FOXM1 Modulates Treatment Outcomes in Acute Myeloid Leukemia

BY

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THESIS

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This thesis is dedicated to my parents who always pushed me to challenge myself, my husband, Hassan Shah, without who's patience, and support it would never have been accomplished and my children, Danyal and Isa Shah, who are my lifelong inspiration.

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CONTRIBUTION OF AUTHORS

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TABLE OF CONTENTS

CHAPTER PAGE

I. INTRODUCTION……1

II. METHODS……...3
- A. Patient Population……3
- B. Instruments and Measurements……3
- C. Statistical Methods……...4

III. RESULTS……5
- A. Patient Characteristics……5
- B. Leukemia-Specific Outcomes ……..……6
- C. Direct Role of FOXM1 in the Development of Resistance……7
- D. Inhibition of FOXM1 Using shRNA or Pharmacologic Agents Induces Apoptosis and Inhibits Proliferation of AML Cells……..13
- E. Ixazomib Inhibits FOXM1 Protein Expression and Transcriptional Activity in Primary Patient-Derived AML Cells……..14
- F. Pharmacologic inhibition of FOXM1 Inhibits Leukemia in Vivo in a Xenograft Model……17

IV. DISCUSSION……20

CITED LITERATURE……23

APPENDIX……...……...27

VITA…….....33

LIST OF TABLES

TABLE PAGE

I.CLINICAL CHARACTERISTICS OF PATIENTS…..…….8

II. COX REGRESSION ANALYSIS FOR PREDICTORS OF RESISTANCE …….8

LIST OF FIGURES

FIGURE PAGE

1. FOXM1 predicts chemotherapy resistance in AML……9

2. FOXM1 confers resistance to standard chemotherapy. 12

3. Targeting FOXM1 using RNA inhibition or pharmacologic agents in vitro…….....15

4. Ixazomib inhibits FOXM1 in primary patient AML cells.....16

5. Pharmacologic inhibition of FOXM1 with ixazomib reduces leukemia disease burden in an in vivo AML model. 19

LIST OF ABBREVIATIONS

AML	Acute Myeloid Leukemia
ARA-C	Cytosine Arabinoside
BM	Bone Marrow
CR	Complete Remission
CI	Confidence Interval
FLT-3	FMS- like tyrosine kinase -3
FOXM1	Forkhead box protein M1
IHC	Immunohistochemistry
MG	Milligrams
ML	Milliliters
NPM	Nucleophosmin
OR	Odds Ratio
OS	Overall Survival
PI	Proteasome Inhibitors
RFS	Relapse Free Survival
µG	Micrograms
µL	Microliters
µM	Micromolar
UIC	University of Illinois at Chicago
WHO	World Health Organization

SUMMARY

Acute Myeloid Leukemia (AML) patients with NPM1 mutations, known to cause aberrant cytoplasmic re-localization, demonstrate a superior response to standard chemotherapy treatment. Early data from my mentor Dr. Gartel's lab established the importance of the physical interaction of these NPM and FOXM1 in cancer cells. We first wanted to link this well-documented favorable outcome of NPM1 mutant AML to the cytoplasmic re-localization and inactivation of FOXM1. We then went on to confirm the important role of FOXM1 in increased chemo-resistance in AML. A multi-institution retrospective study was conducted to link FOXM1 expression to clinical outcomes in AML. We established nuclear FOXM1 as an independent clinical predictor of chemotherapeutic resistance in intermediate-risk AML in a multivariate analysis incorporating standard clinicopathologic risk factors. Using in vitro assays we showed that manipulation of nuclear FOXM1 affected the clonogenic activity of AML cells. In order to further prove a potential role for FOXM1 in AML chemo-resistance, we induced a FOXM1-overexpressing transgenic mouse model of leukemia and demonstrated significantly higher residual disease after standard chemotherapy. This suggests that constitutive overexpression of FOXM1 in this model induces chemo-resistance. Finally, we performed proof of principle experiments using a currently approved drug 'Ixazomib' to target FOXM1 and demonstrated a therapeutic response in patient samples and animal models of AML that correlates with the suppression of FOXM1 and its transcriptional targets. The addition of low doses of Ixazomib increases sensitization of AML cells to standard chemotherapy. Our results underscore the importance of FOXM1

in AML treatment outcomes and suggest targeting it may have therapeutic benefit in combination with standard AML therapies.

I. INTRODUCTION

(Previously published as Khan I, Halasi M, Patel A, Schultz R, Kalakota N, Chen YH, Aardsma N, Liu L, Crispino JD, Mahmud N, Frankfurt O, Gartel AL. (2018) FOXM1 contributes to treatment failure in acute myeloid leukemia. JCI Insight. 2018 Aug 9;3(15) 1-17)

The outcomes for acute myeloid leukemia (AML) have remained abysmally poor for the past 30 years. 20- 40% of patients fail to achieve remission with induction chemotherapy, and 50-70% of patients who achieve a complete remission relapse within 3 years. Therefore, development of novel therapeutic strategies for the treatment of AML is extremely important. Identification of recurrent cytogenetic abnormalities led to the classification of patients into prognostic subgroups which guide the intensity of therapy(1). However, 40% to 50% of AML cases are cytogenetically normal (CN-AML) and, biologically and clinically, are the most heterogeneous group. A major breakthrough in dissecting out prognostic subgroups came with the discovery of the nucleophosmin1 (NPM1) mutation in 50% of cases of CN-AML. Nucleophosmin1 is mutated in 1/3 of cases of AML(2) leading to its aberrant cytoplasmic sequestration. In multiple subsequent analyses patients with AML bearing mutated NPM1 (NPM1c+) showed improved overall survival (OS) and relapse-free survival (RFS)(3, 4). These patients did not benefit from allogeneic transplant due to excellent results with chemotherapy alone. Because of its distinctive molecular, pathologic, and clinical characteristics, NPM1c+ AML was included as a distinct entity in the 2016 WHO classification of hematologic neoplasms (5). Gene expression profiling confirms a distinctive expression profile for NPM1c+ AML(6).

It has been shown that across multiple tumor types, NPM binds to FOXM1 (7) and their interaction is required for sustaining the level and nuclear localization of FOXM1. Although NPM1 mutations were first identified in AML in 2005 (2), the mechanism by which the NPM mutant protein contributes to favorable prognosis remains elusive. We hypothesized that the mechanism underlying the beneficial effect of the NPM1 mutation in AML may be the cytoplasmic relocalization and inactivation of the oncogenic transcription factor FOXM1.

Forkhead box M1 (FOXM1) is a transcription factor of the Forkhead family that induces the expression of genes involved in the execution of the mitotic program (8). Forkhead box protein M1 is expressed in dividing and transformed/tumor cells, but not in terminally differentiated or quiescent cells and contributes to several aspects of oncogenesis.(9). A FOXM1 regulatory network was a major predictor of adverse outcomes in a meta-analysis of expression signatures from ~18 000 human tumors.(10) Also, FOXM1 mRNA was overexpressed in all of 127 examined acute myeloid leukemia (AML) patient samples and immunofluorescence revealed much stronger nuclear FOXM1 expression in leukemic progenitor cells compared with normal counterparts where it was postulated to be driving increased proliferation.(11)

In this study, we examine the clinical relevance of FOXM1 as a predictor of chemo-resistance in intermediate-risk AML, a group comprising >50% of newly diagnosed AML cases. Genomic profiling has attempted to fragment this category to allow more risk-adapted treatment approaches (12). However, interpreting the prognostic effects of individual mutations in AML is impeded by their interactions with other driver mutations.

