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ORIGINAL ARTICLE

Aberrant Mer receptor tyrosine kinase expression contributes to leukemogenesis in acute myeloid leukemia

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Acute myeloid leukemia (AML) continues to be extremely difficult to treat successfully, and the unacceptably low overall survival rates mandate that we assess new potential therapies to ameliorate poor clinical response to conventional therapy. Abnormal tyrosine kinase activation in AML has been associated with poor prognosis and provides strategic targets for novel therapy development. We found that Mer receptor tyrosine kinase was over-expressed in a majority of pediatric (29/36, 80%) and adult (10/10, 100%) primary AML patient blasts at the time of diagnosis, and 100% of patient samples at the time of relapse. Mer was also found to be expressed in 12 of 14 AML cell lines (86%). In contrast, normal bone marrow myeloid precursors expressed little to no Mer. Following AML cell line stimulation with Gas6, a Mer ligand, we observed activation of prosurvival and proliferative signaling pathways, including phosphorylation of ERK1/2, p38, MSK1, CREB, ATF1, AKT and STAT6. To assess the phenotypic role of Mer in AML, two independent short-hairpin RNA (shRNA) constructs were used to decrease Mer expression in the AML cell lines Nomo-1 and Kasumi-1. Reduction of Mer protein levels significantly increased rates of myeloblast apoptosis two to threefold in response to serum starvation. Furthermore, myeloblasts with knocked-down Mer demonstrated decreased colony formation by 67–87%, relative to control cell lines ($P<0.01$). NOD-SCID-gamma mice transplanted with Nomo-1 myeloblasts with reduced levels of Mer had a significant prolongation in survival compared with mice transplanted with the parental or control cell lines (median survival 17 days in parental and control cell lines, versus 32–36 days in Mer knockdown cell lines, $P<0.0001$). These data suggest a role for Mer in acute myeloid leukemogenesis and indicate that targeted inhibition of Mer may be an effective therapeutic strategy in pediatric and adult AML.

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INTRODUCTION

Despite significant improvements over the past 30 years, 5-year survival rates for adults and children with acute myeloid leukemia (AML) are only around 50–60%,[1,2] and those with high-risk features have a predicted survival of less than 35%.[2,3] Using current therapy, patients with relapsed AML have a survival rate of less than 30% and frequently do not survive more than 1 year after relapse diagnosis.[4,5] Unfortunately, other than the use of all-trans retinoic acid in acute promyelocytic leukemia and the option of hematopoietic stem cell transplantation, there have not been any new drug approvals or significant changes in AML therapy in recent years. The toxicity of conventional AML therapy is often problematic for older adult patients. Additionally, concerning long-term effects of AML therapy on pediatric and young adult survivors, include long-term cardiac dysfunction, cognitive deficits and the occurrence of secondary malignancy. AML therapy that targets a specific cancer-promoting signaling pathway could allow a more tailored, efficacious approach leading to a better clinical response with reduced toxicity.

Recent research in AML has revealed only a few mutated or aberrantly expressed protein kinases that serve as potential therapeutic targets. High allelic ratio of the internal tandem duplication mutation of FLT-3 has been associated with poor prognosis, and small molecule inhibitors targeting cells which express FLT-3 have been developed.[6,7] C-Kit positive AML, which is found in a large number of relapsed patients, is associated with poor prognosis and has also been targeted by small molecule inhibitors.[8,9] Further targeted therapy options are needed to provide more efficient AML treatment, or allow for a decrease in high-intensity chemotherapy.

Aberrant expression of the Mer receptor tyrosine kinase has been detected in several human cancers.[10] Preclinical models have demonstrated that Mer is associated with increased cellular proliferation, prosurvival and antiapoptotic signaling and chemoresistance. Mer also exhibits transforming ability *in vitro* and *in vivo*.[11–13] Mer is activated by several ligands including Gas6, which is detectable at significant levels in plasma and bone marrow, and has been shown to activate PI3K/AKT, ERK1/2 and NF-κB.[14–19] Additionally, Mer activation occurs physiologically in the context of Gas6 (or other ligand) binding both Mer and phosphatidyl serine on membrane leaflets of apoptotic cells. The ubiquitous availability of Gas6 and apoptotic material could easily stimulate Mer if it is ectopically expressed on malignant cells. Within the hematopoietic lineage, Mer is normally expressed

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in tissue macrophages, natural killer cells, dendritic cells and megakaryocytes. Mer is not normally present on cells in any stage of lymphocyte differentiation; however, ectopic Mer expression in T-cell lymphoblastic leukemia of childhood has been reported.[11] Mer expression in normal myelopoiesis is largely unknown, and its expression has not been explored in the setting of AML.

In this manuscript we report that Mer is aberrantly over-expressed in a majority of pediatric and adult AML patient samples, when compared with normal myeloid progenitors. Mer is also expressed in the vast majority of AML cell lines. Activation of Mer in AML cell lines, produced prosurvival and proliferation signaling, including phosphorylation of ERK1/2 and AKT, as well as p38 MAPK (p38), MSK1, CREB, ATF1 and STAT6. Lastly, we show that inhibition of Mer via RNA interference in human AML cell lines leads to significantly increased apoptosis, decreased colony forming potential and delayed development of leukemia in immunocompromised mice. Therapeutically targeting aberrant Mer expression in AML is attractive, potentially allowing for better outcomes in high-risk patients, reduced intensity combination therapy in low-risk patients, or a therapeutic option for those who cannot tolerate high-intensity chemotherapy.

RESULTS

Mer is aberrantly expressed in AML

To evaluate Mer expression in AML, we assessed 14 different myeloid cell lines. Mer was detected by immunoblot in the majority of cell lines analyzed as a 150-180 kD protein (size variation has been attributed to differential glycosylation[18]). Flow cytometric analysis confirmed the findings; cells that were positive for Mer demonstrated increased fluorescence intensity compared with mouse immunoglobulin G1 isotype control stained cells, while negative cells did not (Figure 1 and Supplementary Table S1). Our analysis revealed a high degree of correlation between immunoblot and flow cytometric results, and indicated

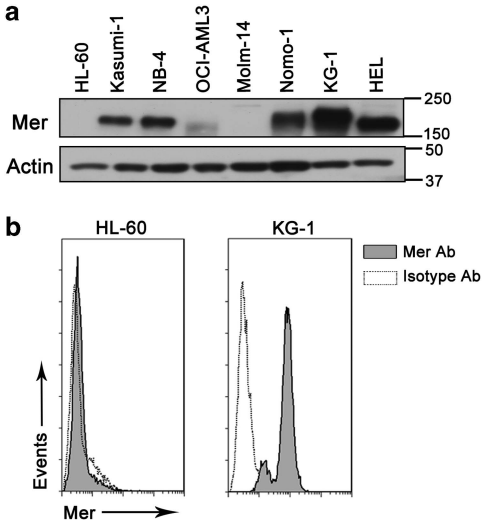


Figure 1. Mer is expressed in AML cell lines. (**a**) Whole-cell lysates derived from AML cell lines were subjected to immunoblot analysis with an anti-Mer antibody. This representative blot shows detection of the Mer protein (150–180 kD) in several AML cell lines. Actin was detected as a loading control. (**b**) Cell surface expression of Mer was determined by flow cytometry after staining with an anti-human Mer primary antibody and a PE-conjugated secondary antibody (gray histogram). Non-specific staining was determined using an IgG1 isotype control primary antibody and PE-conjugated secondary antibody (white histogram). Representative Mer negative (HL-60) and Mer positive (KG-1) cell lines are shown.

there was some degree of Mer expression in 86% (12 of 14) of AML cell lines. In this cell line analysis, Mer expression was not associated with any French-American-British (FAB) or World Health Organization (WHO) classification, cytogenetic abnormality, age of the patient from which the cell line was derived, nor did it correlate with other known clinical features (Supplementary Table S1).

