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14. ABSTRACT
The overall goal of this project is to develop a model for the study of myelodysplasia (MDS), an acquired bone marrow failure syndrome in the aging population and in cancer survivors. MDS is inherently difficult to study. MDS stem cells cannot be grown in culture and in vivo models are thus the gold standard. However, MDS stem cells are diseased and fail to efficiently engraft in current immunodeficient mouse models. We have optimized engraftment of normal adult HSPC into MISTRG. We have determined optimal cell preparation, cell number, pre-engraftment irradiation dose, transplantation route and recipient age. We have optimized the analysis of engrafted humanized mice. We have successfully transplanted primary MDS bone marrow cells into MISTRG mice with successful engraftment and replication of the donor disease status. We have successfully performed secondary transplantation of MDS BM cells to propagate human MDS in MISTRG mice for therapeutic testing.

15. SUBJECT TERMS
Myelodysplasia, Bone marrow failure, Humanized mice, Xenotransplantation

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Table of Contents

Stephanie Halene, M.D., Ph.D.

Progress report

Title: Assessing the Mechanisms of MDS and its Transformation to Leukemia in a Novel Humanized Mouse

Study Site and Personnel:

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Animal Use: Xenotransplantation Studies; IACUC # 11396 approved
Human Subjects: Tissue collection; IRB # 12642 approved

Introduction:

The overall goal of this project is to develop a model for the study of myelodysplasia (MDS), an acquired bone marrow failure syndrome in the aging population and in cancer survivors. MDS is inherently difficult to study. MDS stem cells cannot be grown in culture and in vivo models are thus the gold standard. However, MDS stem cells are diseased and fail to efficiently engraft in current immunodeficient mouse models. We here propose to develop an MDS *in vivo* model using a novel immunodeficient mouse, MISTRG, engineered to express human growth factors instead of murine growth factors, driven by the endogenous murine control elements in a temporally and spatially physiologic way. [1] An *in vivo* model to study MDS biology and aid translation of novel targeted therapeutics into clinic is essential to improve the otherwise dismal outcomes for MDS.

Keywords

Myelodysplasia
Bone marrow failure
Humanized mice
Cytokine knockin
Xenotransplantation

Overall project summary

The progress report summarizes our work during the entire funding period. The proposed studies sought to develop a xenotransplantation model for the study of myelodysplasia (MDS), a MDS-MISTRG patient derived xenograft (PDX) model, which we have successfully accomplished. Cytokines are essential for support of hematopoietic stem and progenitor cells and differentiation to functional mature cells. We hypothesized that replacement of non-cross reactive murine cytokines in the mouse with their respective human counterparts provided diseased MDS stem cells with the essential stimuli to support their maintenance in the murine host as well as differentiation towards mature lineages. We confirmed that human cytokines are indeed expressed in mesenchymal stromal cells (MSCs), which are part of the hematopoietic stem cell (HSC) niche.

We first optimized engraftment of normal adult hematopoietic stem and progenitor cells (HSPCs) into MISTRG and compared efficiency to the current "gold standard", NSG mice.

We subsequently transplanted primary MDS bone marrow cells from patients with high-grade and low grade disease into MISTRG mice and compared engraftment levels and "quality" against engraftment in NSG mice. We optimized T-cell depletion and engraftment; we devised analysis tools, including a targeted exome library amenable to use on mixed human-mouse cell populations, flow-cytometric and histologic characterization; and we established secondary transplantation protocols. We optimized treatment regimens in MDS-MISTRG PDXs including chemotherapeutic and targeted regimens.

Task 1: IRB and HRPO review and approval for studies involving human subjects and IACUC and ACURO review and approval for animal use:
Local Internal Review Board and DoD regulatory review and approval of the human subject protocol to harvest bone marrow, peripheral blood, and buccal swab samples from patients were obtained at the start of the funding period.

Local IACUC and DoD regulatory review and approval for the use of animals (mice) were obtained.

Task 2. Optimize transplantation of primary MDS bone marrow cells into MISTRG mice: Optimize transplantation of primary MDS bone marrow cells into MISTRG mice by modulating pre-transplant irradiation doses, cell numbers and preparation, transplant route, and age of murine recipients

Task 2a. Transplantation of healthy adult stem cells.
The goal is to achieve multilineage engraftment levels of human cells > 10% in the mouse bone marrow with high viability of recipients after transplantation.
We have based our studies with adult hematopoietic stem and progenitor cells (HSPC) on our prior studies with fetal liver derived HSPC. To assess successful engraftment, we have analyzed engraftment in peripheral blood (PB) and in bone marrow (BM) and spleen at 10-15 weeks after transplant.
We have analyzed engraftment by flow cytometric differentiation of human from murine cells and by analyzing human cell subsets based on lineage markers.

(i) Cell numbers and preparation:
Fetal liver CD34+ cells are highly proliferative and as few as 3,000 CD34-selected cells can engraft humanized mice.
Engraftment occurs in 100% of mice with engraftment levels of >90% in mouse bone marrow when 50,000 fetal liver CD34-selected cells are engrafted. Viability of mice engrafted at >90% is compromised due to development of anemia with viability of ~ 50% 8 weeks after transplant. Thus engraftment levels of ~50% are desirable due to greater survival and sufficiently high engraftment levels to study diseases.
We transplanted mice with 100,000 – 150,000 adult CD34+ cells and compared engraftment levels in MISTRG mice versus NSG mice, the current gold standard for xenotransplantation assays:

(ii) Pre-transplant Irradiation Dose:
MISTRG mice can engraft human cells without irradiation due to suboptimal murine stem cell maintenance secondary to humanization of cytokines.
Prior studies have established an irradiation dose of 2x150cGy for engraftment into newborn MISTRG mice. Mice do not exhibit toxicity from this irradiation dose and without transplantation viability is ~100%. Since engraftment with 100,000 – 150,000 adult CD34+ cells with prior irradiation of 2x150cGy gave optimal results with engraftment levels between 10-50% we chose 2x150cGy as the optimal for future studies.

(iii) Transplantation route:

Previous studies have established intrahepatic injection into newborn MISTRG mice as an optimal transplantation route. Intrahepatic injection has several advantages:

1) At time of birth hematopoiesis occurs in the bone marrow and newborn liver with progressive transition from the liver to the bone marrow. The bone marrow niche is primed to accept incoming stem cells, while the liver still continues to provide support for stem cells; this represents an optimal environment for human HSC engraftment into the humanized niche. The niche microenvironment is optimized for stem cell expansion to allow growth of the organism, providing optimal proliferation signals to engrafted stem cells.
2) Engraftment into newborn MISTRG pups is costeffective. MISTRG litters comprise 7-12 pups which are weaned into cages of 3-5mice/cage at 3-4 weeks of age. Viability post transplant is ~50-80%. Engraftment into adult mice retroorbitally requires mice ~ 6 weeks of age and intra-femorally ~8-12 weeks of age due to technical requirements. By injection into newborn mice, maintenance of mice pre-transplant can be avoided with savings of approximated $100-200/litter of ~10 transplant recipients.
3) Intrahepatic transplantation into newborn mice is nearly atraumatic with up to 100% survival post procedure. Injection retro-orbitally into adult mice requires anesthesia, however, it is also rapid with near 100% survival. Intrafemoral injection requires prolonged anesthesia and is technically more difficult and time-consuming. Retro-orbital and intrafemoral injection are nevertheless viable options with positive engraftment when necessary.