III. METHODS

(Previously published as Khan I, Halasi M, Patel A, Schultz R, Kalakota N, Chen YH, Aardsma N, Liu L, Crispino JD, Mahmud N, Frankfurt O, Gartel AL. (2018) FOXM1 contributes to treatment failure in acute myeloid leukemia. JCI Insight. 2018 Aug 9;3(15) 1-17)

A. Patient Population

We identified 111 consecutive adults with the diagnosis of intermediate-risk AML receiving routine medical care at UIC and Northwestern Memorial Hospital between January 2003 and January 2015. The protocol was approved by the Institutional Review Board at both institutions prior to undertaking the chart review. Laboratory and de-identified clinical data at the time of initial presentation were extracted from the electronic medical record charting system, Cerner PowerChart and EPIC at UIC and NMH respectively. Tissue blocks were retrieved at each institution, reviewed by hematopathologist ate each institution for quality of the tissue and then sectioned and 2-4 slides per subject were shipped to the research Histology Core at UIC.

B. Instruments and Measurements

Following staining for FOXM1, slides were scanned on a Aperio AT2 whole slide scanner (Leica Biosystems) at 20× magnification. The images were analyzed using HALO 2.0 software (Indica Labs). The regions of interest (ROI) containing hematopoietic cells were manually selected. Hematoxylin counterstain was used to segment nuclei within the ROIs and to establish an accurate cell count. Threshold values were set for each slide to determine nuclei positive for the FOXM1 marker. Forkhead box protein M1 staining intensity in the cytoplasm of each cell was measured within a radius of approximately 1 μm grown around each nucleus. The nuclear and

cytoplasmic FOXM1 intensity, as well as their ratio, was calculated per each cell and averaged per each slide.

Tissue sections were stained on Bond RX autostainer (Leica Biosystems) following a preset protocol. In brief, sections were deparaffinized, subjected to EDTA-based (Bond ER2 solution, pH 9) antigen retrieval for 40 minutes at 100°C and blocked with hydrogen peroxide for 5 minutes. After washing with Bond Wash Solution, sections were incubated with anti-FOXM1 antibody (1:250, Abcam, ab207298) for 30 minutes. The detection was performed using Bond Polymer Refine Detection kit (Leica Biosystems, DS9800). All slides were counterstained with Mayer's hematoxylin for 5 minutes and mounted with Surgipath Micromount Media (Leica Biosystems).

C. Statistical Methods

For the clinical data on AML patients, OS time was measured from the date of diagnostic BM biopsy to death, with censorship at the date of last contact. Disease-free survival (DFS) was measured from the date of CR to the first relapse or death; patients alive and in CR were censored at the date of last contact. Two-tailed *t* tests (unless otherwise specified) or Kruskal-Wallis tests (for skewed variables) were used to compare means or medians of quantitative variables between response status, and χ_2 test to compare categorical variables. Logistic regression was used to assess associations with CR. The Kaplan Meier method was used to describe OS, event-free survival (EFS), and DFS. Cox regression models were used to assess associations with these outcomes. All statistical analysis was done in collaboration with the Design and Analysis Core at UIC.

IV. RESULTS

(Previously published as Khan I, Halasi M, Patel A, Schultz R, Kalakota N, Chen YH, Aardsma N, Liu L, Crispino JD, Mahmud N, Frankfurt O, Gartel AL. (2018) FOXM1 contributes to treatment failure in acute myeloid leukemia. JCI Insight. 2018 Aug 9;3(15) 1-17)

A. Patient Characteristics and Treatment Patterns

Patients >18 years of age with AML with intermediate-risk cytogenetics(13) were enrolled in this multi-institution retrospective study. We focused on intermediate-risk patients where there is an unmet need for discerning prognostic markers. Clinical characteristics are summarized in Table I. There were a total of 111 patients with a median age of 61 years, with an interquartile range of 51–69 years. There was no sex bias, and the combined population of the 2 institutions had broad racial and ethnic representation, with 51% patients of nonhispanic white, 18% black, 12% hispanic, and 19% subjects reported as other. The median BM blast percentage of 60% reflected that the biopsies were heavily infiltrated with leukemia so the imaging data was reflective of the cells of interest. A total of 87% of the patients had a normal karyotype. Consistent with published prevalence data, 42 of the 100 patients (42%) who underwent molecular testing had an NPM1 mutation, and 31 of 107 patients tested (28%) had a FLT3-ITD mutation.

Induction therapy included cytarabine and an anthracycline in 88 cases and hypomethylating agents (HMA) in 16 cases. Five patients were managed with palliative care, and 2 were transferred to other institutions. Twenty-nine subjects were enrolled on clinical trials for induction therapy. These included SWOG-1203, ECOG-2906, AAML1031, CALGB 10603, and investigator-initiated trials.

Out of 88 patients treated with chemotherapy, 80 (91%) eventually achieved a complete remission with or without count recovery (CR or CRi, respectively) as defined by International Working Group criteria(14) . Lines of induction therapy needed to achieve remission served as a measure of chemotherapy resistance. Patients were stratified as needing 1 or >1 line of induction therapy. FOXM1 expression data was available on the biopsies of 74 patients who achieved a remission, and the corresponding pixilated images were quantified.

B. Leukemia- Specific Outcomes and Correlation with FOXM1 Expression

There were 50 patients of the 74 who achieved a CR with 1 cycle of induction chemotherapy and 24 patients who required more than 1 cycle. We found that patients needing >1 line of induction therapy had more than a 2-fold increase in the percentage of nuclei expressing FOXM1 in their BM biopsy compared with responding patients (mean 25.6% vs. 11.4% nuclei, $P = 0.004$) (Figure 1 A,B). The average nuclear intensity of FOXM1 was also significantly higher in patients who failed their first line of induction (mean OD, 0.22 vs. 0.16; $P = 0.02$). In regression analysis, the percentage of FOXM1-positive nuclei significantly predicted resistance to first-line chemotherapy with an odds ratio (OR) of 1.80 for a 10% increase in positive nuclei ($P = 0.005$). The average nuclear intensity of FOXM1 in the pretreatment BM was also a significant predictor of chemotherapy resistance (OR 2.5 for 0.1 U increase in OD, $P = 0.02$). In the multivariate logistic regression model (Table II) assessing the effects of FOXM1 variables on resistance to first-line chemotherapy, we adjusted clinical-pathologic risk factors including age, WBC count at presentation, and presence of the FLT3-ITD mutation. Due to inter-institution variability in consolidation strategies, survival analysis

was done independently for each institution. FOXM1 nuclear/cytoplasmic (N:C) ratio, as well as average nuclear FOXM1 intensity, were able to predict inferior overall survival (OS) in a single institution cohort (*n* = 43) (Figure 1C) using Cox regression analysis (HR = 4.7 for every 0.1unit increase in N:C ratio, *P* = 0.03; HR = 4.27 for every 0.1 unit increase in OD, *P* = 0.06). In addition, in this single-institution survival analysis, FOXM1 N:C ratio was an independent predictor of OS in a multivariate analysis including FLT3-ITD, NPM1 mutation, BMI, age, and WBC.

C. Direct Role of FOXM1 in the Development of Resistance

The effect of standard-of-care AML therapies on the expression of FOXM1 was investigated. Using a panel of AML cell lines including KG-1 (Figure 2A), HL-60, and THP-1 (data not shown), we show clear evidence of FOXM1 upregulation in the total cell lysate within 24 hours of exposure to chemotherapy. This rapid upregulation of FOXM1 in response to most standard therapies used in the treatment of AML; including doxorubicin, cytarabine and azacitidine - suggests that this may be a common mechanism of resistance utilized by AML cells.