Several AML bone marrow or peripheral blood samples from newly diagnosed patients were analyzed in the same manner, using both immunoblot and flow cytometric analysis. These samples also indicated a high degree of correlation between the whole-cell Mer protein levels visualized on immunoblot and the cell surface expression of Mer demonstrated by flow cytometry, thus we initiated a prospective analysis of newly diagnosed patient samples using flow cytometry alone. Parallel samples were stained with a primary Mer antibody or isotype control, and a secondary antibody. Gating was performed on cells stained with the IgG1 isotype control antibody and secondary antibody, in order to ensure that non-specific antibody binding was excluded from our analysis (Figure 2a). Using this gating strategy, we defined samples that had greater than 20% cells shifted out of the isotype + secondary antibody gating area as 'Mer-positive', and samples with 10–20% cells shifted out of the gate as 'Mer-dim.' Samples that had less than 10% of cells shifted were termed 'Mer-negative,' though samples in this category may still have some low level of Mer expression. Samples were evaluated at their respective institutions using this flow cytometric gating strategy within 24 h of bone marrow or peripheral blood collection. In total, we assessed 36 newly diagnosed pediatric samples and 10 newly diagnosed adult samples (Figure 2b and Supplementary Table S2). Approximately 80% (29 of 36) of samples from newly diagnosed pediatric AML patients, and 100% (10 of 10) of samples from newly diagnosed adult AML patients expressed Mer on leukemic blasts (that is, were Mer-positive or Mer-dim). In addition, 100% (10 of 10 pediatric, and 1 of 1 adult) of samples from patients who relapsed or had disease that was refractory to induction therapy expressed Mer; the majority of these exhibited high levels of cell surface Mer (Supplementary Table S3). There was also a trend towards higher Mer expression in pediatric relapsed and refractory patient samples when compared with the same patient's diagnostic sample; this did not reach statistical significance due to low-sample size.

Importantly, in the data presented, flow cytometry was performed within 24 h of patient sample collection. Frozen AML patient samples obtained from the Children's Oncology Group, which were collected and frozen at inconsistent time points, were subjected to both immunoblot and flow cytometric analysis (Supplementary Figures S1A and B) and showed decreased cell surface Mer expression relative to the samples which were processed within hours of acquisition (Supplementary Figure S1C). Additional control experiments revealed that Mer expression diminished significantly when patient samples were incubated at room temperature for extended periods (Supplementary Figure S1D). Furthermore, freezing and thawing patient samples also decreased surface Mer expression (data not shown). Mer is subject to extracellular domain cleavage and therefore loss of the Mer antibody epitope when cleavage occurs. After 36 h at room temperature, protein cleavage, as well as protein degradation, may contribute to the need to analyze fresh samples.

In contrast to the easily detectable Mer expression in AML blasts, Mer was not detected or detected only at low levels on the cell surface of normal myeloid progenitors. Bone marrow samples from eight healthy donors were obtained and flow cytometry performed within 24 h of collection. Cells were stained with CD34, CD33 and CD14 fluorophore-conjugated antibodies to evaluate each stage of myeloid maturation. Myeloblast and monoblast populations were defined as CD34 + /CD33 +, promyelocytes and promonocytes were defined by high forward scatter and CD33 high/CD14 −, and monocytes were defined as CD33 high/CD14 +. Mer surface expression by flow cytometry was analyzed

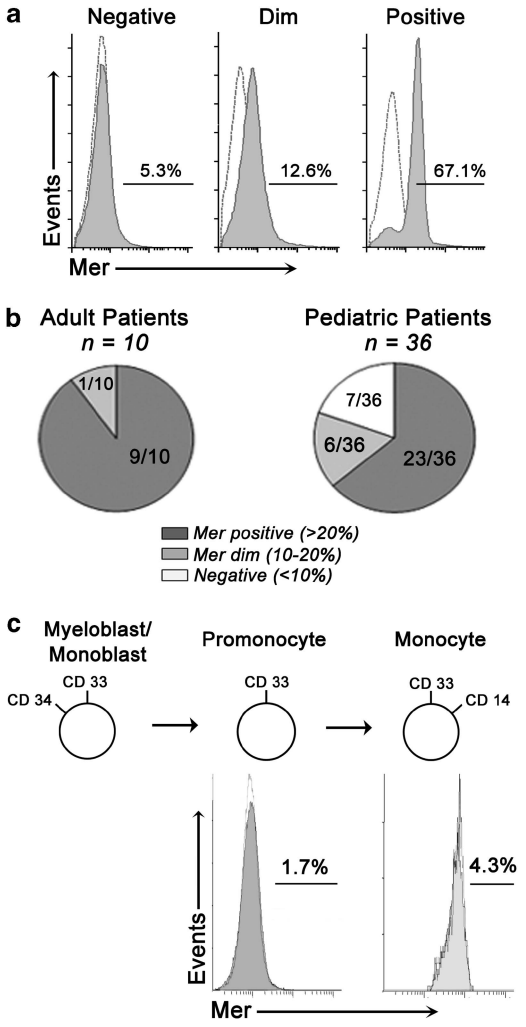


Figure 2. Mer is expressed in a majority of diagnostic bone marrow samples derived from patients with AML, but not in normal bone marrow. Myeloblasts collected at diagnosis from patients with AML were analyzed by flow cytometry for Mer expression. (**a**) Cells that expressed human CD33 and CD45 were analyzed after staining with a mouse anti-human Mer antibody and PE-conjugated secondary antibody to determine Mer expression (gray histogram). Non-specific staining was determined using a mouse IgG1 isotype control antibody and PE-conjugated secondary antibody (white histogram). Representative flow cytometry profiles for Mer-positive (>20% cells positive), Mer-dim (10–20% cells positive) and Mer-negative (<10% cells positive) patient samples are shown. (**b**) Graphic representation showing the fractions of Mer-positive, Mer-dim and Mer-negative pediatric (right panel) and adult (left panel) diagnostic primary patient samples. (**c**) Bone marrow samples from eight healthy donors were evaluated by flow cytometry for Mer RTK expression as described above. Myeloid progenitor populations were identified by gating on the cell surface markers noted in the figure. The fractions of cells expressing Mer in progenitor populations are shown. Myelo/monoblastic populations could not be quantified, due to low-cell number.

using gating as described above excluding non-specific binding of the secondary antibody. Using this gating strategy, only 1.7% of promonocytes and 4.3% of monocytes were Mer expressing (Figure 2c), thus being classified as Mer-negative (<10% Mer positive). It was not possible to accurately determine Mer expression for the normal marrow myeloblast/monoblast populations. Though several of these samples appeared to have no Mer expression, due to the extremely low cell number (203–865 total CD34+/CD33+ cells per sample), we cannot exclude the possibility that this population contains some level of Mer expression.