(iv) Age of murine recipients: Based on our studies with fetal liver derived CD34+ cells have preferably used intrahepatic injection into newborn MISTRG mice.
This methodology has given reliable and multilineage engraftment of human adult CD34+ HSPCs.

MISTRG engraft normal adult hematopoietic stem cells with superior efficiency and support myeloid differentiation.

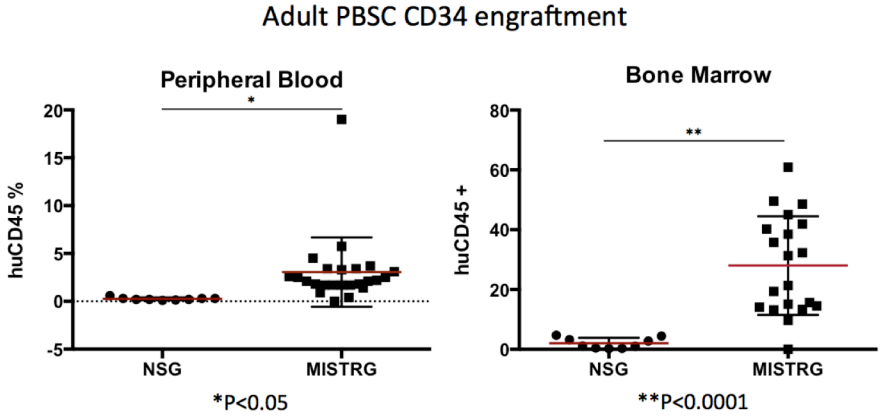

Figure 1: Adult mobilized peripheral blood stem and progenitor cells (PBSCs) successfully engraft MISTRG mice with superior engraftment of peripheral blood (left) and bone marrow (right) when compared to current gold standard NSG mice.

Engraftment levels were significantly higher in MISTRG mice than in NSG mice. For human PBSC engraftment peripheral blood huCD45+ cells comprised 3.05+/-0.7% of all CD45+ cells in MISTRG and 0.26+/-0.05% of all CD45+ cells in NSG ($p<0.05$). In bone marrow human CD45+ cells comprised 28.2+/-3.7% in MISTRG and 2.0+/-0.6% in NSG ($p<0.0001$). For BM CD34+ cell engraftment, PB huCD45+ cells comprised 6.3+/-2.7% of all CD45+ cells in MISTRG and 0.39+/-0.11% of all CD45+ cells in NSG. In bone marrow human CD45+ cells comprised 34.08+/-3.7% in MISTRG and 3.4+/-0.9% in NSG ($p<0.0001$). In addition, MISTRG mice show improved myeloid engraftment and decreased skewing towards the B-lymphocyte lineage (Figure 3). Histologic analysis reveals periosteal engraftment of CD34+ cells and mature myeloid engraftment in MISTRG that is lacking in NSG mice not only for normal CD34+ cells but also for MDS derived CD34+ cells (Figure 4).

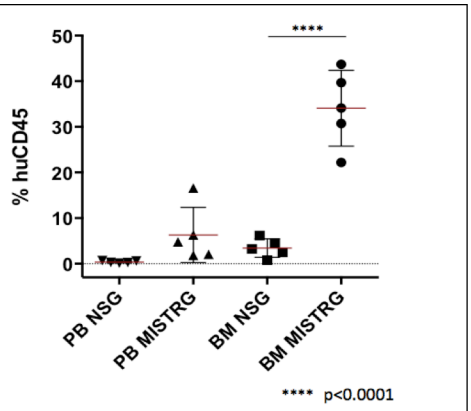

Figure 2: Adult BM derived CD34+ cells successfully engraft MISTRG mice with superior engraftment when compared to NSG mice.

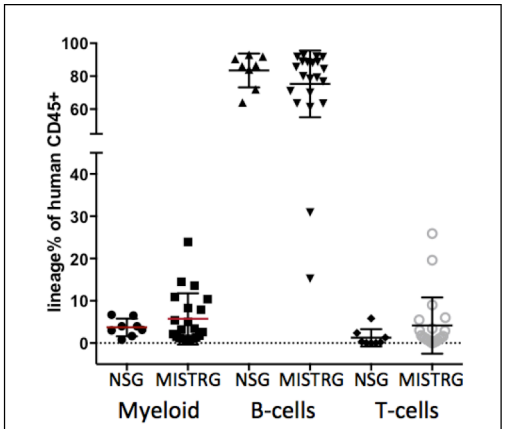

Figure 3: MISTRG provide improved myeloid engraftment over NSG mice

Task 2b. Transplantation of MDS HSPCs.

We have used MDS hematopoietic stem and progenitor cells from primary patient bone marrow to establish optimal:

(i) Cell numbers and preparation:

Primary human bone marrow samples vary greatly in "quality" due to 1) patient's disease and bone marrow status and 2) technical quality. Bone marrow for research purposes is taken at the time that the bone marrow procedure is performed for clinical purposes. Optimal sample delivery to the clinical labs is assured.

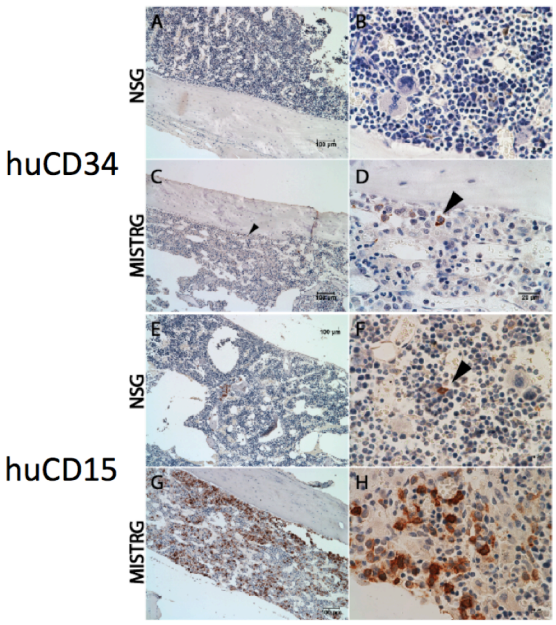

Figure 4: Histologic examination of MISTRG and NSG bone marrow engrafted with human MDS.

Cell numbers: primary MDS bone marrow samples vary between $0.3x10^6$ to $1x10^9$ cells/sample. Clinical factors determining cell number obtained are:

Bone marrow cellularity

Presence of absence of fibrosis

MDS subtype – lower grade MDS generally yields lower cell numbers that higher grade MDS.