To establish whether FOXM1 regulates chemotherapy resistance in myeloid leukemia, we utilized previously developed transgenic (Tg) mice lacking the alternative reading frame (ARF) tumor suppressor: (a) $Arf^{-/-}$ C57BL/6 and (b) Rosa26-FoxM1b Tg;$Arf^{-/-}$ C57BL/6 (15). We are utilizing FOXM1 expression in the $Arf^{-/-}$ background to overcome sustained expression of ARF, which is a potent inhibitor of FOXM1 transcriptional activity. Following 5-fluorouracil (5-FU) treatment, hematopoietic progenitor cells (HPCs) from both $Arf^{-/-}$ and FoxM1b Tg;$Arf^{-/-}$ mice were collected and transduced with FLT3-ITD retrovirus and transplanted into lethally irradiated recipients.

Table I: CLINICAL CHARACTERISTICS OF PATIENTS

Characteristics	Number (%) / Median (IQR)	N analyzed
Institution	NMH: 45 UIH: 66	111
Age (years)	61; IQR=51-69	111
Gender (male:female)	54%: 46%	111
Race (Caucasian, Black, Hispanic, Other)	C: 57 (51%) B: 20 (18%) H: 13 (12%) O: 21 (19%)	111
WBC ($x10_3$/dl)	14.6; IQR=3-49.4	111
BMI	28.2; IQR=24.4-33.2	108
Bone marrow blasts %	60%; IQR= 33-78	105
Hemoglobin (g/dl)	8.8; IQR=7.8-10.1	111
Platelets	61; IQR=34-110	111
LDH	318; IQR=225-531.5	111
Molecular abnormalities	$NPM1_{mut}$: 42 (42%) FLT3-ITD: 30 (28%) CEBPa (biallelic): 6 (5.9%)	100 107
Cytogenetics (int risk)	Normal: 99 (90%) Del20q: 2 (2%) Trisomy 8: 3 (2.7%) [a]Other: 10 (9%)	110
Cytarabine /Anthracycline induction (n%) Enrolled on clinical trials (ECOG2906, SWOG1203, AAML1031)	88 (84) 29 (28)	105
Hypomethylator, n (%)	16 (15)	105
Complete Remission Rate, n (%)	81 (77)	105
• CR/CRi after one cycle of induction chemotherapy	52 (66)	105
• Number of patients needing>one cycle induction	21(26)	105
Refractory Disease, n (%)	6 (8)	105

[a] Other cytogenetic abnormalities included inv9, t(8;16),+ 4, t(9;11),+14, +6,+13,t (1;22), - Y

Table II: COX REGRESSION ANALYSIS FOR PREDICTORS OF RESISTANCE

Variable	**Hazard Ratio**	**95% CI**	**p-value**
Age at diagnosis (>60)	1.6	0.48-5.42	0.683
WBC	0.99	0.97-1.01	0.184
FLT3-ITD mutation	0.47	0.09-2.49	0.374
% nuclei FOXM1 positive	**1.70**	**1.1-2.6**	**0.018**

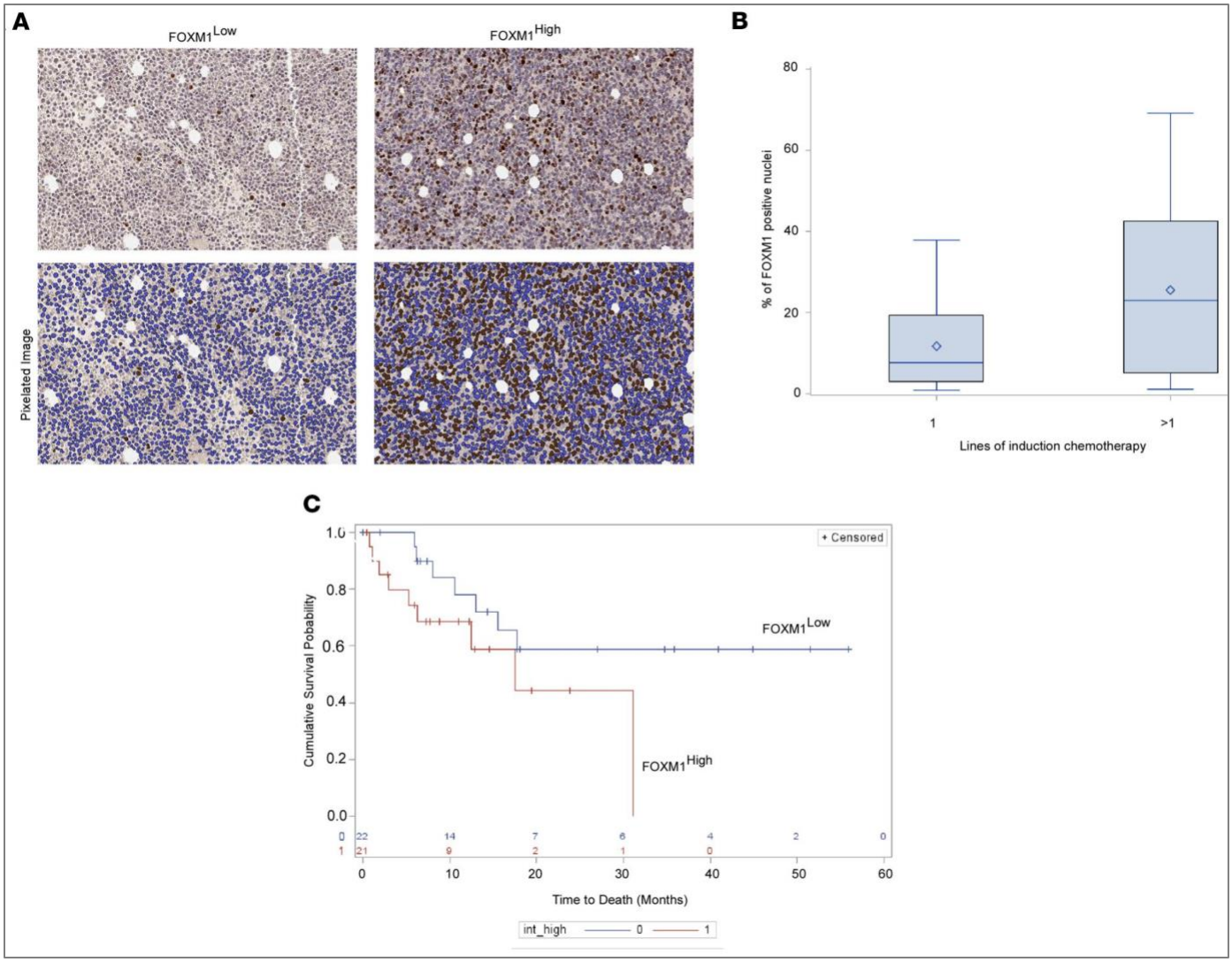

Figure 1. FOXM1 predicts chemotherapy resistance in AML. (**A**) Two patient BM slides (200× magnification) with the corresponding markup images below (**B**) Nuclear FOXM1 expression levels stratified by clinical chemoresistance (**C**) Kaplan-Meier analysis for overall survival stratified based on average nuclear intensity of FOXM1.

All the animals developed a myeloproliferative neoplasm (MPN) characterized by leukocytosis and splenomegaly, as previously described (16). Engraftment was similar between genotypes, as confirmed by peripheral blood (PB) chimerism on day 14, and animals were randomized to treatment with vehicle versus cytarabine 75 mg/kg intra-peritoneally. for 5 consecutive days. The animals were sacrificed 3 weeks following treatment, and blood, BM, and spleen were analyzed for disease assessment. The MPN developed on a FOXM1-overexpressing background showed significantly higher resistance to standard chemotherapy, as evidenced by increased leukemic burden in the BM, spleen, and blood by GFP assessment using flow cytometry analysis of mononuclear cells (Figure 2, B and C) and by larger spleen size. Peripheral smears and blood counts showed persistently elevated circulating myeloid cells following treatment in the FOXM1-overexpressing model.

