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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this research proposal is to provide better treatments for Fanconi Anemia (FA), an inherited bone marrow failure disorder that affects approximately 1 in 100,000 children. The combination of hematopoietic stress and inherent genomic instability leads to cancer and accumulation of genetic defects is likely the cause of AML progression. We proposed to study primary human cell defective in the FA pathway to delineate pathways of leukemia progression and eventually prevent progression to bone marrow failure or progression to leukemia. Our two aims are to 1) identify molecular vulnerabilities and genetic changes promoting oncogenesis in FA deficient CD34+ cells *in vitro* and to 2) determine molecular changes at the root of disease progression in primary human FA bone marrow and test potential therapeutic approaches *in vivo* in MISTRG-kit^{MUT} mice. To achieve this goal we have to i) obtain primary FA patient cells and ii) generate human FANC gene KO CD34+ cells. Note that the COVID pandemic has significantly impaired our progress since 3/15/2020. We have focused our efforts on generating FA defective cells via two mechanisms: a) shRNA mediated knockdown and b) via CRISPR/Cas9 mediated deletion. We have encountered 2 difficulties which we are still addressing: inefficiency of deleting FA genes and selection against deleted cells; silencing of rescue lentiviral vectors in primary hematopoietic cells. With COVID all work had to halt and mouse work was minimal – we are expanding colonies and actively transplanting primary FA samples with goal to further optimize engineering of FA samples and transplantation in MISTRG mice.

15. SUBJECT TERMS
None listed.

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INTRODUCTION: The goal of this proposal is to identify factors in Fanconi Anemia that accelerate or delay oncogenesis. We propose to generate *in vitro* and *in vivo* models of FA via genetic engineering of primary human CD34+ hematopoietic stem and progenitor cells (HSPC) and transplantation of genetically engineered and primary patient-derived FA HSPC into humanized MISTRG-kit^{MUT} mice.

KEYWORDS: Fanconi Anemia, bone marrow failure, leukemia, CRISPR/Cas9, humanized mice, xenotransplantation.

OVERALL PROJECT SUMMARY: The goal of this research proposal is to provide better treatments for Fanconi Anemia (FA), an inherited bone marrow failure disorder that affects approximately 1 in 100,000 children. The combination of hematopoietic stress and inherent genomic instability leads to cancer and accumulation of genetic defects is likely the cause of AML progression. We proposed to study primary human cell defective in the FA pathway to delineate pathways of leukemia progression and eventually prevent progression to bone marrow failure or progression to leukemia. Our two aims are to 1) identify molecular vulnerabilities and genetic changes promoting oncogenesis in FA deficient CD34+ cells *in vitro* and to 2) determine molecular changes at the root of disease progression in primary human FA bone marrow and test potential therapeutic approaches *in vivo* in MISTRG-kit^{MUT} mice. To achieve this goal we first have to i) obtain primary FA patient cells and ii) generate human FANC gene KO CD34+ cells. As discussed, during year 1 of our award Dr. Nalepa's unexpected passing has slowed down our efforts to obtain primary patient samples. Year 2 has been cut short due to COVID, extending into the NCE period. We have focused our efforts on generating FA defective cells via two mechanisms: a) shRNA mediated knockdown and b) via CRISPR/Cas9 mediated deletion. We generated all tools for FA gene deletion, including FANC gene cDNAs with modified PAM sites to allow temporary rescue of FA deficient cells. In vitro deletion was optimized. When we engrafted MISTRG-kit^{MUT} mice with CD34+ cells modified via CRISPR, FA gene deleted cells experienced significant growth disadvantage. Temporarily inducible expression of FANC genes via lentiviral transduction to rescue cells at time of engraftment failed due to silencing of the inducible vector in hematopoietic cells. To further understand FA pathology and advance treatments, we have conducted synthetic lethal screens and validation for targets and therapeutics detrimental to FA deficient cells *in vitro*.

ACCOMPLISHMENTS: (according to approved SOW)

The **major goals and accomplishments** are stated under each task.
This project was not intended to provide **training and professional development o**pportunities, but the postdoctocal associate, Dr. Wei Liu, in the Halene laboratory has learnt about the biology of FA and xenotransplantation into humanized mice. He has enhanced his skills in CRISPR/Cas9 technology and lentiviral vector design as well as work with primary human hematopoietic stem and progenitor cells.
The data has **not yet been disseminated** at meetings or in publication.