Based on our studies, when >$1x10^7$ mononuclear cells are obtained xenotransplantation of ~5 recipients is feasible. Samples with <1x107 cells are unlikely to yield significant engraftment in a significant number of mice (>/= 5).

Cell preparation: All bone marrow samples are ficolled to obtain the mononuclear cell fraction and cryopreserved in aliquots of 1-5 $x10^7$ cells/vial; at time of xenotransplantation samples are rapidly thawed and processed for transplantation.

Based on our studies and studies by Wunderlich et al.[2], we pre-incubated primary bone marrow samples with an anti-CD3 antibody to prevent engraftment of alloreactive T-cells. In MISTRG engraftment of T-cells is thus minimized and survival of engrafted mice thus not limited by graft versus host disease.

1) Transplantation of unsorted bone marrow is feasible with pre-incubation of anti-CD3 antibody
2) T-cell depletion and engraftment of the CD3 negative fraction is feasible with or without pre-incubation of anti-CD3 antibody

3) CD34 selection and engraftment of the CD34-selected fraction gives optimal engraftment results with multilineage hematopoiesis (absent T-cells with pre-incubation with anti-CD3 antibody).

(ii) Pre-transplant Irradiation Dose:
Based on studies with normal CD34+ HSPC engraftment a pre-transplant irradiation dose of 2x150cGy administered 4-8 hours apart was chosen.
(iii) Transplantation route:
Based on studies with normal CD34+ HSPCs intrahepatic engraftment into newborn MISTRG mice was chosen.
(iv) Age of murine recipients: Based on our studies with fetal liver derived CD34+ cells and adult HSPCs intrahepatic engraftment into newborn mice was chosen. The major risk of intrahepatic injection in newborn mice is rejection by the mom. We have extended the injection time from postnatal day 1 to day 3. MISTRG mice successfully engraft up to day 3 postnatally.

Low- and high-grade MDS efficiently engraft in MISTRG but not NSG mice.

Based on these techniques we have established an efficient and reliable MDS xenotransplantation model. Examples of successful MDS xenotransplantation results are given in Figure 5:

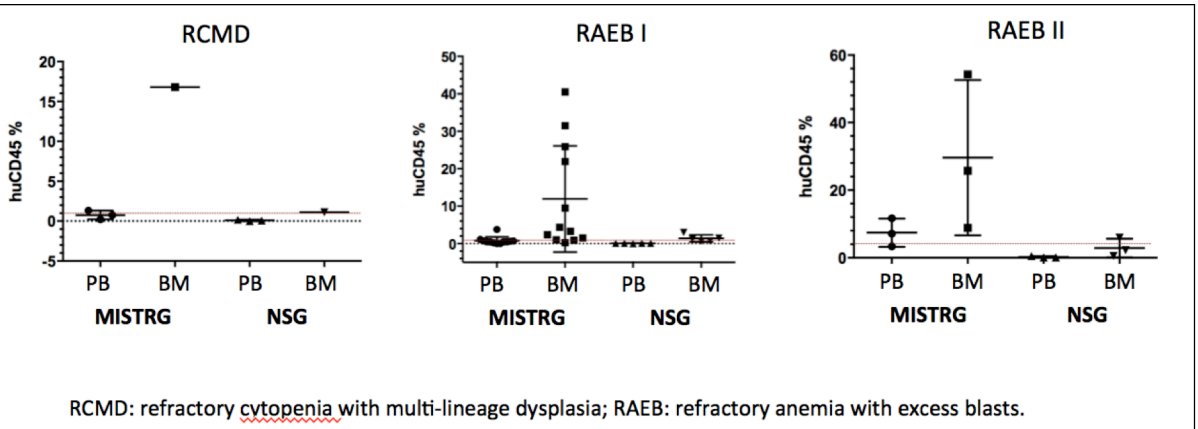

Figure 5: Low and high grade MDS hematopoietic stem cells engraft in MISTRG mice. MDS engraftment in MISTRG is superior to engraftment in NSG mice.

Given the successful engraftment of normal CD34+ HSPCs and first MDS samples, we tested engraftment of primary MDS BM derived CD34+ cells from patients with low and high-grade MDS. With the goal to develop

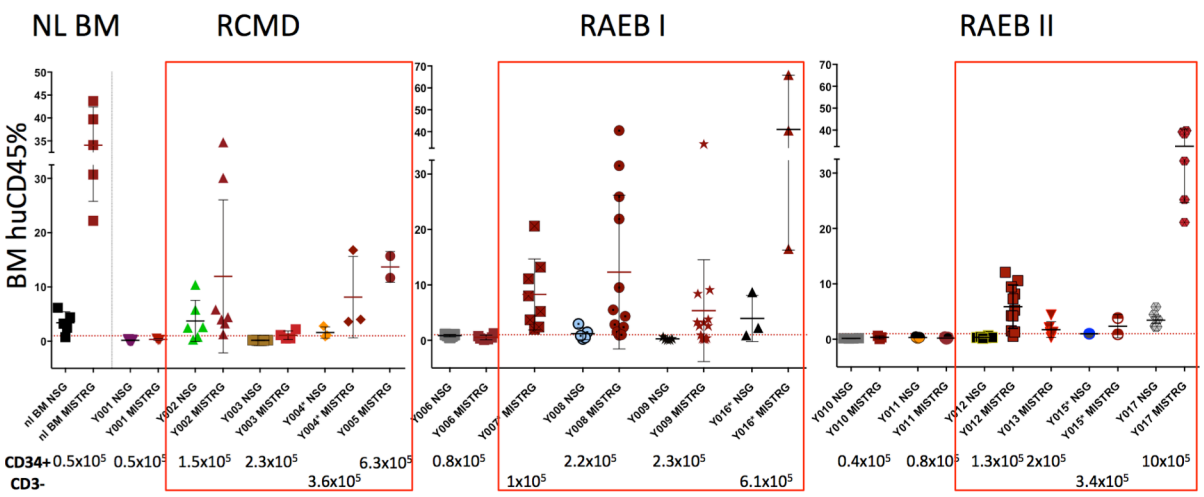

Figure 6: Low- and high-grade MDS efficiently engraft in MISTRG but not NSG mice

a pre-clinical model we engrafted CD34+ cell numbers based on availability of cells and newborn NSG and MISTRG mice with cell numbers ranging from $5x10^4$ – 10^6 CD34+ enriched cells/mouse. Whenever available MISTRG and NSG newborn mice were engrafted at the same time. As evident by the data presented in Figure 6, a minimum of 100,000 CD34+ MDS HSPCs are required for measurable engraftment > 1% (red line) in MISTRG and NSG mice. MISTRG mice promoted significantly higher engraftment in BM and PB (PB not shown).

Task 3. Study MDS disease progression MISTRG mice (mths 10-24):
Study disease progression in MISTRG mice, such as clonal evolution and progression to AML

Task 3a. Analyze MDS-engrafted primary and secondary MISTRG recipients for disease phenotype and clonality.