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Given the putative role of the leukemia-initiating cells (LICs) in mediating relapse, we also explored the effect of chemotherapy on the LIC population in the context of FOXM1 overexpression. In a parallel experiment, BM mononuclear cells (MNCs) from FOXM1-overexpressing and control FLT3-ITD leukemic mice were plated in colony assays 1 week following cytarabine treatment. To assess the residual burden of LICs in the FoxM1b Tg;Arf$^{-/-}$ mice compared with Arf$^{-/-}$ littermates, we did serial replating assays. Serial re-ating resulted in increasing plating efficiency in the cells overexpressing FOXM1 that may contribute to the chemotherapeutic resistance in these animals (Figure 2, D and E). The rapid resurgence of disease and increased re-plating ability within weeks of chemotherapy treatment suggests FOXM1 is a critical mediator in the emergence of resistant leukemic clones.

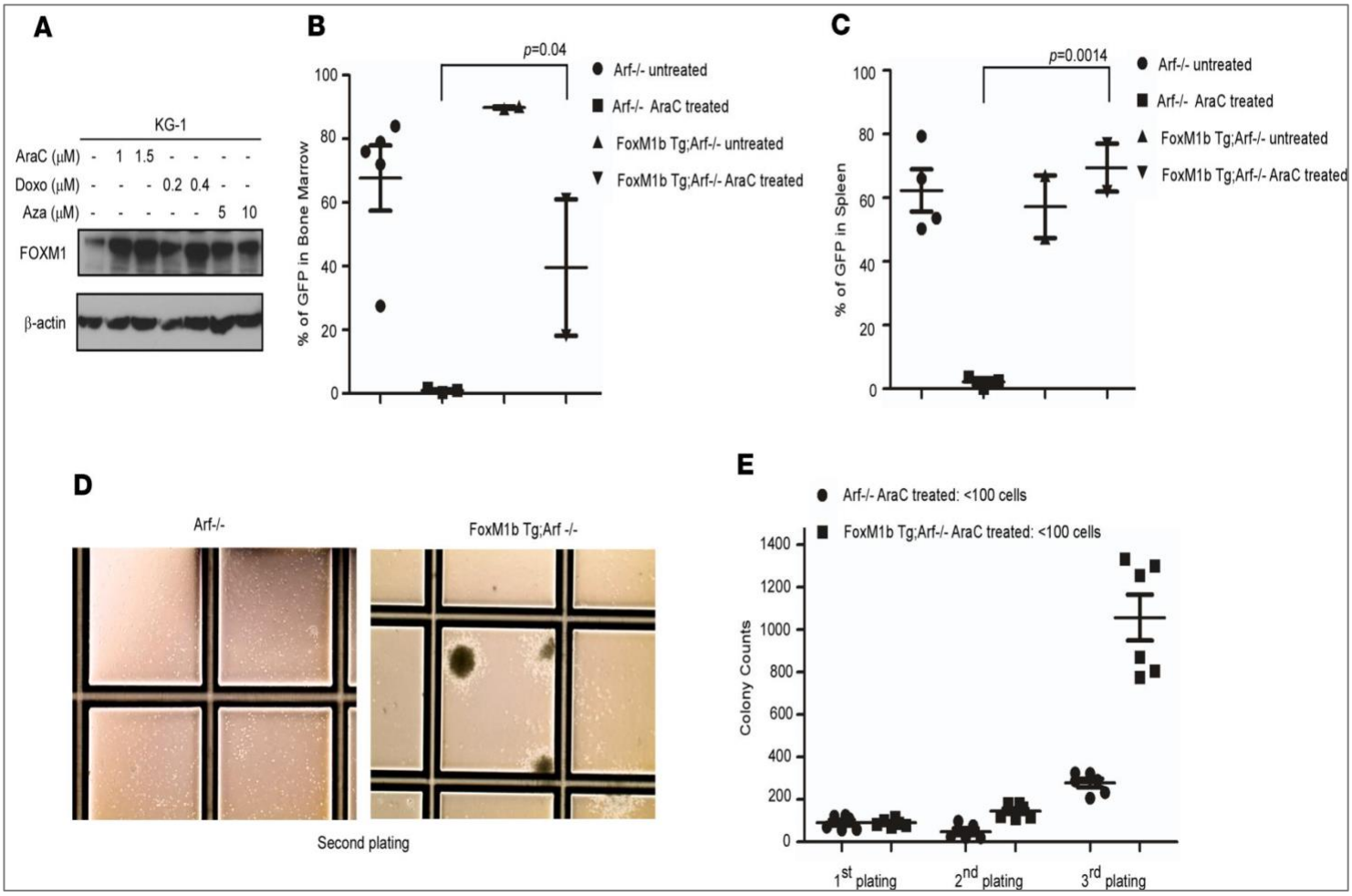

Figure 2. FOXM1 confers resistance to standard chemotherapy. (**A**) Western blotting analysis of FOXM1 levels. GFP expression in the BM (**B**) and the spleens (**C**) following treatment in transgenic murine AML model (*n* = 4/group). (**D, E**) Serial replating colony assays. Colonies were imaged with the EVOS XL Core Imaging System (4X)

D. Inhibition of FOXM1 Using sh RNA or Pharmacologic Agents Induces Apoptosis and Inhibits Proliferation of AML Cells

Inhibition of FOXM1 expression using short hairpin RNA results in reduced clonogenic activity of AML cells (Figure 3,A and B). Our lab has previously shown that FOXM1 is a general target of proteasome inhibitors (PIs).(17) The mechanism underlying this effect is stabilization of HSP70, which is a negative regulator of FOXM1. (18) Ixazomib is an oral PI that is approved for the treatment of relapsed multiple myeloma and is very well tolerated. (19). Second, the PI ixazomib was tested for in vitro effect on FOXM1 in AML cells. Ixazomib inhibits the transcriptional activity of FOXM1 using a luciferase reporter osteosarcoma cell line with inducible FOXM1 (Figure 3C).

We then assessed the mRNA levels of FOXM1 and its transcriptional targets. Treatment with ixazomib resulted in dose-dependent inhibition of FOXM1 and its transcriptional targets aurora kinase B (AurkB), Cdc25B, and polo-like kinase 1 (Plk1) (Figure 3D). Forkhead box protein M1 binds to its own responsive regulatory elements as part of an autoregulation loop (20), and therefore, FOXM1 mRNA levels are also downregulated following ixazomib treatment. Dose-dependent FOXM1 protein inhibition by ixazomib is accompanied by stabilization of HSP-70 and increasing levels of apoptosis in KG-1, HL-60 (Figure 3, E and F) cell lines. Our data suggest that, in AML, ixazomib at nanomolar doses is a potent FOXM1 inhibitor, which may be contributing to its antileukemic activity.

We did additional experiments using BM MNCs from FLT3-ITD–induced leukemia in FOXM1-overexpressing and control transgenic mice. The GFP-positive transformed cells were sorted and subsequently exposed to a range of doses of

ixazomib from 1–30 nM and plated in colony-forming assays (Figure 3G), and they were examined for cell death at 24 hours by flow cytometry (Figure 3H). The results show that overexpression of FOXM1 reverses the anti-leukemic activity of ixazomib, thereby implicating FOXM1 in mediating the anti-leukemic activity of this drug.