Mer activates prosurvival and antiapoptotic cellular pathways

To assess the function of Mer signaling in AML cells, we investigated downstream signaling pathways in two Mer expressing AML cell lines, which are negative for tyrosine kinase family members Tyro-3 and Axl: Nomo-1 and Kasumi-1 (Figure 3a). These cell lines were selected as representative of two different AML subtypes; Nomo-1 was derived from an adult acute monoblastic leukemia and Kasumi-1 was derived from a pediatric acute myeloblastic leukemia, with granulocytic maturation. Serum-starved Nomo-1 and Kasumi-1 cells were stimulated with Gas6 (a known Mer ligand), and phosphorylation of Mer was confirmed by immunoprecipitation (Figure 3b). A phospho-kinase array was used to screen for activation of signaling pathways following stimulation of AML cells with Gas6, compared with buffer-treated cells. Two independent replicates were performed, and relative changes in phosphorylation were determined. Following Gas6 treatment, the arrays suggested a 1.5-fold or greater increased phosphorylation of MSK1, AMPKα, AKT, mTOR, CREB, SRC family kinases SRC, LYN, YES, FGR and HCK, and STAT6 (Figures 3c and d). Immunoblot analysis was used to confirm phosphorylation of these proteins and was also used to assess several other downstream signaling candidates, including ERK1/2, p38 and AFT1, after Gas6 stimulation (Figure 3e). We confirmed previous analysis, which demonstrated phosphorylation of ERK1/2, AKT and p38 in response to Mer activation but stimulation of MSK1, CREB, ATF1 and STAT6 phosphorylation revealed new potential pathways downstream of Mer. These data indicate the potential for Mer activation to contribute to malignant cell survival and proliferation (Figure 3f).

Mer inhibition with Mer shRNA knockdown

To study the functional role of Mer in AML, we introduced short-hairpin RNA (shRNA) directed against Mer into the two cell lines used above. Nomo-1 and Kasumi-1 were transduced with lentivirus encoding one of two different shRNA constructs (shMer1 and shMer4) to decrease Mer expression, or a non-silencing shRNA (shControl), and stable clones were developed. Immunoblot analysis of whole-cell lysates was performed to assess the efficacy of Mer inhibition. Both knockdown clones efficiently decreased expression of Mer, with the shMer4 clone of each cell line having slightly more efficient Mer knockdown than the shMer1 clone (Figure 4a). These findings were confirmed by flow cytometric analysis for cell surface Mer expression, which demonstrated efficient knockdown for both constructs, with slightly more inhibition in cells with shMer4 (70–80% knockdown) than shMer1 (60% knockdown) compared with shControl (Figure 4b).

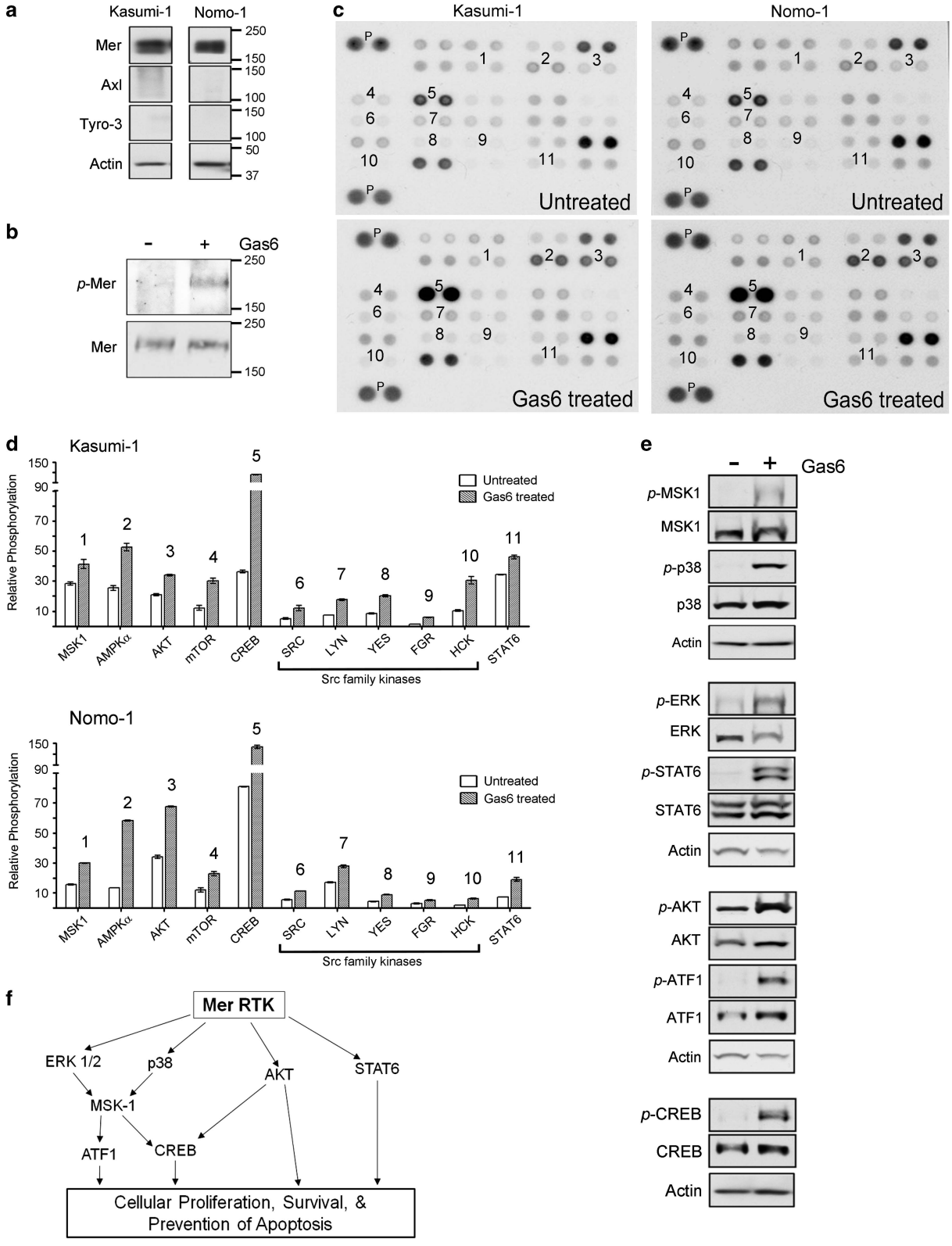
Mer inhibition using shRNA decreases p38, ERK and CREB phosphorylation

To assess which downstream pathways were most affected by Mer inhibition using shRNA, we further analyzed the proteins which demonstrated increased phosphorylation after Gas6 stimulation (that is, MSK1, p38, ERK, AKT, CREB, ATF1 and STAT6) using our Mer knockdown Nomo-1 and Kasumi-1 cell lines. When serum-starved cells were stimulated with Gas6, knockdown cell lines consistently demonstrated a concomitant decrease in phosphorylation of p38, ERK and CREB, compared with those treated with buffer alone (Figure 4c).