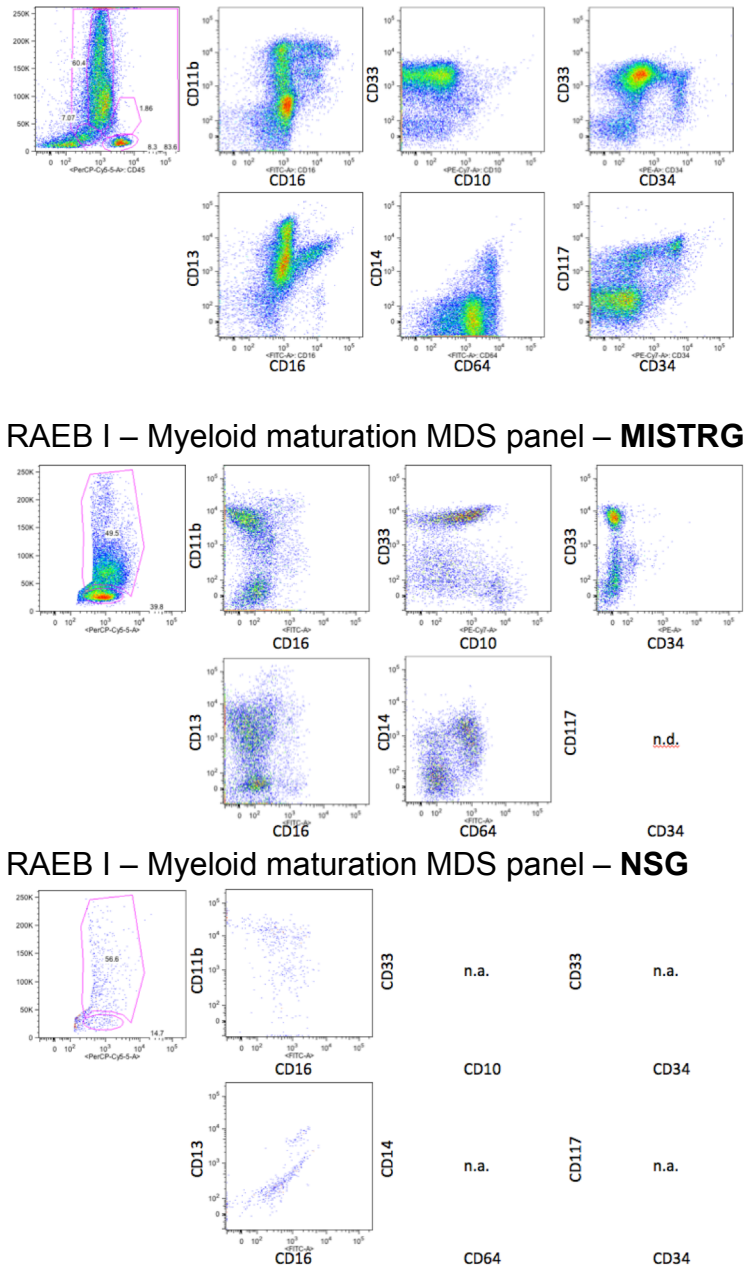

Figure 7: Abnormal myeloid maturation is a hallmark of myelodysplasia in patients' bone marrow (top). MISTRG mice show robust myeloid engraftment and replicate the patient's disease phenotype (middle) while myeloid engraftment in NSG mice is suboptimal (bottom).

We transplanted MDS derived HSPC into MISTRG mice with the protocol established from task 2a. and 2b. We transplanted MDS derived HSPC from patients with different subtypes of MDS (according to the World Health Organization (WHO) classification). Due to the heterogeneity of the disease we transplanted as many patient samples as possible to establish a solid model and to understand the different subtypes of the disease, contribution of different genetic and epigenetic abnormalities, and patient history.

MISTRG mice replicate the disease phenotype in patients.
We engrafted MISTRG and NSG mice with primary bone marrow cells from MDS patients. We engrafted CD34-selected cells with pre-incubation with anti-T-cell antibody to prevent acute graft versus host disease.
MISTRG replicate the disease phenotype in regards to myeloid differentiation and blast percentage encountered in human samples.

MDS clonal hematopoiesis is represented and preserved in MISTRG mice.

MDS is a clonal disorder of the stem cell. Diseased and normal stem cells can coexist in a patient's bone marrow and normal, healthy stem cells may preferentially engraft and repopulate the murine host in xenotransplantation studies. MDS stem cell are carry genetic aberrations, such as chromosomal rearrangements and gene mutations. To determine whether the patient's disease clone(s) engrafted in MISTRG and NSG mice, we sorted human hematopoietic cells from MISTRG and NSG bone marrow an submitted these to chromosomal and FISH analysis. In addition, we designed a targeted exome library to identify mutations in ~ 200 genes frequently mutated in MDS and other myeloid malignancies (Figure 9).

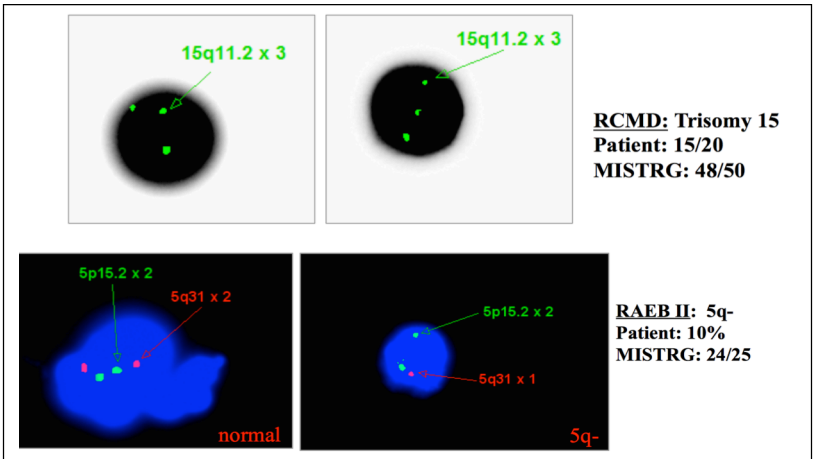

Figure 8: MDS clonal hematopoiesis is represented and preserved in MISTRG mice: FISH.