E. Ixazomib Inhibits FOXM1 Protein Expression and Transcriptional Activity in Primary Patient-Derived AML cells

Ixazomib inhibits FOXM1 in primary AML samples and induces apoptosis. Mononuclear Cells were used in ex vivo studies to evaluate the effects of ixazomib on inhibition of FOXM1 in patient-derived AML cells. We assessed 13 patient samples treated in liquid culture. Cells were incubated with DMSO or 75 nM ixazomib for 24 hours and then RNA extracted. The mean of 13 samples is shown, with FOXM1 mRNA expression normalized to DMSO-treated cells. Ixazomib causes significant downregulation of FOXM1 mRNA (Figure 4A), as well as FOXM1 canonical targets AurkB, Cdc25B, and Plk1 in primary AML cells. Inhibition of FOXM1 protein expression was confirmed by Western blot with associated induction of apoptosis, as detected by caspase 3 cleavage (Figure 4B). Cytospin preparations of AML MNCs treated ex vivo with ixazomib show significant downregulation of nuclear FOXM1 by IHC (Figure 4, C and D).

Treatment of patient samples (n = 5) with ixazomib resulted in a 2-fold increase in cell death by annexin staining (Figure 4, E and F). Colony assays show inhibition of colony-forming activity in 4 patient samples (Figure 4, G and H). These findings in primary AML cells recapitulate the observation in cell lines that ixazomib inhibits FOXM1, resulting in inhibition of colony-forming activity and induction of apoptosis.

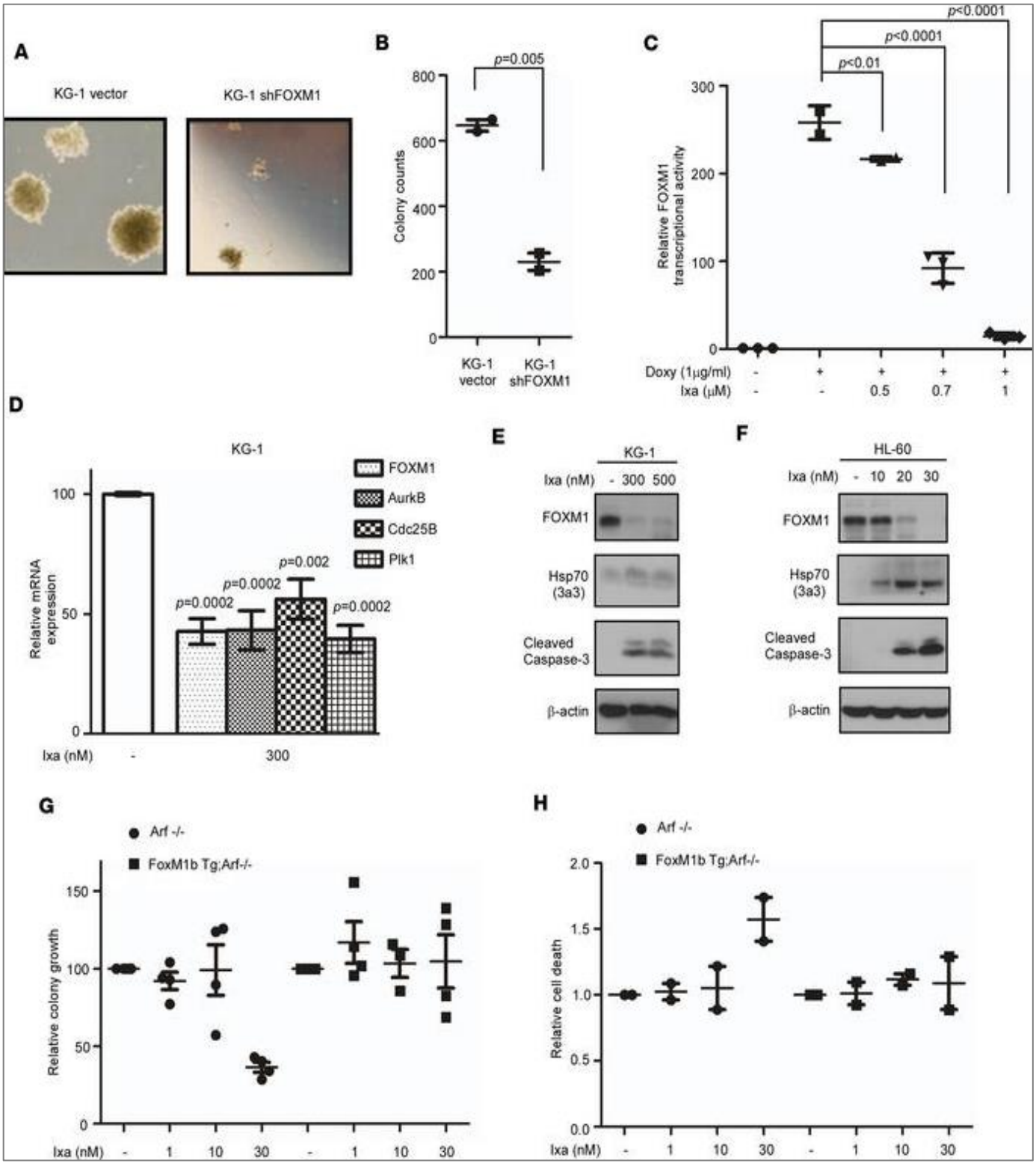

Figure 3. Targeting FOXM1 using RNA inhibition or pharmacologic agents in vitro. (A,B) Colony size and numbers after FOXM1 knockdown. **(C)** FOXM1 transcriptional activity using a luciferase assay. **(D)** Quantitative PCR for FOXM1, AurkB, Cdc25B, and Plk1. **(E,F)** Suppression of FOXM1 protein expression with stabilization of HSP-70 and caspase-3 cleavage. **(G,H)** Colony forming activity and cell death by annexin after ixazomib.