Inhibition of Mer induces apoptosis myeloblasts *in vitro*

The phenotypic effects of Mer inhibition in AML were assessed *in vitro*. Nomo-1 and Kasumi-1 cells with or without the shRNA

constructs were stained with Yo-Pro-1 iodide and propidium iodide after 18 h of serum starvation, and subjected to flow cytometric analysis. Cells taking up Yo-Pro-1 iodide alone (early apoptosis), and those taking up both Yo-Pro-1 iodide and propidium iodide (late apoptosis and necrosis) were included in the analysis; cells taking up only propidium iodide (dead cells) were excluded to eliminate the possibility of analyzing cells that had died before analysis. Cell lines with reduced levels of Mer demonstrated a higher degree of apoptosis; ~35% (shMer1) and 55% (shMer4) of cells were considered apoptotic in response to serum starvation. This was significantly greater than the 15–20% apoptosis observed in control cell lines ($P<0.05$ and $P<0.01$,



Figures 5a and b). The increase in apoptosis correlated with the level of Mer knockdown; cells containing the shMer4 construct exhibited more apoptosis (~50–60%) than those containing shMer1 (~35%).

Inhibition of Mer decreases proliferation of myeloblasts in vitro

To assess another malignant characteristic of myeloid leukemia cells, the shMer1, shMer4 or shControl cells were evaluated for colony forming potential. Cells were plated in equal number in methylcellulose, and myeloblast colony formation was assessed after 13 days. Compared with control cell lines, AML cell lines with reduced levels of Mer demonstrated a 67–87% decrease in total colony forming units ($P<0.01$, Figures 6a, b and d).

Reintroduction of Mer rescues myeloblast ability to evade apoptosis and form colonies

Kasumi-1 shMer1 cells were transduced with a retroviral vector containing a wild-type (WT) Mer gene (shMer1 + Mer WT), a kinase domain mutant Mer gene (shMer1 + Mer K619R), or a green fluorescence protein gene (shMer1 + NTV) (Figure 5c). Apoptosis was analyzed by flow cytometric analysis of the shControl and shMer1 cell lines along with the Mer WT add-back and K619R mutant Mer cell lines after 18 h serum starvation, as described above. Cells containing the green fluorescence protein were excluded in this assay due to fluorophore interference with the Yo-Pro-1 iodide. Reintroduction of WT Mer protein rescued the myeloblast's ability to evade stress-induced apoptosis to levels comparable to shControl cells (difference not significant), which was a significant decrease in apoptosis compared with shMer1 cells ($P<0.05$, Figure 5d). In contrast, shMer1 cells containing the Mer K619R kinase mutant demonstrated apoptosis levels similar to shMer1 (difference not significant). Additionally, Kasumi-1 shMer1 + Mer WT, shMer1 + Mer K619R and shMer1 + NTV cells were analyzed in methylcellulose. Reintroduction of WT Mer rescued the myeloblast's ability to form colonies comparable to parental and shControl cells (difference not significant, Figures 6b and c), which was a significant increase in the number of colonies formed compared with shMer1 + Mer K619R and shMer1 + NTV control cells ($P<0.01$) and shMer1 ($P<0.01$).

Mer inhibition increases leukemia-free survival *in vivo*

A mouse xenograft model of AML was developed to explore how decreased Mer expression affects myeloblast survival *in vivo*. Sublethally irradiated NOD-SCID-gamma mice were injected intravenously with the parental Nomo-1 cell line or Nomo-1 cell lines containing shControl or shMer constructs. Following injection of leukemic cells, mice were monitored for clinical signs of leukemia (weight loss exceeding 15% of body weight, inability to move effectively, lethargy). When mice became ill or at the end of the experiment (45 days post-injection) they were euthanized and relevant tissues were extracted. Single cell suspensions were prepared, stained with antibodies specific for human leukocyte markers, and analyzed by flow cytometry to confirm engraftment. Engraftment was defined as greater than 20% CD33+ and CD45+ cells in the bone marrow and/or spleen (data not shown). Symptom-free survival was determined for all mice with confirmed engraftment, and Kaplan–Meier curves demonstrated a statistically significant prolongation in survival ($P<0.0001$, Figure 7) in mice injected with Nomo-1 Mer knockdown cells relative to those injected with parental or shControl cells (median survival for parental and shControl was 17 days, 32 days for shMer1 and 36 days for shMer4).

DISCUSSION

We demonstrate here that the Mer tyrosine kinase is over-expressed in the vast majority of AML cell lines and leukemic patient AML blasts, when compared with the level of Mer expression in myeloid precursors from normal bone marrow. Mer was found on the surface of 80–100% of adult and pediatric patient samples at diagnosis, independent of the FAB, WHO or cytogenetic risk classification. Additionally, Mer cell surface expression appears to be higher in AML patient samples at the time of relapse or induction failure, though this data set is too small to determine statistical significance. The relation of Mer to patient prognosis was not determined in this study as the recent sample collection makes the time to follow-up insufficiently short to observe clinical course and relapse for the majority of our patients. Collection of samples from patients with AML and analysis of Mer expression is ongoing in order to address this question more effectively.

In contrast, normal promonocytes and monocytes in the bone marrow express undetectable to minimal levels of Mer. Though we were not able to fully determine the extent of Mer expression of myeloblasts and monoblasts, genetic loss of Mer in myeloid progenitors does not appear to have major clinical consequences, based on the observation that Mer knock-out mice exhibit no deleterious effects on myeloid cell production.[20]

The mechanism of Mer over-expression in AML is not clear; possible mechanisms include adaptations at the level of transcription or translation such as altered promoter methylation or microRNA regulation. Increased expression of Axl, another member of the Tyro-3/Axl/Mer tyrosine kinase family, in myeloid leukemia after treatment with chemotherapy has been found to be coincident with increased Axl promoter methylation.[21] In breast cancer, silencing of Mer-suppressive microRNA has been associated with increased metastasis.[22] Abnormal Mer expression in leukemia has not yet been linked to specific epigenetic alterations, and further evaluation of the mechanism of Mer over-expression in AML is warranted.

Figure 3. Mer stimulation in AML cells activates oncogenic signaling pathways. (**a**) Immunoblot analysis of Kasumi-1 and Nomo-1 cell lines confirmed they were positive for Mer (~180 kD), but negative for other Tyro-3/Axl/Mer family tyrosine kinases Axl (140 kD) and Tyro-3 (140 kD). (**b**) Kasumi-1 and Nomo-1 cells were incubated in serum-free RPMI medium then treated with 200 nM rhGas6 (Gas6 treated) or buffer (Untreated). Immunoprecipitate analysis demonstrating phosphorylation of Mer after 200 nM Gas6 treatment (+) compared with treatment with buffer (−). Total Mer was used as a loading control. (**c**) Similarly treated diluted lysates were incubated with human phospho-kinase array membranes and bound phospho-proteins were detected according to kit instructions. Each membrane contains positive control (P) antibodies spotted in duplicate. Proteins which demonstrated a 1.5-fold or greater increase in phosphorylation after stimulation with Gas6 (Gas6 treated) relative to buffer treated (Untreated) are marked by numbers between duplicate spots, which correlate with the identification numbers shown in (**d**). (**d**) Levels of the indicated phospho-proteins were quantified after normalizing pixel density of each positive control to 100. Untreated samples are denoted in white, and Gas6 treated cells are denoted in gray hatched lines, showing the difference in relative phosphorylation between the two conditions. Each bar represents the mean ± s.e. of duplicate spots. Increases were found in levels of phosphorylated MSK1 (S376/S360), AMPKα (T174), AKT (S473), mTOR (S2448), CREB (S133), SRC family kinases SRC (Y419), LYN (Y397), YES (Y426), FGR (Y412) and HCK (Y411) and STAT6 (Y641). (**e**) Kasumi-1 and Nomo-1 cells were incubated in serum-free media for 2–3 h followed by treatment with 200 nM rhGas6 (+) or buffer (−). Whole-cell lysates were subjected to immunoblot analysis with antibodies specific for the phosphorylated and total MSK1, p38, ERK1/2, STAT6, AKT, ATF1 and CREB proteins. Representative Nomo-1 immunoblot shown here. (**f**) Oncogenic downstream signaling pathways found to be activated in Mer-positive AML cells following stimulation with Gas6.

