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An accessible patient-derived xenograft model of low-risk myelodysplastic syndromes

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Authorship Contributions: PT wrote the manuscript, performed research, analyzed data. SP analyzed sequencing data. IC designed, performed and analyzed intron retention experiments. SC performed library preps for sequencing experiments. WBD designed, performed and analyzed splicing experiments. LPG designed, performed and analyzed sequencing experiments. AED contributed patient samples. GG designed the project. All authors critically reviewed the manuscript.

Conflict of interests/Disclosures: GG received research support from Menarini Ricerche, Abbie, Inc. and serves on advisory board of Syros, Inc. WBD received research support from Abbvie, Inc.
**Data Sharing Statement:** The essential data supporting our findings are within the article and supplementary materials. The corresponding author upon reasonable request can provide raw data and additional details. All shared data will be anonymized for privacy.

Myelodysplastic syndromes (MDS) are one of the most common myeloid malignancies. (1) They encompass a spectrum of clonal bone marrow (BM) failure diseases characterized by ineffective hematopoiesis and progression to acute myeloid leukemia (AML). (2) The clinical course of MDS varies widely from indolent, requiring only monitoring/supportive care to aggressive disease with AML-like biology. (1) Recurrent somatic mutations contribute to the pathophysiology, being recently incorporated into prognostic scoring systems. (2, 3) Akin to targeted therapy in AML, somatic mutations might be therapeutically targeted in MDS. Unfortunately, testing this hypothesis has been challenging due to the lack of preclinical models that truly recapitulate disease biology, particularly in lower-risk disease. This limitation has resulted in a dearth of novel targeted therapeutics and slow drug development in the field. (4)

Genetically engineered mouse models (GEMMs) of recurrent somatic mutations present in MDS hold promise for advances in understanding the biology of these diseases. However, given that introns are generally not conserved between mice and humans, there is potential for significant differences in intron retention and cryptic splicing in human and mouse hematopoietic cells bearing spliceosome mutation. (4) The lack of readily available patient-derived xenograft (PDX) models remains the major barrier to drug development. NSG/NSG-S mice lack functional B/T/NK cells and immunoglobulins (5), and are unreliable to establish PDX
of low-risk MDS.(4) Our previous experience with condition-regimens based exclusively on XRT did not result in engraftment of MDS cells (data not shown). Recently, PDX models of MDS, based on intra-bone co-injection of human mesenchymal stroma cells (hMSCs)(6) or subcutaneous generation of ossicles using hMSCs(7) mitigated this shortcoming. Moreover, intra-hepatic injection of OKT3-depleted MDS BM into double-irradiated newborn MISTRG mice results in robust and reliable engraftment.(8) However, these models are not widely available and require technical skills that reduce their utility. Hence, the opportunity to develop a PDX model more amenable to testing novel targeted therapies and one that allows for high throughput drug screening in low-risk diseases.