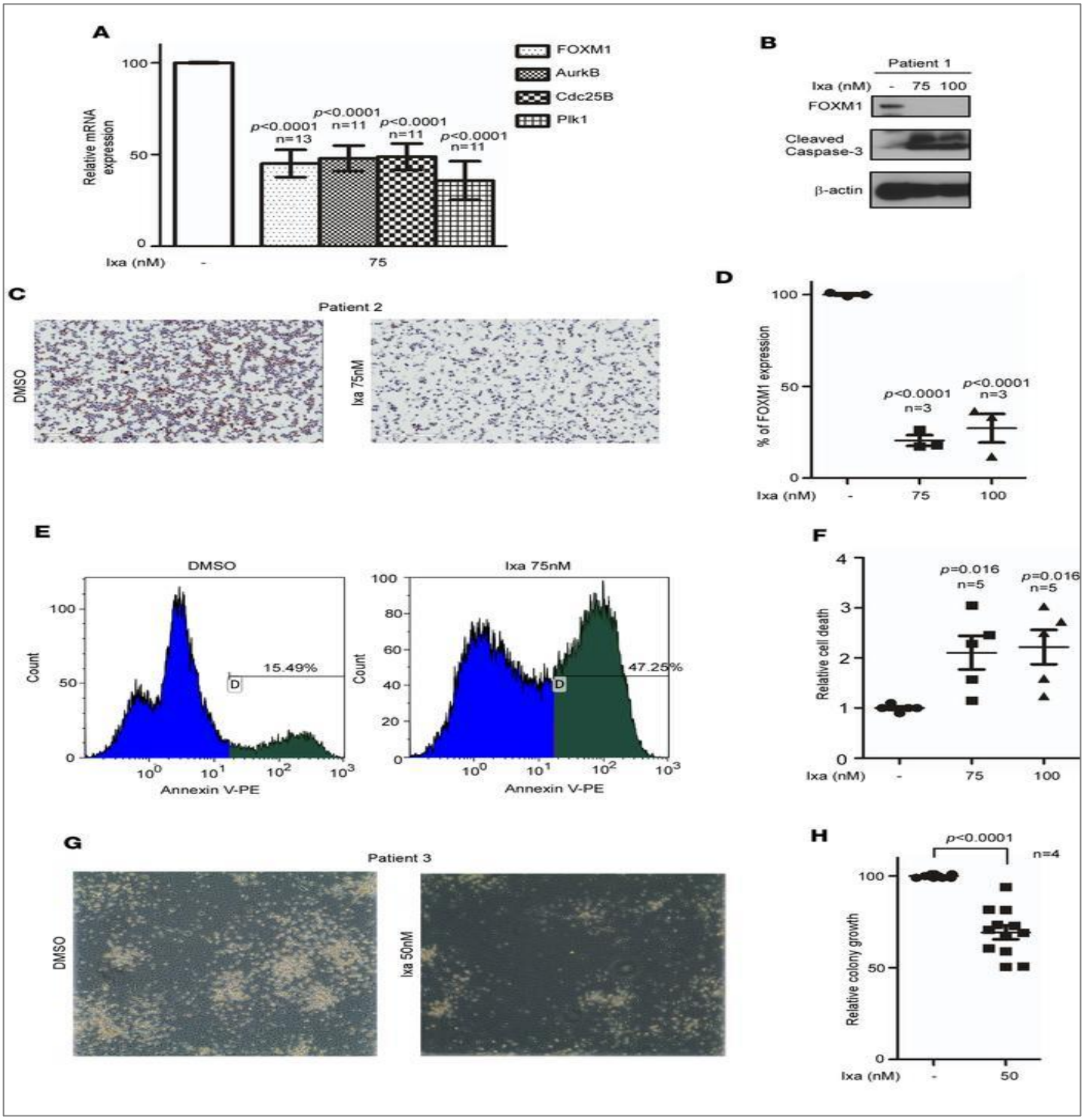

Figure 4. Ixazomib inhibits FOXM1 in primary patient AML cells. (A) RNA expression of FOXM1 and its targets AurkB, Cdc25B, and Plk1. FOXM1 protein inhibition and caspase cleavage **(B).** Quantification of nuclear FOXM1 % in cytospin slides **(C,D).** Cell death by flow cytometry after annexin V–PE staining **(E,F)** and colony-forming activity **(G,H)** of AML mononuclear cells after treatment with ixazomib.

F. Pharmacologic Inhibition of FOXM1 Inhibits AML In Vivo in a Xenograft Model

We next determined whether ixazomib could decrease leukemic burden and improve outcomes in an orthotopic murine model of AML. KG-1 cells were injected into the tail veins of sub-lethally radiated immunodeficient (NOD-SCID-γ–null; NSG) mice. At day 14, the PB was analyzed for human CD45 expression (huCD45) by flow cytometry, and any animal with >2% engraftment was included. Animals were randomized into control and treatment groups, and the treatment group received ixazomib (8 mg/kg) i.v. twice a week for 4 weeks. At the end of treatment, the entire cohort was sacrificed, and BM and PB involvement by AML cells was quantified by flow cytometry for huCD45 expression.

Ixazomib reduced the leukemia burden in the BM of treated animals (Figure 5, A and B). There was substantial reduction of leukemic disease burden in the BM as assessed by CD45 expression. Representative flow plots are shown from each group. Plot shows quantification of CD45 expression in vehicle- and ixazomib-treated animals. Data are expressed as the mean ± SEM (n = 8–10/group); $P < 0.05$ by unpaired 2-tailed t test. Peripheral blood was analyzed to study the effects on normal blood production. Treated animals showed a higher hemoglobin count, with an increase in mean hemoglobin from 7.75 g/dl to 11.25 g/dl (Figure 5C) in the treated animals, suggesting improved hematopoiesis. The dot plot shows the hemoglobin range in the vehicle-treated animals with several animals with hemoglobin < 5g/dl. In comparison, none of the drug-treated animals had such profound anemia. The mean platelet count was similar between groups (data not shown), suggesting ixazomib did not suppress

normal hematopoiesis. The improved anemia levels make this drug a promising agent in the treatment of AML.

To further establish that ixazomib does not suppress normal hematopoiesis, healthy 6-week-old NSG mice were randomized and treated with 8 mg/kg ixazomib or vehicle i.v. twice a week for 4 weeks. The treated animals showed no perturbations in weight or hematological parameters in the PB compared with their vehicle-treated counterparts. This suggests that doses of ixazomib that suppress FOXM1 and attenuate leukemia disease severity do not disrupt normal hematopoiesis.

Following treatment, BM cells were collected for RNA and protein extraction (Figure 5D). FOXM1, Cdc25B, and Plk1 mRNA expression levels using quantitative PCR were quantified as percentage of mRNA expression levels in treated cells compared with control cells. Data are expressed as the mean ± SEM (n = 7/group); $P < 0.05$ by 1-way ANOVA followed by Tukey's multiple comparison post test. (Figure 5E) FOXM1 inhibition in the BM cells is shown by Western blotting. (Figure 5F) Cytospins from the BM mononuclear cells were prepared, and representative images are shown (200× magnification). (Figure 5G) Nuclear FOXM1 was quantified in ixazomib- and vehicle-treated animals. Animals treated with ixazomib had significant downregulation of nuclear FOXM1. Data are expressed as the mean ± SEM (n = 8/group); $P = 0.058$ by unpaired 1-tailed t test.