Task 1. IRB and HRPO review and approval for studies involving human subjects and IACUC and ACURO review and approval for animal use (mths 1-3):
1.1 After selection for the award, local Internal Review Board approval of the human subject protocol to harvest bone marrow from patients has been obtained. DoD regulatory review of the submitted HRPO protocol has been performed and approved. The latter approval was delayed due to the sudden passing of our collaborator, Dr. Nalepa, at IU. Our goal was to recruit an estimated total of 10 human subjects for the studies proposed in this award during the funding period. As we expected delay and potentially lower accrual we are emphasizing genetically engineered FA cells. De-identified peripheral blood mobilized and umbilical cord blood CD34+ were obtained as fee for service through a core facility at the Yale Cooperative Center for Experimental Hematology.

1.2 After selection for the award, local IACUC and DoD regulatory review and approval for the use of animals (mice) were obtained.
MISTRG-kit^{W41} mice were bred to homozygosity and the colony was expanded to serve for transplantation studies for this grant. During COVID breeding had to be significantly reduced with re-expansion once laboratory operations could resume.

Task 2. In vitro studies of FA
2.1 Production of shRNA knockout and CRISPR-altered FANC gene knockout cells

Given the rarity of FA patient samples and the very low CD34+ cell number we sought to generate FA models by inducing gene deletion via shRNAs or CRISPR/Cas9 deletion of FANC genes.
CRISPR/Cas9 mediated gene deletion is highly efficient >>50%, but does not occur in <u>all</u> cells. Non-modified cells are highly likely to outgrow deleted cells even if present at a low ratio.
To overcome this shortcoming we devised a rescue strategy with the hope to circumvent outgrowth of wildtype cell and preferential loss of FANC gene deleted cells by cloning an inducible FANC gene overexpression vector described in more detail below. Our goal was to modify cells with shRNA or CRISPR and at the same time transduce cell with the inducible overexpression vector; and ultimately turn off FANC gene rescue once mice are engrafted (**Fig. 1**).

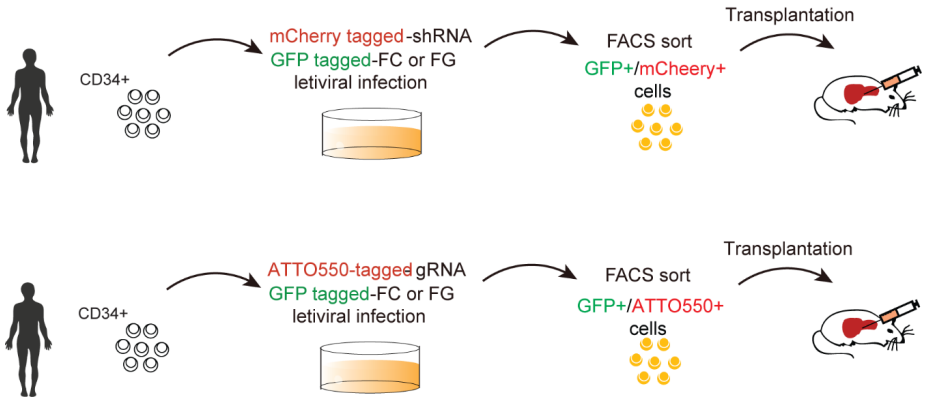

Fig. 1 Transplantation strategy of genetically engineered CD34+ cells. CD34+ cells are either transduced with mCherry tagged shRNA vectors or ATTO550 tagged gRNA and at the same time transduced with an inducible lentiviral vector expressing FANC C or FANC G; shRNAs would target the 3'UTR, absent in cDNA or PAM site would be deleted without altering protein sequence. Cells FACS sorted for both markers are transplanted into MISTRG mice.

In addition, to increase our chance at success we have targeted several FANC genes in parallel; FANCD2 is a very large protein and hyperrecombinogenic. We have therefore chosen FANCC and FANCG, also mutant in patients, for our validation studies.