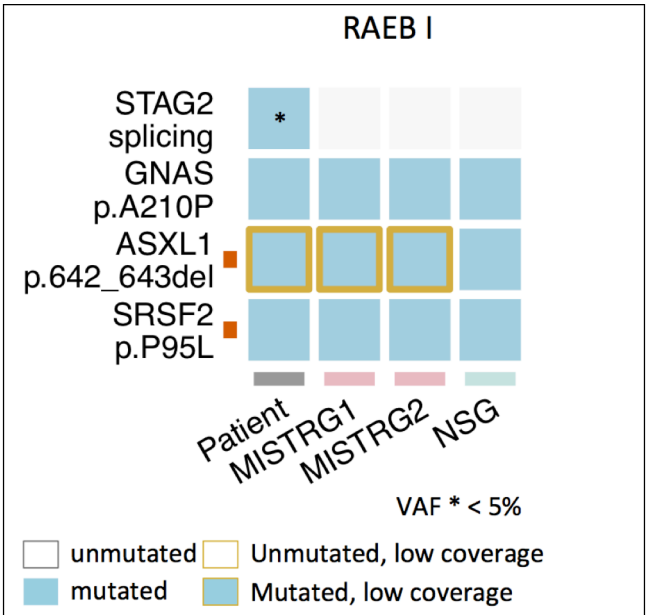

Figure 9: MDS clonal hematopoiesis is represented and preserved in MISTRG mice: targeted exome sequencing.

MISTRG mice replicate the hallmark of MDS: myeloid dysplasia.

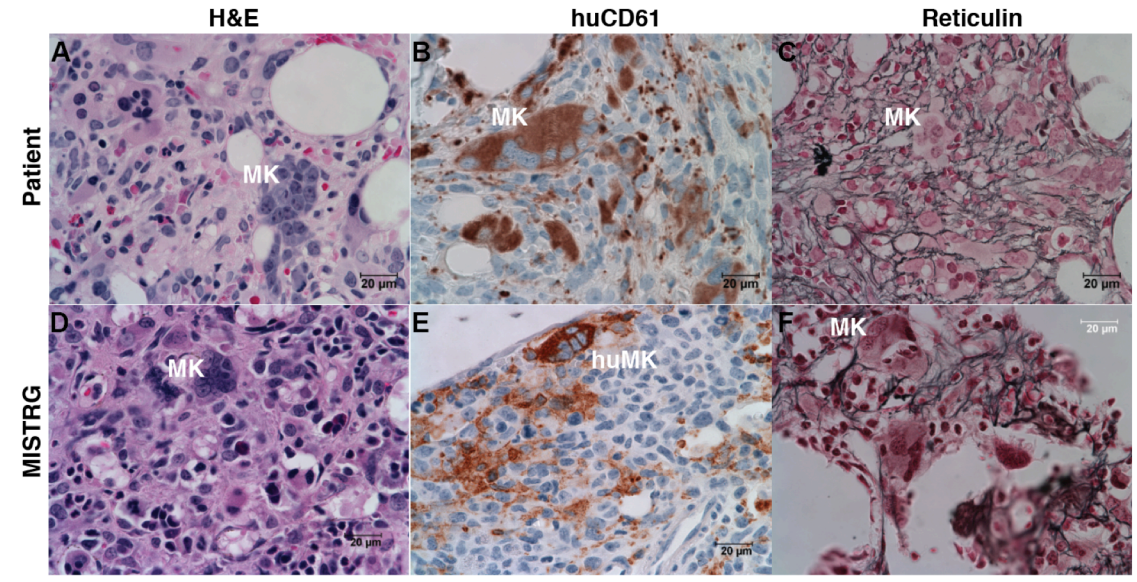

Figure 10: MISTRG mice replicate the hallmark of MDS: myeloid dysplasia. **A, D** H&E. **B, E** huCD61 staining human megakaryocytes. **C, F** Reticulin staining revealing fibrosis.

MISTRG mice were engrafted with a primary MDS sample, RAEB I, with DNMT3a mutation. Mice were allowed to engraft for 16-20 weeks and analyzed for engraftment levels and signs of dysplasia. As evident in Figure

10, histologic findings in engrafted MISTRG mice were those encountered in the patient at time of diagnosis. H&E reveals the presence of dysplastic megakaryocytes (MK) and the presence of fibrosis as well as absence of mature myeloid elements. Dysplastic megakaryocytes are highlighted by anti-huCD61 staining in the patient sample (top) and in engrafted MISTRG bone marrow. Reticulin fibrosis is markedly increase in the patient's and MISTRG bone marrow.

Task 3b. Establish treatment protocols in (i) non-engrafted and then (ii) engrafted MISTRG mice .
Drug treatment: We have successfully established a treatment protocol using standard chemotherapy in MISTRG mice.
As opposed to NSG mice, MISTRG mice do not carry an inherent DNA repair defect, thus allowing the use of cytotoxic drugs. First studies have been performed in non-engrafted mice to assess toxicity of chemotherapeutic agents in MISTRG mice. MISTRG mice tolerated cytarabine (50mg/kg vs 100mg/kg, daily x 5 days) and doxorubicin (1.5mg/kg vs 3mg/kg, daily x 3days) alone and in combination without significant liver toxicity unlike NSG mice. However, main toxicity in MISTRG mice is hematopoietic toxicity and survival after chemotherapy administration is approximately 50%.