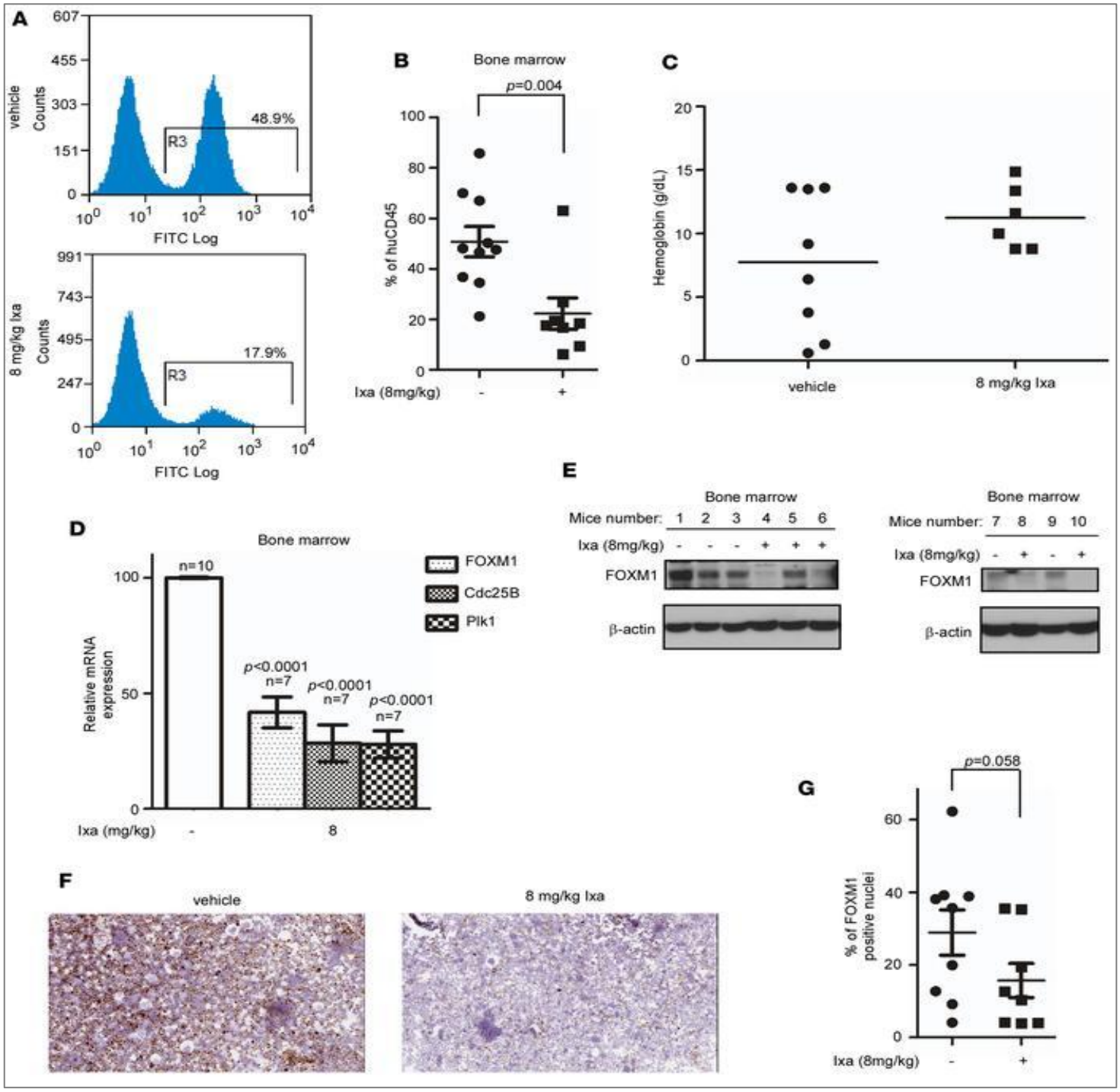

Figure 5. Pharmacologic inhibition of FOXM1 with ixazomib reduces leukemia disease burden in an in vivo AML model. Leukemic burden in the BM by CD45 expression by flow cytometry**(A,B)** and blood Hb levels **(C)**. FOXM1, Cdc25B, and Plk1 mRNA **(D)** and **(E, F)** FOXM1 protein suppression in the BM mononuclear cells by immunoblot and IHC (200X). **(G)** Nuclear FOXM1 was quantified.

V. DISCUSSION

(Previously published as Khan I, Halasi M, Patel A, Schultz R, Kalakota N, Chen YH, Aardsma N, Liu L, Crispino JD, Mahmud N, Frankfurt O, Gartel AL. (2018) FOXM1 contributes to treatment failure in acute myeloid leukemia. JCI Insight. 2018 Aug 9;3(15) 1-17)

The application of targeted therapies in specific molecular subsets of AML patients is allowing a survival benefit to emerge, resulting in several new drug approvals in the past 2 years. (12) However, the setback in relying purely on genomic classification to allocate patients to therapeutic pathways is the complexity of AML genomes, the observation that most patients harbor multiple gene mutations, and the dynamic patterns of disease evolution. (21) Instead of targeting specific genetic aberrations, an alternate strategy for treatment would be to target more commonly dysregulated pathways that are implicated across AML subtypes.

The recent success of a liposomal particle with fixed-ratio delivery of cytarabine and daunorubicin (Vyxeos) shows that utilizing innovative approaches, currently approved agents can be dramatically enhanced in their efficacy. Recognizing a potential mechanism of resistance in the patient at diagnosis would help tailor the treatment regimen to be more effective and increase remission rates. Current genomic predictors of resistance to induction chemotherapy in AML include mutations in RUNX1, ASXL1, and TP53; elevated SNP-A–based genomic complexity; and specific recurrent copy number aberrations/loss of heterozygosity (22).

Our work draws attention to a potentially novel predictor of chemotherapeutic resistance in AML. Forkhead box protein M1 is a transcription factor expressed in proliferating cells, but not in quiescent or terminally differentiated cells, making it an

attractive target. The relevance of FOXM1 in AML is supported by an important bedside to bench discovery, where we previously showed that the favorable NPM1 mutant subset of AML had reduced nuclear levels of FOXM1. (23) The current work establishes the prognostic relevance of nuclear FOXM1 in AML in a clinical retrospective analysis of over 100 patients. Previous publications have observed increased FOXM1 in high-risk molecular subsets of AML(24) , but it has not been validated as a prognostic marker in a clinical cohort until now.

Using quantitative microscopy on the diagnostic BM biopsies of a multi-institution cohort, we confirmed the clinical significance of the nuclear expression of this protein in predicting outcomes. When nuclear FOXM1 expression was combined in a multivariate analysis with conventional clinical and molecular predictors of outcome, it emerged as an independent predictor of upfront chemotherapeutic resistance. Moreover, when the institutions were considered individually, nuclear FOXM1 expression emerged as a predictor of inferior OS (Figure 1).

Using a transgenic murine model of FOXM1 overexpression, we provide proof of concept that FOXM1 overexpression induces resistance to the AML chemotherapy backbone cytarabine (Figure 2). Transcription factors have conventionally been considered as undruggable targets. Our study has challenged this dogma and provides evidence that targeting FOXM1 has anti-leukemic effects. We used 2 methods to decrease FOXM1 activity in AML: RNA interference, which is specific, and proteasome inhibitors, which are clinically approved but relatively nonspecific. Proteasome inhibitors act by stabilizing HSP70, which our lab has shown to be a negative regulator of FOXM1. (18)

Here, we establish that ixazomib, an oral PI, has anti-leukemic activity that correlates with the inhibition of FOXM1 (Figures 3 and 4). Decreasing FOXM1 activity in human and murine leukemia cells with WT NPM1 led to decreased clonogenicity and increased apoptosis. Additionally, treatment with ixazomib was very well tolerated and reduced tumor burden in vivo in an AML model (Figures 5). Recent work has demonstrated a critical role of FOXM1 in maintaining hematopoietic stem cell quiescence. (25) We postulate that there may be differential levels of FOXM1 expression in leukemic stem cells compared with hematopoietic stem cells that would allow for a therapeutic window to target FOXM1. This is the subject of ongoing work. PIs have already entered the clinical realm of AML. Previous trials have demonstrated therapeutic benefit by adding bortezomib to cytarabine-based chemotherapy (26-28), but they increased toxicity. In addition to being the first oral PI, ixazomib is well tolerated with minimal neurotoxicity and myelosuppression.(29) We show that low-dose ixazomib induces sensitization of AML cell lines and primary human and murine AML cells to the chemotherapy backbone drugs cytarabine and 5-azacitidine.

The putative mechanism of action of PIs is the inhibitory effect on transcription factor NF-κB through stabilization of its inhibitor, I-κB.(30) We present an alternate mechanism of action of ixazomib and link its antileukemic activity to its effects on FOXM1. Using overexpression of FOXM1, we show the anti-leukemic activity of ixazomib is, at least in part, through its inhibition of FOXM1.

In summary, our study provides proof of principle for the inhibition of the transcription factor FOXM1 as a potentially novel strategy in the treatment of AML. Currently approved therapies such as the PI ixazomib could be harnessed to overcome

FOXM1-mediated resistance in AML. Moreover, our findings demonstrate the need for the development of more specific and potent FOXM1 inhibitors in the treatment of leukemia.

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ACADEMIC APPOINTMENT: Assistant Professor of Medicine, University of Illinois at Chicago, Department of Medicine, Section of Hematology-Oncology, 2013 –

LICENSURE: Illinois

BOARD EXAMINATIONS: Hematology, 2012
Oncology, 2012
Internal Medicine 2008

AWARD AND RESEARCH SUPPORT:

Active

Leukemia Research Foundation New Investigator Grant Khan (PI)
7/1/19-6/30/20
Targeting FOXM1 to improve treatment responses in AML

University of Illinois Cancer Center Director's Pilot Program Khan (PI)
7/1/19-6/30/20
Structural violence as a contributor to disparities in leukemia outcomes

Completed

KL2TR002002-01 Khan (Scholar) DiPietro (PI)
9/16/16-9/15/18
FOXM1 is a critical downstream target of the FLT3-ITD mutation in AML.