<u>CRISPR/Cas9 deletion of FANC genes:</u>
We designed a series of guide (g)RNAs against FANCC and FANCG. These guide RNAs directed against were transfected into 293T and human leukemia cell lines, and the resulting populations of cells were tested for (a) decrease in FANCC and FANCG transcript (**Fig. 2**) (b) knockdown of FANCC and FANCG protein expression and (c) decrease of FANCD2 monoubiquitination, which depends on an intact FA core complex, which contains

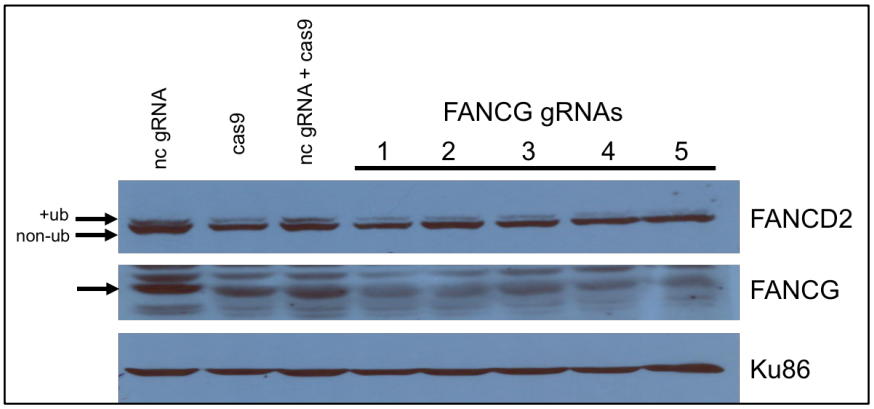

Fig. 2 CRISPR knockdown of FANCG expression results in decreased FANCD2 mono-ubiquitination

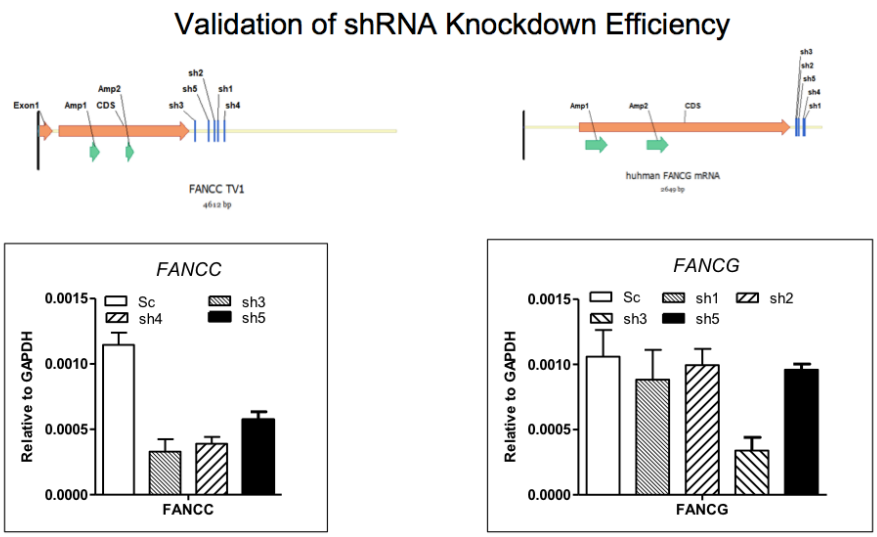

Fig. 3 Validation of FANCC (left) and FANCG (right) shRNA knockdown efficiency.

FANCC/G.
We have identified several gRNAs for both FANCC and FANCG that efficiently disrupt the respective genes, leading to reduced transcript and protein levels.
The results for FANCG targeting are shown. Immunoblot reveals roughly 50% decrease of FANCG with concomitant diminution of FANCD2 monoubiquitination, suggesting successful disruption of FANCG expression (**Fig 2**).
As an alternative to CRISPR/Cas9 mediated FANCC/G disruption we have also devised a shRNA mediated strategy. The shRNA approach will allow flow storing of stably transduced cells (**Fig. 3**). While with shRNA 100% knockdown can generally not be achieved, shRNAs are stably expressed and >50% knockdown can reliably be achieved giving rise to FA deficient cells and phenotypes.
Given that deletion of FANC genes will result in a significant growth disadvantage we screened whether the PAM sites for the efficient gRNAs could be mutated without changing protein coding (silent mutation) of FANCC or FANCG. We successfully cloned FANCC^{SilMut} and FANCG^{SilMut} into a dox-inducible lentiviral vector and validated expression for the FANC G gene (**Fig. 4**) . To test whether concurrent transduction of CAS9/gRNA and FANC G^{SilMut} carrying lentiviral vectors would rescue the proliferative disadvantage conferred by FANC G deletion we performed colony forming assays.
We expect efficient deletion of FANC genes and expansion of mutant cells that are FANC deficient upon withdrawal of doxycycline (**Fig. 5**). gRNA against FANC G significantly reduced colony formation (lanes 4&5) while induction of FANC G expression rescued in FANC G deleted cells rescued colony formation (lane 6). Of note, overexpression of FANC G in