MISTRG mice are similar to other xenograft models in the sense that they are immunocompromised and express human cytokines (knocked-in the case of MISTRG mice). Nevertheless, one key feature of MISRTG mice is their expression of human SIRPα. Their ability to recognize human CD47 (“don’t eat me” signal) may explain the more reliable engraftment of human cells in these mice.(9) One way to reproduce this feature in NSG/NSG-S mice, is by depleting macrophages with clodronate. Administering 100µl of clodronate liposomes (FormuMax), intraperitoneal, to 6-8 weeks old mice (NOD.Cg-PrkdcscidIl2rgtm1WjlNSG-Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ, Jackson Laboratories), led to 83% macrophage-reduction in the BM, and >99% reduction in spleen, after 48hrs (Supplementary Figure 1A and B). 7 days later, the depletion persisted, with 97% reduction in the BM, and still >99% in the spleen (Supplementary Figure 1A and B). In contrast, administering 2Gy of XRT (classically used for conditioning) did not affect the presence of macrophages in the BM or spleen (Supplementary Figure 1A and B). To test if depleting macrophages allows for engraftment of patient-derived
MDS cells, we conditioned 6-8 weeks old NSG/NSG-S mice with 100µl of clodronate liposomes, on day -2 and 2Gy irradiation on day 0. 6 hours later, we injected CD34+ cells, via tail vein, selected as previously published(7), from the BM of a patient with low-risk MDS (Figure 1A). At the time of sample collection, the patient had 1-2% CD34+ blasts (Table). BM samples were collected from MDS patients consented in accordance with the Declaration of Helsinki, under a research protocol approved by the Johns Hopkins Institutional Review Board. Flow-cytometry analysis of PB collected from transplanted mice at 1, 2, and 3 months post-transplant showed the presence of human leukocytes [mouse CD45^−(mCD45^−)hCD45^+] (Figure 1B). At 3 months post-transplant, NSG mice showed reliable engraftment in the BM (Supplementary Figure 1C). NSG-S mice demonstrated consistent engraftment in the PB, spleen, and BM (Supplementary Figure 1C, Table). More so, NSG-S mice showed detectable CD34^+ leukocytes (mCD45^-hCD45^-hCD3^+) as well as human erythroid cells (mCD45^-hCD45^-glycophorin A-GPA^+) in the BM at 3 months post-transplant. We selected hCD45^+ cells, using hCD45 beads and a magnetic column (Miltenyi), from the BM of these mice. hCD45^+ cells were pooled together and DNA was extracted using the Quick-DNA Miniprep Plus kit (Zymo Research). Further, library prep was done using xGen Prism DNA Library prep kit (IDT) and IDT hybridization capture-based targeted Duplex sequencing was done on a NOVASeq6000 platform in an SP flow cell. Data analyzed using DRAGEN Enrichment (v4.0.3) showed that selected hCD45^+ cells had a similar clonal architecture as the transplanted CD34^+ cells and the clinical samples (Figure 2A). Secondary transplantation using 10^6 hCD45^+ cells isolated from the BM of NSG/NSG-S mice resulted in detectable human engraftment, albeit at lower levels compared to primary recipients (data not shown). We tested the reproducibility of the NSG-S model using various conditions: CD34^+
numbers, and fresh vs. frozen cells (Table); and clinical specimens from low-risk MDS patients with different molecular profiles (Table). Human leukocytes were present in all mice in the PB at 1 (0.52%-14%) and 3 months (3.1%-20.7%) post-transplant (Table). Similarly, we observed successful engraftment in the BM (18.1%-99.3%) and spleen (8.1%-31.3%) at 3-6 months post-transplant (engraftment levels for each individual mouse are shown in Supplementary Figure 1C). Human cells were mostly CD33+ myeloid cells (38.2%-90.7% of hCD45+). B cell engraftment as detected by CD19+/CD20+ (0.11%-2.1% of hCD45+) were present in a subset of animals. No mice showed CD3+ T cell engraftment. Human erythroid engraftment is particularly difficult to achieve in xenograft models, as erythroid precursor cells are constantly removed by host macrophages. The depletion of macrophages with clodronate resulted in erythropoietic cells engraftment including hCD45-hCD71+ and hCD45-hCD71+GPA+ in the BM and spleen of recipient mice (Figure 1B and Table). In addition, hCD45-hCD41+ (0.14%-0.47% of hCD45+) and hCD45− hCD41+ (0.1%-0.63% of mCD45−hCD45−) megakaryocyte lineage cells were present in the BM and spleen.