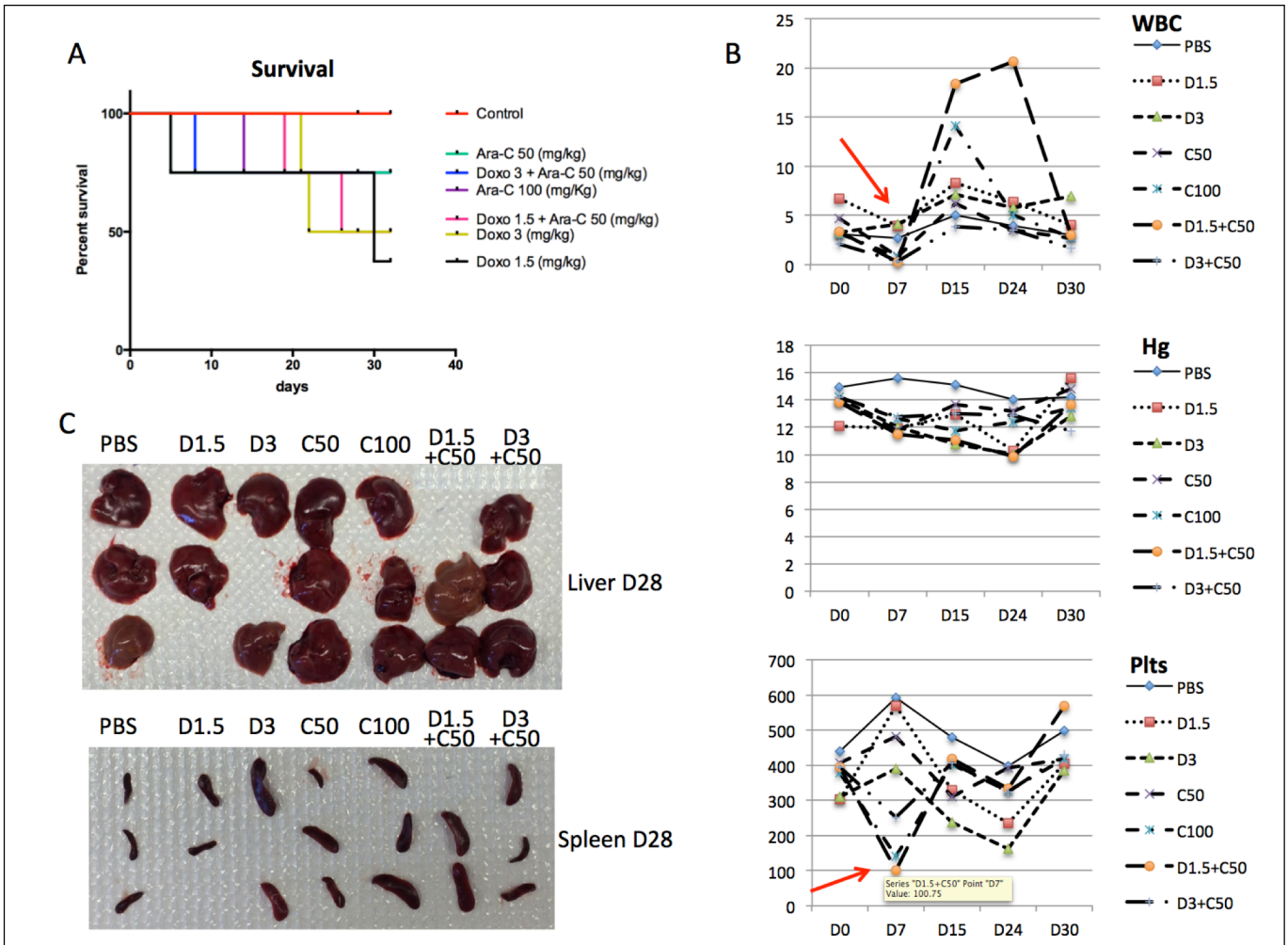

Figure 11: Chemotherapy treatment in MISTRG mice. Non-engrafted MISTRG mice were treated with vehicle, Doxorubicin, Cytarabine, or a combination of Doxorubicin and Cytarabine. Approximately 50% of mice survive chemotherapy treatment (**A**) due to significant cytopenias (**B**, white blood cell count (top), hemoglobin (middle), and platelet count (bottom)). MISTRG mice do not display liver toxicity (**C**). Enlarged spleens are a mark of splenic erythropoiesis in response to chemotherapy.

Task 4. Develop a model to study MDS "disease-driving" mutations (mths 1-24):
Study individual mutations identified in our primary MDS samples by introduction of these mutations into human CD34+ stem and progenitor cells followed by in vitro and in vivo analysis

2a. Generation of inducible lentiviral vectors and *in vitro* analysis of transduced CD34+ cells.

We have successfully cloned lentiviral vectors with inducible expression of WT and MUT SRSF2 and U2AF1, key splicing factors carrying point mutations in MDS. However, as our and the research of others has revealed, exogenous expression of mutant splicing factors does not provide the expected clonal advantage over wildtype cells. This has significantly limited our ability to engraft engineered CD34+ cells into MISTRG or NSG mice (data not shown)

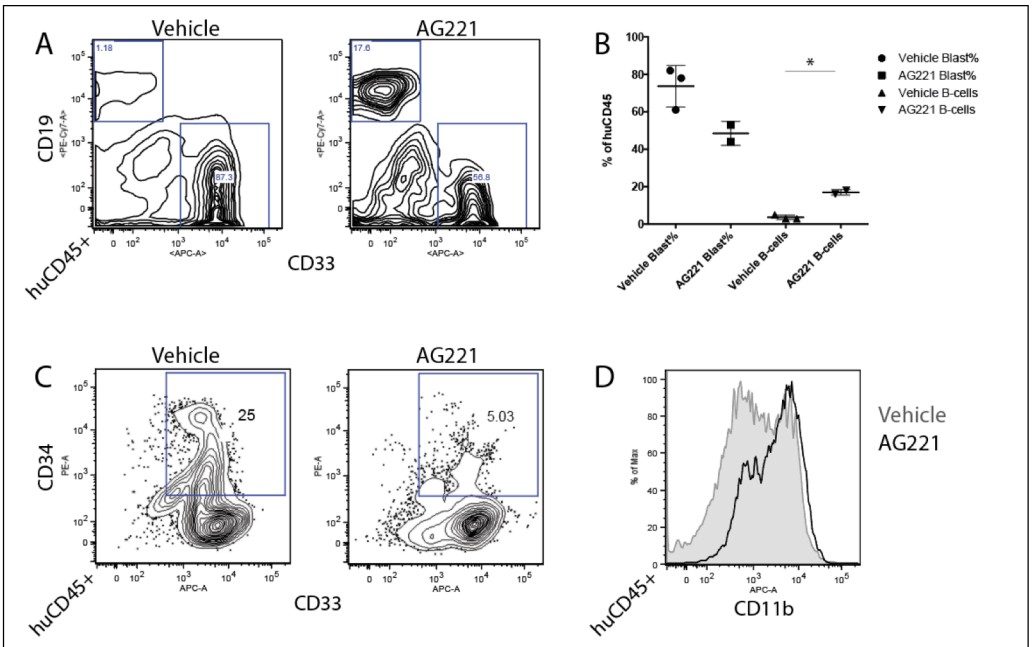

Figure 12: Treatment of IDH2 mutant AML and MDS with IDH2 inhibitors in MDS-MISTRG PDX. **A, B** AG221 treated AML engrafted MISTRG show a reduction in blast percentage and resurgence of multi-lineage engraftment (CD19+ B-cells). **C** AG221 treatment results in reduction in MDS CD34+ blast percentage and **D** myeloid differentiation.

CRISPR/Cas mediated gene editing represents an attractive alternative to exogenous expression of mutant proteins and we have pursued this as an alternative strategy. These studies are now ongoing in follow-up to this proposal.

To determine, whether MDS-MISTRG PDX allow the study of disease of specific driving mutations we selected MDS samples specifically based on their mutational spectrum and tested the effects of targeted therapeutics on MDS engraftment, blast percentage, and lineage differentiation.

IDH2 mutations occur in approximately 20% of AML and 5% of MDS patients. They occur early in the disease process and promote loss of differentiation and progenitor expansion. Promising IDH2 inhibitors have been developed that are in clinical trials.

We engrafted MISTRG mice with IDH2 mutant AML and MDS and subjected mice to short- and long-term treatments with vehicle or the IDH2 inhibitor AG221.

IDH2 inhibition results in reduction in blast % and concomitant multilineage differentiation (Figure 12).

Future projects aim at understanding the cooperativity between IDH mutations and other driver mutations as well as the effects of IDH2 inhibition on the MDS stem cell compartment.