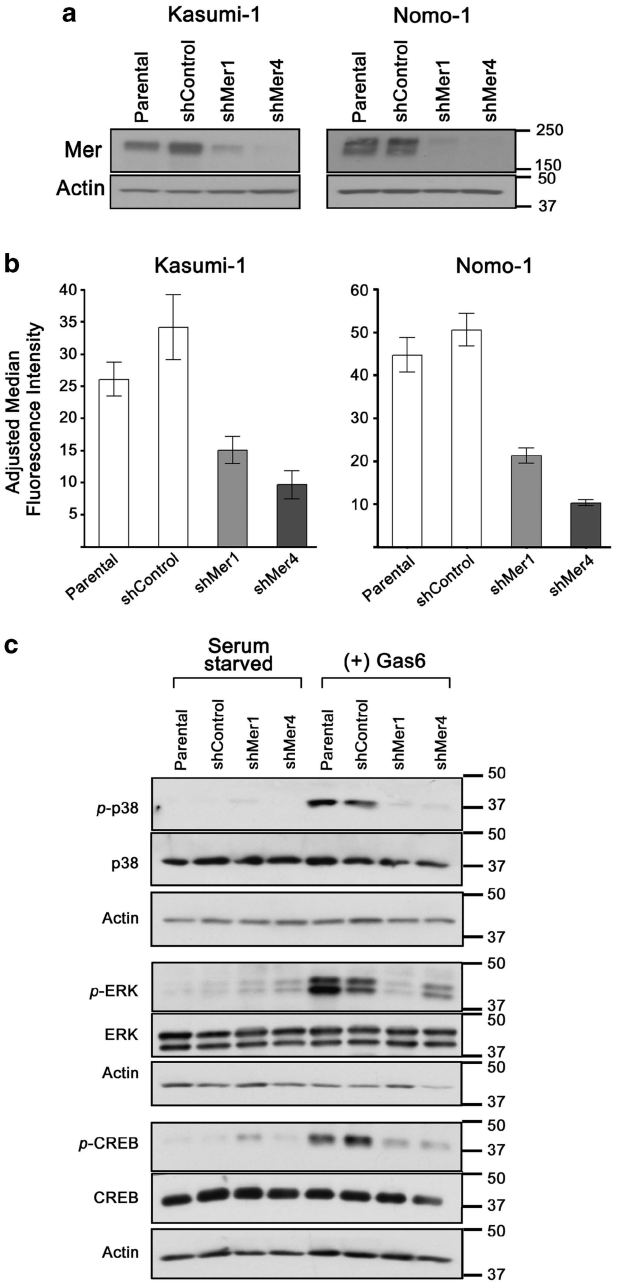


Figure 4. Mer protein expression is reduced by shRNA expression in AML cell lines. Kasumi-1 and Nomo-1 cells were infected with lentiviral particles containing short hairpin RNA (shRNA) constructs targeting Mer (shMer1, shMer4), or GFP as a non-silencing control (shControl). Knockdown of Mer was confirmed by (**a**) immunoblot analysis of whole-cell lysates and (**b**) flow cytometry using anti-Mer antibody. Values represent means and s.e. derived from three independent experiments. (**c**) Kasumi-1 and Nomo-1 parental and shRNA containing cells were incubated in serum-free media for 2–3h followed by a 10 min treatment with 200 nM rhGas6 ((+) Gas6) or buffer (Serum Starved). Whole-cell lysates were subjected to immunoblot analysis with antibodies specific for the phosphorylated and total proteins analyzed in Figure 3e. Of those, p38, ERK1/2, and CREB consistently demonstrated decreased phosphorylation corresponding to level of Mer, when stimulated with Gas6.

Mer activation in AML cell lines stimulates prosurvival pathways that are known to be important in AML pathogenesis. These studies demonstrate that Gas6 stimulated Mer-expressing AML cell lines increased phosphorylation of ERK1/2 and AKT, confirming earlier findings that these pathways are downstream of Mer in other cancer types.[10] Previous studies have emphasized the importance of the ERK1/2 and AKT pathways in maintenance of myeloblast survival, proliferation and evasion of apoptosis.[23,24] Our work also demonstrates several signaling molecules, which have not been previously described downstream of Mer and/or in Mer-positive human leukemias including p38, MSK1, CREB, ATF1 and STAT6. Activation of p38 has previously been described downstream of a Mer chimeric receptor consisting of the extracellular and transmembrane epidermal growth factor receptor domains and the Mer cytoplasmic domain.[25] Downstream of ERK1/2 and p38, MSK1 has been associated with malignant proliferation in AML,[26] but has not previously been assessed as a part of the Mer signaling cascade in malignancy. Activation of the ERK1/2 and p38 pathways in these AML cell lines by Gas6 ultimately led to phosphorylation of transcription factors CREB and ATF1, which have not previously been reported as part of Mer signaling. Phosphorylation and over-expression of CREB has been described in adult and pediatric AML, and has been linked to abnormal proliferation and survival of myeloid cells, and poor prognosis.[27–29] Similar in function to CREB, the ATF1 transcription factor has rarely been assessed in AML, though its activation has been associated with poor prognosis in AML in one study.[30] STAT6 is a member of a family of transcription factors that regulate cellular processes such as proliferation, differentiation and survival. STAT6 deficiency has been associated with increased numbers of committed myeloid progenitors in the bone marrow,[31] suggesting the possibility that STAT6 activation maintains myeloid progenitor immaturity. These data expand our knowledge of Mer activation by Gas6, suggesting a mechanism for proliferation and survival in myeloblasts.

Also reported here are the effects that inhibition of Mer has on myeloblast survival and proliferation *in vitro* and on leukemogenesis *in vivo*. Using shRNA, we effectively decreased Mer protein levels. This in turn decreased myeloblast cell line ability to stave off starvation-induced apoptosis, indicating that Mer functions to promote survival in AML cells. Consistent with this observation, colony formation in methylcellulose was also decreased in AML cells expressing shMer. Most importantly, reducing Mer expression significantly prolonged survival in a mouse model of AML. Interestingly, our data show correlation between Mer levels and phenotypes; whole-cell lysate and surface Mer expression was reduced to a greater extent in the shMer4 clone and had a greater effect on these measured phenotypes than the shMer1 clone. These results suggest that development of therapies directed against Mer should aim for the most effective degree of inhibition. Additionally, we demonstrate that when Mer knockdown cell lines are stimulated with Gas6, there was a decrease in phosphorylation of p38, ERK and CREB. These data suggest that these pathways may contribute to the growth and proliferation differences observed between the WT and shControl cell lines, and the shMer knockdown cell lines in our experiments.