Clonal architecture of engrafted cells recapitulated the clinical BM sample and enriched CD34+ cells (Figure 2A, B and D). In lieu of morphologic analysis of engrafted human cells, we tested if these cells show preserved cryptic intron retention similar to the original sample. To this end, we extracted mRNA (from the pooled hCD45+ cells sorted from the mouse BM) using the Monarch Total RNA Miniprep Kit and synthesized cDNA with the High-Capacity cDNA Reverse Transcription Kit in order to do splicing assays of the splicing factors mutated samples (SF3B1 and ZRSR2). For the SF3B1 splicing assay, isoform-competitive endpoint PCR for canonical cryptic acceptors in exon 2 of TMEM14C was performed with primers
GACACCTCGCAGTCATTCCT and TGATCCCACCAGAAGCAACC. For the ZRSR1 splicing assay, qPCR using KAPA SYBR FAST qPCR Master Mix (2X) Kit was performed with primers CCCGCTCCAGCTACTTTGAA and CAAACAAGTAGAGCGAGTCTCT for canonical LZTR1 and primers CCCGCTCCAGCTACTTTGAA and AGTTCACTGGGGAGTGAGGAT for LZTR1 intron 18 retention. The expression ratio was calculated with $2^{-\Delta \Delta Ct}$ (canonical cycle threshold–cryptic cycle threshold).

mRNA analysis of engrafted MDS cells with SF3B1 (patient 2) and ZRSR2 (patient 3) mutations validated the persistence of physiologically impactful (10, 11) missplicing events in TMEM14C and LZTR1, respectively (Figure 2C and E).

For further disease relevance, after confirming engraftment, PDXs derived from patient 3 were treated with either azacitidine (five daily injections 5mg/kg, 2 cycles, 4 weeks apart) or saline. PDXs treated with azacitidine showed decreased levels of BM engraftment at 6 months post-transplant (4 weeks after the completion of the 2nd cycle) (Table). Although not definitive (because of the small number of mice), these results serve as proof of concept of the model’s applicability to study therapeutic interventions.

Recent years have seen the emergence of new technologies for augmentation of murine modeling of MDS, including GEMMs and innovative PDX models. The PDX model described here complements currently available analytic tools in MDS. It makes use of relatively inexpensive and widely available reagents (i.e. NSGS mice, clodronate, and radiation) and engraftment is tracked via non-invasive methods. Through its simplicity of workflow: no need for other moving parts (i.e. timed pregnancies, pre-engraftment of other cell populations or bone fragments); the use of viably frozen CD34+cells and simple conditioning regimen followed by intravenous injection; this model is amenable for large-scale drug testing in low-grade MDS. Typically, 20-
30mL of BM aspirate from a low-grade MDS sample may be sufficient to generate 50-60 PDX mice. Such scale allows for testing of single agent regimens (i.e. spliceosome inhibitors) and combination therapies (i.e. with hypomethylating agents) thus, informing early stage clinical development.
References:


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<th>Sample</th>
<th>Sample type</th>
<th>No. of cells injected (number of mice per group)</th>
<th>PB</th>
<th>BM – final</th>
<th>Spleen – final</th>
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<td>Fresh</td>
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### Table

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<td>ASXL1</td>
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<td>ASXL1</td>
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<td>NRAS</td>
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<tr>
<td>ZRSR2</td>
<td>p.V253</td>
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### Notes
- **Patient 1**: 74 years, 47,XY+8 [4]/46,XY[17] <2% 9.4 338 2.45 3 points/ Low risk 0.23/ Moderate high risk
- **Patient 2**: 64 years, 46, XY <2% 12.4 299 1.36 1 point/ Very low risk -2.3/ Very low risk
- **Patient 3**: 74 years, 46, XY 3% 8.8 88 1 3.5 points/ Intermediate risk 0.45/ Moderate high risk

### Additional Table

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Figure Legends

Figure 1. Clodronate and XRT conditioning allow for reconstitution of human low-risk MDS hematopoiesis in NSGS mice
(A) Graphic representation of mouse engraftment technique and assessment. (B) Representative flow cytometry analysis of the human graft in an NSGS mouse. Data was analyzed using the BD FACSDiva software v8.0.1.

