secures the survival of their precursor populations that may or may not include leukaemic stem cells.

Notwithstanding the potential commercial value inherent in the ability to better culture ESCs and IPS cells, the key questions that remain include the verification that these processes have in vivo relevance. The correlative data with respect to prognosis in AML together with the correlation between Nm23-H1, IL-1β and IL-6 expression in AML mononuclear cells suggest that the in vitro observations demonstrated by Okabe-Kado and Honma group and ourselves have at the very least pathological in vivo relevance. The fact that Nm23-M1 and Nm23-M2 knockout mice are viable and that the double knockout mice die perinatally due to severe anaemia suggest that Nm23 proteins may not have an important role in regulating the homeostasis of ESCs during early embryonic development (Arnaud-Dabernat et al. 2003; Di et al. 2010; Postel et al. 2009). Finally, Fig. 4 summarises our interpretation of the known data regarding AML. The model proposes both paracrine and autocrine stimuli in both immature blast populations which most likely include leukaemic stem cells and other more mature cells within the AML clone.

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Conflict of interest The authors declare that they have no conflicts of interest.

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