These results also suggest that AML cell lines are reliant on Mer signaling to sustain viability under stress and *in vivo*. Additionally, these data demonstrate a rationale for anti-Mer targeted therapy in both adult and pediatric AML. Potentially, Mer inhibition could have a clinically significant effect in patients at both ends of the AML age spectrum, as either monotherapy or in combination with current AML therapies.

Currently, there are a few translational agents targeting Mer in preclinical development. One of these is a recently described Mer monoclonal antibody, which showed decreased tumor migration and survival in glioblastoma multiforme cell lines.[32] There are also selective Mer small molecule tyrosine kinase inhibitors in early development that effectively decrease Mer phosphorylation *in vitro* and *in vivo*.[33]

Our data provide proof of concept that Mer is a therapeutic target in AML and will likely lead to further development of Mer

targeted agents, including additional antibodies and/or specific small molecule inhibitors. These novel therapies have the potential to impact patients with AML, as well as patients with other malignancies characterized by Mer dependency, and may provide a means to improve survival with decreased toxicity relative to current standard chemotherapeutic regimens.

MATERIALS AND METHODS

Patient samples and cell culture

Bone marrow or peripheral blood samples were obtained from the Children's Hospital Colorado Oncology Tissue Bank, the University of North Carolina Hematolymphoid Malignancies Tissue Procurement Facility, or the Children's Oncology Group repository of AML patient samples. De-identified primary patient samples were collected after written consent was obtained in accordance with the Declaration of Helsinki, and all experiments were approved by the Colorado Multiple Institutional Review Board or the University of North Carolina Office of Human Research Ethics Institutional Review Board. AML samples that were analyzed when left out at room temperature were resuspended in 800 μl complete RPMI, then evenly distributed into four tubes (accounting for 12, 24, 36 and 48 h time points). When cells were harvested, both the cell pellet and supernatant were treated with lysis buffer as describe below (*Immunobloting and Signaling Assays*). Equal quantities of supernatant were loaded into SDS-polyacrylamide gel electrophoresis gels, though no loading protein was detected. Cell lines EOL-1, HEL, HL-60, K-562, KG-1, ML-2, MV4-11, NB-4, Nomo-1, OCI-AML3, THP-1 and UT-7 were obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany); Kasumi-1 and U-937 were obtained from the American Type Culture Collection (Manassas, VA, USA); Molm14 was a gift from Robert Arceci (Johns Hopkins, Baltimore, MD, USA). All cell lines except UT-7 were maintained in RPMI 1640 media (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C in 5% CO_2; UT-7 was maintained in 80% alpha-MEM with 5 ng/ml granulocyte-macrophage colony-stimulating factor and 20% FBS. For apoptosis and proliferation assays, cells were maintained in culture at logarithmic growth phase. All human cell lines were subjected to genomic fingerprinting via short tandem repeat microsatellite loci analysis to verify their identity.

Flow cytometry

At relapse or induction failure, flow cytometry was performed on patient bone marrow or peripheral blood within 24 h of sample collection at the institution ,in which the patient was diagnosed using identical protocols and antibodies. Pediatric samples were obtained and flow cytometric analysis performed at the Children's Hospital Colorado. Adult samples were obtained at the North Carolina Cancer Hospital and flow cytometric analysis performed at Lineberger Comprehensive Cancer Center. Samples were prepared using Immunoprep Reagent System (Beckman Coulter, Brea, CA, USA) or Ficoll-Paque separation. Cells were then stained with a mouse-anti-human Mer antibody or mouse IgG1 isotype control, washed with phosphate-buffered saline (PBS), stained with a PE conjugated donkey-anti-mouse secondary antibody, washed with PBS, stained with anti-human CD33 and anti-human CD45 fluorophore-conjugated antibodies, washed with PBS, then analyzed. During flow cytometric data acquisition, patient blasts populations (CD33 + /CD45 +) were indentified, and from these populations gating was set using the sample stained with