Millenium/Takeda 083434-00001 Khan (PI)
9/1/16-8/30/18
Combination treatment with proteasome inhibitors to target FOXM1 in AML.

NIH 1R21CA194608-01 Gartel/Khan (PI/Co-I)
5/1/15-4/30/17

Cytoplasmic FOXM1 contributes to the higher complete remission and longer overall survival of AML patients after chemotherapy

UIC Cancer Center Pilot grant Khan (PI)
3/1/ 14 –2/28/15
Cytoplasmic relocalization of FOXM1 by mutant NPM may correlate with a favorable outcome for AML patients.

5T32CA079447-12 Khan (Trainee) Rosen (PI)
7/1/10- 6/30/12
Targeting the PI3 kinase pathway in Myeloproliferative Neoplasms.

PUBLICATIONS:

1. Management of hyperleukocytosis and impact of leukapheresis among patients with acute myeloid leukemia (AML) on short- and long-term clinical outcomes: a large, retrospective, multicenter, international study. Stahl M, Shallis RM,..**Khan I,** … Gore S, Zeidan AM. Leukemia. 2020 Mar 4. doi: 10.1038/s41375-020-0783-3.
2. Shallis RM, Stahl M, Sekeres MA, Fathi AT, …**Khan I, …**Gore S, Zeidan AM et al. Patterns of care and clinical outcomes of patients with newly diagnosed AML presenting with hyperleukocytosis who do not receive intensive chemotherapy. Leuk Lymphoma. 2020 Feb 26:1-6.
3. **Khan I**, Halasi M, Patel A, Schultz R, Kalakota N, Chen YH, Aardsma N, Liu L, Crispino JD, Mahmud N, Frankfurt O, Gartel AL. FOXM1 contributes to treatment failure in acute myeloid leukemia. JCI Insight. 2018 Aug 9;3(15). pii: 121583. doi: 10.1172/jci.insight.
4. Saraf SL, Oh AL, Patel PR, Sweiss K, Koshy M, Campbell-Lee S, Gowhari M, Jain S, Peace D, Quigley JG, **Khan I**, Molokie RE, Mahmud N, Gordeuk VR, Rondelli D. Haploidentical Peripheral Blood Stem Cell Transplantation Demonstrates Stable Engraftment in Adults with Sickle Cell Disease. Biol Blood Marrow Transplant. 2018 Aug;24(8):1759-1765
5. Patel P, Oh AL, Koshy M, Sweiss K, Saraf SL, Quigley JG, **Khan I**, Mahmud N, Hacker E, Ozer H, Peace DJ, Weichselbaum RR, Aydogan B, Rondelli D. A phase 1 trial of autologous stem cell transplantation conditioned with melphalan 200 mg/m2 and total marrow irradiation (TMI) in patients with relapsed/refractory multiple myeloma. Leuk Lymphoma. 2018 Jul;59(7):1666-1671
6. Sweiss K, Anderson J, Wirth S, Oh A, Quigley JG, **Khan I**, Saraf S, Mactal-Haaf C, Rondelli D, Patel P. A prospective study of intravenous pentamidine for PJP prophylaxis in adult patients undergoing intensive chemotherapy or hematopoietic stem cell transplant. Bone Marrow Transplant. 2018 Mar;53(3):300-306.
7. Halasi M, Hitchinson B, Shah BN, Váraljai R, **Khan I**, Benevolenskaya EV, Gaponenko V, Arbiser JL, Gartel AL. Honokiol is a FOXM1 antagonist. Cell Death Dis. 2018 Jan 24;9(2):84
8. Brinda B, **Khan I**, Parkin B, Konig H. The rocky road to personalized medicine in acute myeloid leukaemia. J Cell Mol Med. 2018 Jan 12: 1-17.
9. **Khan I**, Mariana Halasi, Mariam F. Zia, Peter Gann, Sujata Gaitonde, Nadim Mahmud, Andrei L. Gartel . FOXM1 drives chemoresistance in AML *Leukemia*.

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10. **Khan I**, Saraf SL, Shergill A, Chen YF, Patel PR, Quigley JG, Peace D Gordeuk VR, Hoffman R, Rondelli Damiano. Outcome Disparities in Caucasian and non-Caucasian Patients with Myeloproliferative Neoplasms. *Clin Lymph Myeloma Leuk* 2016 Mar 8.
11. Saraf SL, Oh AL, Patel PR, Peace D, Quigley JG, **Khan I**, Molokie RE, Hsu LL, Mahmud N, Levinson DJ, Pickard AS, Garcia JG, Gordeuk VR, Rondelli D et al. Nonmyeloablative Stem Cell Transplantation with Alemtuzumab/Low-Dose Irradiation to Cure and Improve the Quality of Life of Adults with Sickle Cell Disease*. Biol Blood Marrow Transplant*. 2015 Sep 5.
12. Patel P, Aydogan B, Koshy M, Mahmud D, Oh A, Saraf SL, Quigley JG, **Khan I**, Weichselbaum RR, Rondelli D et al. Combination of linear accelerator-based intensity- modulated total marrow irradiation and myeloablative fludarabine/busulfan: a phase I study. *Biol Blood Marrow Transplant*. 2014; 20(12):2034-41.
13. **Khan** I, Huang Z, Wen Q, Stankiewicz MJ, Gilles L, Goldenson B, Schultz R, Diebold L, Gurbuxani S, Finke CM, Lasho TL, Koppikar P, Pardanani A, Stein B, Altman JK, Levine RL, Tefferi A, Crispino JD. AKT is a therapeutic target in myeloproliferative neoplasms. *Leukemia*. 2013 Sep;27(9):1882-90
14. **Khan I**, Altman JK, Licht JD. New Strategies in Acute Myeloid Leukemia: Redefining prognostic markers to guide therapy. *Clin Cancer Res*. 2012;18(19): 5163-71
15. **Khan I**, Malinge S, Crispino JD. Myeloid Leukemia in Down Syndrome. *Critical reviews in Oncogenesis.* 2011;16(1-2):25-36
16. Querfeld C, **Khan I**, Mahon B, Nelson BP, Rosen ST, Evens AM. Primary cutaneous and systemic anaplastic large cell lymphoma: clinicopathologic aspects and therapeutic options.*Oncology*. 2010; 24(7):574-87.
17. Dose M, **Khan I**, Guo Z, Kovalovsky D, von Boehmer H, Khazaie K, Gounari F. c-Myc mediates pre-TCR-induced proliferation but not developmental progression. *Blood.* 2006;108(8):2669-77

Book Chapters

1) *ASH Self-Assessment Program (ASH-SAP)* 7th edition. Chapter 7. Cellular basis of hematopoiesis and stem cell transplantation. Irum Khan and Kim-Hien T. Dao (2019)
2) Cancer Consult: Expertise for Clinical Practice. Chapter 30*.* Hematopoietic Cell Transplantation in Primary Myelofibrosis. Khan I, Rondelli D. September 2014, *Wiley-Blackwell.*
3) Contemporary Management of Myeloproliferative Neoplasms. Chapter 7. Hematopoietic Stem Cell Transplant in Myelofibrosis. Khan I, Rondelli D. Nov 2014, *Jaypee Brothers Medical Publishers.*

PROFESSIONAL MEMBERSHIP:

American Society of Hematology (ASH)