Research Timeline	Year 1				Year 2			
Review and approval of studies involving human subjects (IRB; HRPO) and animals (IACUC; ACURO)	✓							
Aim 1. Optimize transplantation of primary MDS bone marrow cells into MISTRG mice		✓	✓	✓	✓			
Aim 1.1 Transplantation of healthy adult stem cells		✓	✓					
Aim 1.2 Transplantation of MDS HSC			✓	✓	✓	✓		
Aim 2. Study MDS disease progression in MISTRG mice				✓	✓	✓	✓	✓
Aim 2.1a Analyze MDS-engrafted primary and secondary MISTRG recipients for disease phenotype and clonality				✓	✓	✓	✓	✓
Aim 2.1b Establish treatment protocols in non-engrafted and then engrafted MISTRG mice		✓	✓	✓	✓	✓		
Aim 2.2 Develop a model to study MDS "disease-driving" mutations	✓	✓	✓	✓	✓	✓	✓	✓

Key Research Accomplishments:

- Optimal transplantation technique into MISTRG for adult HSPCs has been established in regards to cell preparation, cell number, pre-transplant irradiation, and intrahepatic injection in d1- d3 old pups.
- Engraftment of adult HSPCs in MISTRG mice is superior to standard immunosufficient mice.
- Patient MDS bone marrow derived HSPCs successfully engraft MISTRG mice. An optimal transplantation model has been established.
- MISTRG give rise to multilineage engraftment and successful myeloid maturation.
- Engraftment of MDS HSPCs in MISTRG replicates the patient's immunophenotype.
- MISTRG mice lack the DNA repair defect inherent to NSG mice and are thus tolerant of chemotherapeutic agents allowing the testing of combination therapies.
- MISTRG mice engraft clonal MDS hematopoiesis as evident by cytogenetics, FISH, and targeted exome sequencing. Clonal distribution and mutant allele frequencies found in patients were preserved in our MDS-PDX model.
- MISTRG mice supported robust myeloid engraftment and replicated the patients' MDS immunophenotypes while NSG mice had a higher frequency of lymphoid predominant grafts. Interestingly, overall MDS CD34 + cells result in higher myeloid engraftment than normal bone marrow in NSG and MISTRG (samples not age matched) as has been previously noted.
- Engrafted MISTRG bone marrows replicated the corresponding patient bone marrow findings, such as marked dysplasia and clustering of human CD61 positive megakaryocytes with resultant myelofibrosis as well as myeloid differentiation.
- MISTRG mice represent the ideal model for the development and testing of therapeutics targeted at MDS driver mutations. Inhibitors of mutant IDH2 reduce blast percentage in MISTRG mice transplanted with IDH2 mutant AML and high risk MDS and induce differentiation of myeloblasts as evident by upregulation of CD11b and CD15 (tested by flow Cytometry and histologic analysis).

Conclusion

MISTRG mice engraft low and high grade MDS with replication of the disease genotypes and phenotypes, supporting higher engraftment than NSG mice for all MDS samples assessed. We have developed a highly successful MDS xenotransplantation model in humanized immunodeficient mice that promises to become the new gold standard for *in vivo* MDS studies. This model represents the ideal platform to understand MDS biology and to decipher the response at the MDS stem cell level to targeted therapeutics.

List of Personnel

Stephanie Halene
Yuanbin Song
Ashley Taylor
Yang (Stephen) Liang

Publications, Abstracts and Presentations

Manuscripts are in preparation

Oral Presentations:

International/National:

- 2015: Sir William Osler Interurban Clinical Club; Young Investigator Award; 211th Meeting of the Interurban Clinical Club (Baltimore, Boston, New Haven, New York, Philadelphia); New Haven, CT ; "Molecular Mechanisms of MDS and the Development of a Xenotransplantation Model"
- 2015: 13th International Symposium on Myelodysplastic Syndromes (MDS), Washington D.C.. "Humanized Mice Afford Efficient Engraftment and Disease Replication of Myelodysplastic Syndromes."
- 2016: International Working Group on Humanized Mice (IWHM), Zuerich, Switzerland. "Efficient Engraftment and Disease Replication of Myelodysplastic Syndromes Using a Novel Humanized Mouse Model."

Regional:

- 2014: Yale Cancer Center Research in Progress. Xenotransplantation Studies in MDS; The role of Splicing Factor Mutations in MDS
- 2015: Cardiovascular Research Group Series. Modeling Myelodysplasia.

Abstracts:

IWHM Zuerich 1/2016:

Efficient Engraftment and Disease Replication of Myelodysplastic Syndromes Using a Novel Humanized Mice Model

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Myelodysplastic Syndromes (MDS) are a heterogeneous disorder of the hematopoietic stem cell caused by a large number of genetic and epigenetic alterations. MDS is inherently difficult to study *in vitro*. We established a MDS xenotransplantation model in humanized immunodeficient mice amenable to mechanistic *in vivo* studies and therapeutic testing.

"MISTRG" mice express several essential human, non-crossreactive cytokines, namely *M*-CSF, *I*L-3, GM-CSF, and *T*hrombopoietin as well as human macrophage receptor signal regulatory protein-alpha (SIRPα) from the respective murine loci, in the *Rag2*$^{-/-}$*IL2rγ*$^{-/-}$ background (Rongvaux et al. Nature Biotech 32(4): 364 – 372, 2014).

We optimized host irradiation dose, transplantation route, CD34^{+} cell number and cell preparation. Mice were allowed to engraft for >10 weeks. Peripheral blood (PB), bone marrow (BM), and spleen were analyzed for engraftment by flow cytometry and tissues submitted for histologic analysis. HuCD45^{+} cells from engrafted MISTRG BM were subjected to targeted exome sequencing.

MISTRG mice consistently supported higher engraftment in PB and BM than NSG mice for low and high grade MDS. On average, huCD45 engraftment was 7.3-fold higher in MISTRG than in NSG mice (17.78% vs. 2.45%) and 56.1% of MISTRG versus 26.7% of NSG mice were engrafted with improved preservation of clonal distribution and mutant allele frequencies. MISTRG as opposed to NSG mice supported robust myeloid engraftment and replicated the patients' MDS immunophenotype. Engrafted MISTRG bone marrows replicated the corresponding patient bone marrow findings, such as marked dysplasia and clustering of human CD61 positive megakaryocytes with resultant myelofibrosis.

In conclusion, MISTRG mice engraft low and high grade MDS with replication of the disease genotypes and phenotypes, supporting higher engraftment than NSG mice for all MDS samples assessed. With lack of the DNA repair defect inherent to NSG mice MISTRG represent a promising "co-clinical" model of MDS to dissect disease mechanisms and for therapeutic testing.

ASH 12/2015:

Efficient Engraftment and Disease Replication of Myelodysplastic Syndromes Using a Novel Humanized Mice Model

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Myelodysplastic Syndromes (MDS) are a heterogeneous disorder of the hematopoietic stem cell caused by a large number of genetic and epigenetic alterations. With the development of novel therapeutics a reliable model to test the drugs' efficacy in correlation with genetic and epigenetic alterations and disease phenotype is essential. Recent advances in the field of MDS xenotransplantation have been achieved by transgenic expression of human cytokines in the murine host as well as by co-transplantation of primary patient derived mesenchymal stromal cells (MSCs) concurrent with MDS stem cells. However, neither model to date affords efficient transplantation of MDS at a scale that allows in vivo mechanistic studies or provides a platform to develop and test novel therapeutics.