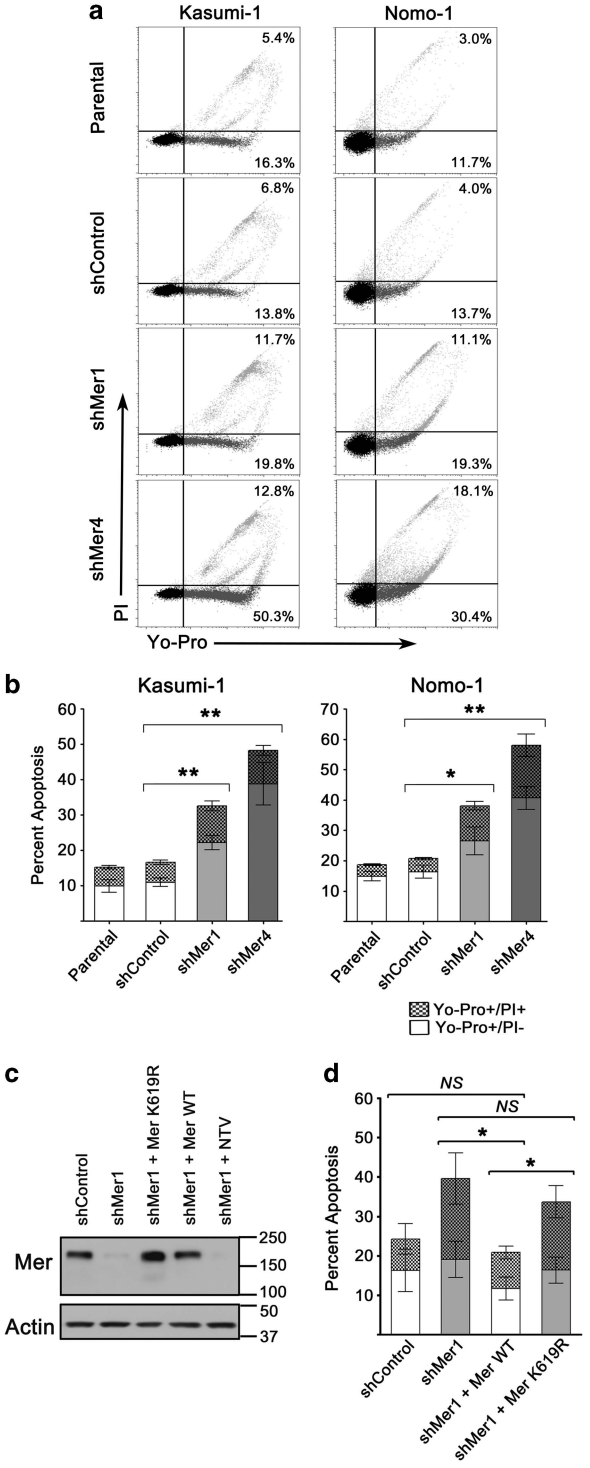


Figure 5. Mer knockdown confers susceptibility to apoptosis. (**a**) Parental, non-silencing control shRNA (shControl) and Mer knockdown (shMer1, shMer4) Kasumi-1 and Nomo-1 cells were serum starved or cultured in complete medium for 18 h before staining with propidium iodide and Yo-Pro-1 iodide. Apoptotic cells were identified by flow cytometry. Representative flow cytometry profiles are shown. The percentages of early apoptotic (lower right quadrant; Yo-Pro + /PI −) and late apoptosis/necrosis (upper right quadrant; Yo-Pro + /PI +) cells are shown. (**b**) Cumulative data demonstrate a significant accumulation of apoptotic cells in Kasumi-1 and Nomo-1 cells when Mer is inhibited by shRNA knockdown (*$P < 0.05$ and **$P < 0.01$ *versus* shControl). No significant differences between parental and shControl cells were observed. (**c**) Reintroduction of WT Mer into Kasumi-1 shMer1 knockdown cells rescues Mer protein level after knockdown (shMer1 + Mer WT). Additional control cell lines were generated by transduction of a gene for a kinase mutant Mer (shMer1 + Mer K619R), which maintains the extracellular epitope that binds the anti-Mer antibody, or a non-targeting vector (shMer1 + NTV) into Kasumi-1 shMer1 cells. Representative immunoblot of Mer protein levels shown here. (**d**) Apoptosis of Kasumi-1 WT add-back (shMer1 + Mer WT) and mutant Mer add-back (shMer1 + Mer K619R) cells were analyzed in comparison to shControl and shMer1 cells after serum starvation as described above. Mer WT add-back rescues myeloblast ability to stave off apoptosis comparable to shControl cell lines (*NS* = not significant compared to shControl, *$P = 0.05$ compared with shMer1 and shMer1 + K619R). Mean values and s.e. derived from at least three independent experiments are shown.

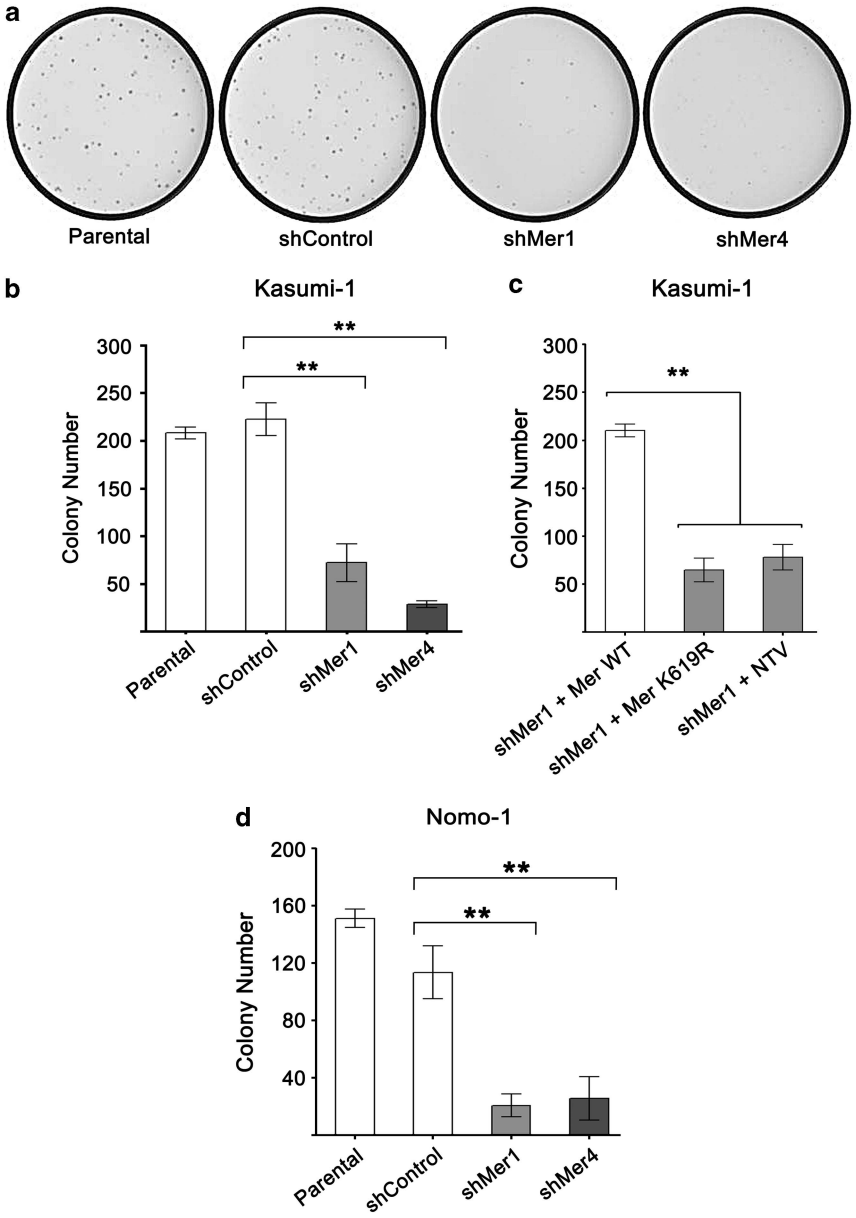


Figure 6. Mer knockdown decreases AML colony forming potential. Parental, non-silencing control shRNA (shControl), and Mer knockdown (shMer1, shMer4) Nomo-1 and Kasumi-1 cells were cultured in methylcellulose for 13 days. (**a**) Images of representative plates from the Nomo-1 cell line are shown. (**b** and **d**) Total colony number was determined demonstrating a statistically significant decrease in colony formation when Mer is inhibited (**$P<0.01$). (**c**) When Mer is reintroduced into shMer1 cells, colony forming potential is restored to shControl levels seen in (**b**) (difference not significant), which does not occur when Mer K619R mutant (shMer1 + Mer K619R) or non-targeting vector (shMer1 + NTV) are introduced into shMer1 cells (**$P<0.01$). Mean values and s.e. derived from at least three independent experiments are shown.

mouse IgG1 isotype control and PE secondary, such that two percent or less of the cells were outside the gating area. This gate was then applied to the patient sample stained with the Mer primary and PE secondary antibodies to determine the percent of cells expressing Mer. When assessing normal bone marrow samples, myeloid progenitor cells were identified by serial gating using forward and side scatter, CD33, CD34 and CD14 surface expression. Three populations were identified: CD34+, CD33+ and CD14− myeloblasts and monoblasts; high forward scatter, CD34+/−, CD33 high and CD14− promyelocytes and promonocytes; and CD34−, CD33 high and CD14+ monocytes. AML cell lines were assessed after staining with either a mouse IgG1 isotype control or a mouse anti-human Mer antibody and APC conjugated donkey-anti-mouse secondary antibody. The following antibodies were used for flow cytometric analysis of cell surface proteins: CD33 PE-Cy7, CD34 FITC (BD Biosciences, San Jose, CA, USA); CD45 PE-Texas Red, CD14 PE-Cy5 (Beckman Coulter); donkey-anti-mouse APC, donkey-anti-mouse PE (Jackson ImmunoResearch, West Grove, PA, USA); mouse IgG1 isotype control (R&D Systems, Minneapolis, MN, USA). Additionally a mouse-anti-human Mer monoclonal antibody (Mer 590) produced by the Graham laboratory as previously described[32] was used for all flow cytometric analysis of Mer expression. Fluorescence was assessed and analyzed using an FC-500 flow cytometer (Beckman Coulter) for pediatric patient and normal marrow samples, a MACSQuant flow cytometer (Miltenyi Biotec Inc., Cambridge, MA, USA) for adult patient samples, or a Gallios flow cytometer (Beckman Coulter) for cell lines, with CXP data analysis software (Beckman Coulter).