Figure 2. Low-risk MDS engrafted cells maintain the cytogenetics (and their functional impact) of the original patient samples
(A) Comparison between the mutational profiles identified in the clinical report of Patient 1 vs. analysis of CD34+ cells sorted pre-transplant and pooled hCD45+ cells sorted from the mouse bone marrows 3 months after transplant. (B) Comparison between the mutational profile in the CD34+ cells sorted from Patient 2 before transplant and the pooled hCD45+ cells isolated for the BM of mice engrafted with cells from patient 2, at 3 months after transplant. (C) Splicing assay showing abnormal intron retention in the hCD45+ cells of patient 2 prior to engraftment (MDS preENG, column 2) and those isolated from the BM of mice engrafted with cells from this patient at 3 months after transplant (MDS postENG, column 3) as predicted in SF3B1 mutant cells (NBM=Normal Bone Marrow). (D) Comparison between the mutational profile of CD34+ cells sorted from Patient 3 before transplant and the pooled hCD45+ cells sorted from the BM of mice engrafted with cells from patient 3 at 6 months after transplant. (E) Ratio of cryptical (abnormal intron retention) to canonical LTZR1 splicing in the pooled hCD45+ cells isolated from the BM of mice engrafted with cells from patient 3 as predicted in ZRSR1 mutant cells (NBM=Normal Bone Marrow). Each dot represents an independent experiment, horizontal lines represent the mean of four experiments.

Table. Engraftment of primary low-risk MDS samples into NSGS mice, after macrophage depletion, is reliable regardless of the cytogenetics or type of sample used.
Table showing patient characteristics for the samples used for engraftment (TOP) and human cell populations and percentages in peripheral blood (at different time points), BM (bone marrow) and spleen, in NSGS mice (BOTTOM).
A. 100μl Clodronate, i.p. XRT, 2 Gy hCD34+ cells, tail-vein injection Peripheral blood Peripheral blood Peripheral blood Flow cytometry

NSG/NSG-S mouse

Day -2 Day 0 1 mo 2 mo Spleen 3 mo

B.

Propidium Iodine

SCC

Mouse CD45

Human CD45

Human CD34

Human CD33

Human CD71

Glycophorin A

Human CD41

Human CD19

Human CD3
Figure 2

A. Patient 1 - Clinical
   - U2AF1 p.S34F
   - NRAS p.A91G
   - ASXL1 p.G646fs
   - ASXL1 p.F1005fs

Patient 1 – CD34+
   - U2AF1 p.S34F
   - NRAS p.A91G
   - ASXL1 p.G646fs
   - ASXL1 p.F1005fs

Patient 1 – mouse-derived hCD45+
   - U2AF1 p.S34F
   - NRAS p.A91G
   - ASXL1 p.G646fs
   - ASXL1 p.F1005fs

B. Patient 2 – CD34+
   - TET2 p.F683fs
   - SF3B1 p.K700E

Patient 2 – mouse-derived hCD45+
   - TET2 p.F683fs
   - SF3B1 p.K700E

C. Protein expression
   - TMEM14C

D. Patient 3 – CD34+
   - ZRSR2 p.V253X
   - TET2 p.Q1030fs
   - EZH2 p.K634N
   - ASXL1 p.G646fs

Patient 3 – mouse-derived hCD45+ (no Aza)
   - ZRSR2 p.V253X
   - TET2 p.Q1030fs
   - EZH2 p.K634N
   - ASXL1 p.G646fs

E. Cytosolic/canonical LZTR1
   - ZRSRS-wt (NBM)
   - ZRSRS-mut (pre-engaft)
   - ZRSRS-mut (post-engaft)
   - ZRSRS-mut (post-engaft with Aza)
Supplemental Figure 1. Clodronate depletes macrophages in the spleen and BM of NSGS mice, allowing for engraftment of primary low-risk MDS

(A) Clodronate, but not XRT, depletes >99% of mouse spleen macrophages at 2 and 7 days. (B) Clodronate, but not XRT, depletes 83% of mouse BM macrophages at 2 days, and 97% at 7 days. (C) Table showing levels of engraftment in individual mice, in PB, BM and spleen, at the time of sacrificing. Percentages represent hCD45+ cells out of (mCD45+ + hCD45+) cells.