We sought to establish a MDS xenotransplantation model in humanized immunodeficient mice amenable to mechanistic *in vivo* studies and therapeutic testing.

Several murine cytokines essential for hematopoiesis are non-crossreactive with their human counterpart. "MISTRG" mice express several human, non-crossreactive cytokines, namely *M*-CSF, *I*L-3, GM-CSF, and *T*hrombopoietin from the respective murine loci, as well as human macrophage receptor signal regulatory protein-alpha (*S* IRPα) to prevent murine macrophage-mediated immune rejection in the *Rag2*$^{-/-}$ *IL2rγ*$^{-/-}$ background (Rongvaux et al. Nature Biotech 32(4): 364 - 372, 2014).

To establish a reliable, efficient MDS xenotransplantation model we optimized the host irradiation dose, transplantation route, CD34^{+} cell number and cell preparation. Mice were allowed to engraft for >10 weeks. Peripheral blood (PB), bone marrow (BM), and spleen were analyzed for engraftment by flow cytometry. BM, spleen, and liver were also fixed and sectioned for histologic analysis. Human CD45^{+} cells were sorted from engrafted MISTRG bone marrow and genomic testing was performed by cytogenetics, FISH, and/or targeted exome sequencing.

MISTRG mice consistently supported higher engraftment in peripheral blood and bone marrow than NSG mice for the majority of MDS samples assessed. Out of 25 different patient's BM samples, including 6 RCMD, 5 RAEB I, 12 RAEB II, and 2 CMML patient samples, 23 samples engrafted in MISTRG mice, while 19 of the samples were transplanted concurrently into NSG mice (6 RCMD, 3 RAEB I, and 11 RAEB II patient samples) out of which 12 samples engrafted. Mice were classified as engrafted when huCD45^{+} cells accounted for over 1% of all nucleated cells in BM. On average, huCD45 engraftment was 7.3-fold higher in MISTRG than in NSG mice (17.78% vs. 2.45%). 56.1% of all MISTRG mice compared with 26.7% of NSG mice transplanted with MDS were engrafted. The number of engrafted MISTRG mice per sample ranged from 2-10 mice could be further improved with optimal bone marrow sample collection.

We verified engraftment of the MDS clone via cytogenetics, FISH, and/or targeted exome sequencing, also revealing preserved clonal distribution and mutant allele frequencies in engrafted mice. Flow cytometric analysis of lineage differentiation revealed robust myeloid engraftment in MISTRG mice as opposed to NSG mice. In addition terminal differentiation of myeloid cells was markedly improved in MISTRG over NSG mice, with immunophenotypic concordance between engrafted MISTRG mice and the patient's primary bone marrow. Histologic analysis showed striking similarities between engrafted MISTRG bone marrow and the concurrent patient's bone marrow, such as marked dysplasia and clustering of human CD61 positive megakarocytes with resultant myelofibrosis as evident by reticulin staining. MISTRG mice lack the DNA repair defect inherent to

NSG mice and are thus tolerant of chemotherapeutic agents. Studies testing hypomethylating drugs and targeted agents are now underway to establish MISTRG as promising "co-clinical" model for MDS.

In conclusion, physiologic expression of essential non-crossreactive human cytokines greatly facilitates long-term engraftment of MDS patient derived CD34+ HSPCs in the murine immunodeficient host. MISTRG mice engraft lower and higher grade MDS with replication of the disease genotypes and phenotypes, supporting higher engraftment in bone marrow and blood than NSG mice for all MDS samples assessed. MISTRG mice may provide a xenotransplantation model for MDS allowing us to study the biology of the disease and to test therapeutics *in vivo*.

MDS Meeting May 2015

Humanized Mice Afford Efficient Engraftment and Disease Replication of Myelodysplastic Syndromes

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Background Myelodysplasia is a heterogeneous disorder of the hematopoietic stem cell caused by a large number of genetic and epigenetic alterations. With the development of novel therapeutics a reliable model to test their efficacy in correlation with genetic and epigenetic alterations and disease phenotype is essential.

Introduction Interesting advances have been made in the field of MDS xenotransplantation by modifying the host via transgenic expression of human cytokines as well as by improving cell preparation by co-transplantation of primary patient derived MSCs with MDS CD34+ stem and progenitor cells. However, neither model to date affords efficient transplantation of MDS at a scale that allows in vivo mechanistic studies or provides a platform to develop and test novel therapeutics.

Purpose We sought to establish a MDS xenotransplantation model in humanized immunodeficient mice amenable to mechanistic *in vivo* studies and therapeutic testing.

Materials and Methods We optimized xenotransplantation into *Rag2*$^{-/-}$*IL2rγ*$^{-/-}$ mice, named MISTRG, that express human macrophage receptor signal regulatory protein-alpha (SIRPα) to prevent murine macrophage-mediated immune rejection as well as several human, non-crossreactive cytokines, namely M-CSF, IL-3, GM-CSF, and thrombopoietin from the respective murine loci (Rongvaux et al. Nature Biotech 32(4): 364 – 372, 2014). We optimized irradiation dose, transplantation route, CD34+ cell number and preparation prior to injection for MDS engraftment. We performed direct comparison between NOD/scid/*IL2rg*$^{-/-}$ (NSG) and MISTRG mice. Mice were allowed to engraft for >10 weeks and peripheral blood, bone marrow and spleen were analyzed for engraftment by flow cytometry, histology, and genomics.

Results MISTRG mice consistently supported higher engraftment in peripheral blood and bone marrow than NSG mice for all MDS samples assessed, including RCMD, RAEB I, and RAEB II. Over 70% of all MDS samples engrafted in MISTRG mice, with detection of the abnormal clone via genetic testing. MISTRG mice support myeloid engraftment with improved terminal differentiation as determined by flow cytometry, with improved immunophentypic concordance between MISTRG mice and the patient's primary bone marrow. Bone marrow histology of engrafted MISTRG mice replicates histologic findings in patient bone marrows, such as dysplastic megakaryocytic differentiation with resultant myelofibrosis. The number of

engrafted MISTRG mice per sample ranged from 2-10 mice which may be improved with optimal bone marrow sample collection.

Conclusions Physiologic expression of essential non-crossreactive human cytokines greatly facilitates long-term engraftment of MDS patient derived CD34+ HSPCs in the murine immunodeficient host. MISTRG mice engraft lower and higher grade MDS with replication of the disease geno- and phenotypes.

Inventions, Patents and Licences
N/A

Reportable Outcomes
N/A

Other achievements
N/A

References
N/A

References:

1. Rongvaux, A., et al., *Development and function of human innate immune cells in a humanized mouse model.* Nat Biotechnol, 2014. **32**(4): p. 364-72.
2. Wunderlich, M., et al., *OKT3 prevents xenogeneic GVHD and allows reliable xenograft initiation from unfractionated human hematopoietic tissues.* Blood, 2014. **123**(24): p. e134-44.