Immunoblotting and signaling assays

R&D Systems Human Phospho-Kinase Antibody Array (ARY003) was used to detect relative changes in kinase phosphorylation. Cells were plated at

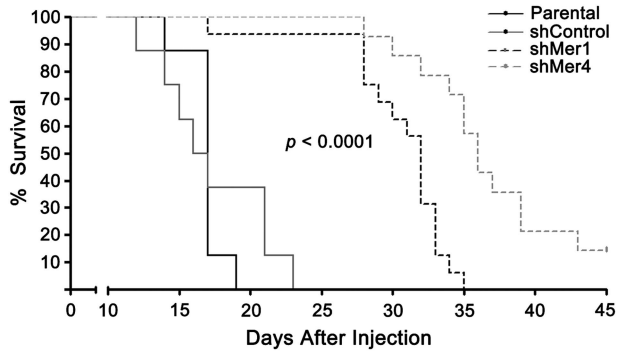


Figure 7. Inhibition of Mer expression prolongs survival in a murine model of AML. NOD-SCID-gamma mice were radiated with 200 cGy and transplanted with five million Nomo-1 parental (Parental, $n = 8$), non-targeting shRNA (shControl, $n = 8$) or Mer knockdown (shMer1 $n = 16$, shMer4 $n = 14$) cells by intravenous injection. Kaplan–Meier analysis comparing survival is shown demonstrating a statistically significant prolongation ($P < 0.001$) in symptom-free survival in mice injected with Mer knockdown cells compared with parental and shControl cells. No significant differences between parental and shControl cells were observed. Median survival was 17 days for mice injected with parental and shControl cells, compared with 32 days for mice injected with shMer1-expressing cells and 36 days for mice injected with shMer4-expressing cells.

3×10^6 in RPMI without FBS for 2–3 h, and treated with 200 nM recombinant human Gas6 (R&D Systems) or buffer for 10 min at room temperature. Remaining steps were performed according to manufacturer instructions. To determine relative changes in phosphorylation between buffer-and Gas6-treated cells, densities of individual spots were measured using ImageJ software (National Institutes of Health). For immunoblots, whole-cell lysates were prepared in a lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate and protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Lysates were incubated on ice for 15–20 min, then protein supernatant was collected after centrifugation. Lysates were resolved on 8% Tris-Glycine SDS-polyacrylamide gel electrophoresis gels (Invitrogen, Grand Island, NY, USA) and transferred onto polyvinylidene difluoride membranes. Membranes were blocked in a buffer of tris buffered saline with 0.1% Tween-20 containing 5% milk. The following antibodies were used for immunoblot analysis according to manufacturer recommendations: anti-human Mer (Epitomics Inc., Burlingame, CA, USA); anti-human Axl (R&D Systems); anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Tyro-3, anti-phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204), anti-p44/42 MAPK, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-p38 MAPK, anti-phospho-MSK1 (Ser376), anti-MSK1, anti-phospho-AKT (Ser473), anti-AKT, anti-phospho-CREB (Ser133), anti-CREB, anti-phospho-STAT6, anti-STAT6 (Tyr641) (Cell Signaling Technology, Danvers, MA, USA); anti-ATF1 (Up-state Millipore, Billerica, MA, USA); goat-anti-mouse IgG-HRP, goat-anti-rabbit IgG-HRP (BioRad, Hercules, CA, USA). Proteins were visualized by horseradish peroxidase chemiluminescence detection (Perkin-Elmer, Waltham, MA, USA).

Immunoprecipitation

Cells were plated at 3×10^6 in RPMI without FBS for 2–3 h, treated with 200 nM recombinant human Gas6 (R&D Systems) or buffer for 4 min, then 1:100 diluted hydrogen peroxide (a mild phosphatase inhibitor) was added for an addition 4 min. Cells were then resuspended in lysis buffer as described above. Anti-human Mer antibody (R&D Systems) and rec-Protein G-sepharose beads (Invitrogen) were then added, and lysates were incubated overnight on a rocking platform. Cells were washed twice, then resolved on 8% SDS-polyacrylamide gel electrophoresis gels, and transferred onto nitrocellulose membranes. Membranes were blocked in tris buffered saline with 0.1% Tween-20 containing 5% milk, then probed with an anti-phospho-Mer antibody (Phosphosolutions, Aurora, CO, USA). Membranes were then stripped and reprobed for anti-human Mer (Epitomics).

Production of shRNA clones

Two independent shRNA constructs targeting human Mer (shMer1, shMer4) and one non-silencing control shRNA construct directed against GFP (shControl) were used as previously described.[34] The shMer1 construct compliments the 3′ untranslated region of the Mer RNA, whereas the shMer4 sequence complime0nts the kinase-encoding region of the RNA. Stable clonal lines were derived from heterogeneous Mer knockdown populations by single-cell flow cytometry sorting. The shMer1 and shMer4 clones were selected for decrease in Mer expression. The shControl clone was selected for similar Mer expression to the parental cell line. Additionally, Kasumi-1 shMer1 clones were used to reintroduce WT Mer protein levels (shMer1 + Mer WT) using pLNCX2 add-back vectors (Clontech, Mountain View, CA, USA) which lack the 3′ untranslated region to evade shMer1 binding, as previously described.[32,35] Similarly, shMer1 cells were transduced with pLNCX2 vectors coding for Mer with a kinase domain mutation (shMer1 + Mer K619R) or green fluorescence protein (shMer1 + NTV) as controls.

Apoptosis and cell survival assays

Cells were counted by trypan blue exclusion using a Cedex XS Analyzer (Roche Applied Science). For apoptosis assays following shRNA knock-down, cells were cultured in medium with or without serum for 18 h. Cells were collected, washed twice with PBS and stained with propidium iodide and Yo-Pro-1-iodide (Invitrogen). The percentage of apoptotic cells was determined by flow cytometry using a FC-500 flow cytometer. For methylcellulose colony forming assays, cells were plated in triplicate at a density of 500 cells/plate in Colony Gel Human Base Medium methylcellulose (Reach Bio, Seattle, WA, USA) with RPMI media containing 10% FBS added per manufacturer instructions. Plates were incubated for 13 days at 37 °C with 5% CO_2, and colonies were counted using a GelCount automated colony counter (Oxford Optronix, Oxford, UK).

Mouse xenograft studies

Six- to 10-week-old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NOD-scid-gamma) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) or bred in-house and maintained under sterile conditions. Animals were irradiated with 200 cGy using a RS-2000 irradiator (Rad Source, Suwanee, GA, USA) within 24 h before tail vein injection with 5×10^6 viable human Nomo-1 AML cells in 0.1 ml sterile PBS. Mice were monitored daily and weighed twice weekly. Once mice appeared clinically ill or lost >15% of their body weight, they were euthanized and peripheral blood, bone marrow, spleen and any tumors or involved lymph nodes were harvested. Presence of AML was confirmed by flow cytometry after staining with anti-human CD45 FITC and CD33 PE-Cy7 conjugated antibodies (BD Biosciences). All experiments were conducted under the supervision of the facility's Institutional Animal Care and Use Committee according to an Institutional Animal Care and Use Committee–approved protocol.

Statistical analysis

Analysis was performed using Graphpad Prism 4 Software (La Jolla, CA, USA). Phospho-kinase array data represent means of two independent replicates of each cell line. Remaining *in vitro* data represent means of at least three independent replicates, and a paired *t*-test was used to compare knockdown samples to control shRNA samples. *In vivo* survival curve data were analyzed using a log-rank test. Results were considered statistically significant if the *P*-value was calculated at less than 0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)