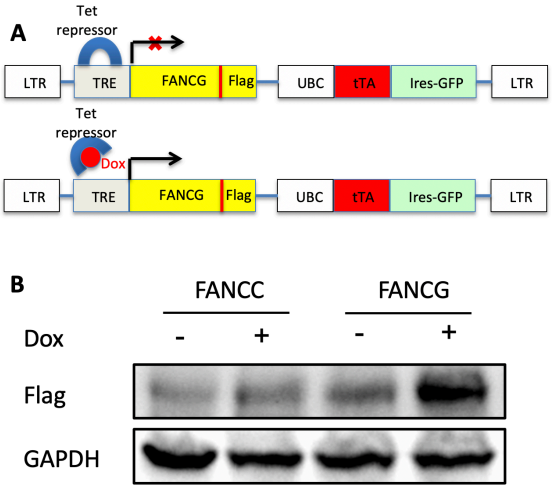

Fig. 4 Validation of Dox-inducible overexpression Vectors carrying modified FANC C or FANC G genes

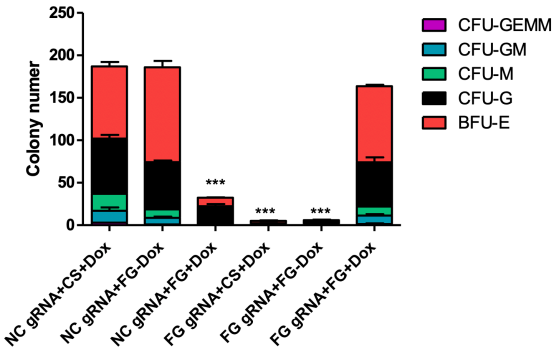

Fig. 5 Colony formation assay in control and FANC G gRNA and control and FANC G overexpressing lentiviral vectors

control gRNA-transfected cells also limited colony formation (lane 3) suggesting the need for tight regulation of FANC G expression.
Next, engraftment of GFP/mCherry sorted Cord blood CD34+ cells were engrafted in MISTRG-kit^{MUT} mice with good engraftement efficiency, given the need for in vitro manipulation and culture (**Fig. 6**). Of note, when engrafted cells were tested for GFP expression, none was detected. Since FACS sorted cells were transplanted, we isolated human cells and checked for the presence of the GFP DNA, which was present (data not shown). This suggested to us, that while transduced cells were engrafted, the inducible lentiviral vector was silenced. This is plausible since the Tet-inducible promoter is CMV based. We are now pursuing studies with alternative methods to deliver the FANC G message to allow engraftmend of CRISPR modified cells.

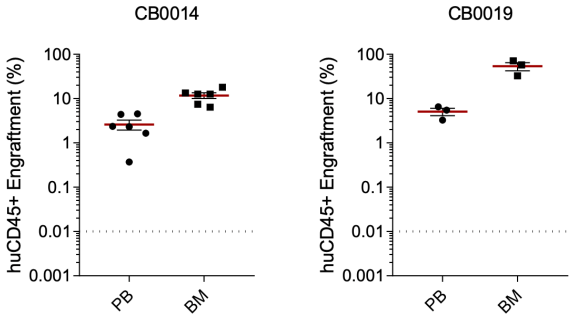

Fig. 6 gRNA and inducible lenti-viral vector transduced CD34+ cells engraft in MISTRG kit mutant mice at 12 weeks. Cells are GFP negative (data not shown).

<u>ShRNA mediated deletion of FANC genes:</u>
Hematopoietic cells easily silence lenti- and retroviral vectors. To permanently silence FANCD2, we tested 2 shRNA vectors from Dharmacon with the GIPZ shRNA backbone and the U4 promoter driving the shRNA (**Fig. 7**) with successful deletion of FANCD2. Current studies are underway to also test the Sigma Mission Bio shRNA HIV-based pLKO.1 vector backbone, with the U6 promoter driving the shRNA for its efficient transcription, and precise initation and termination.

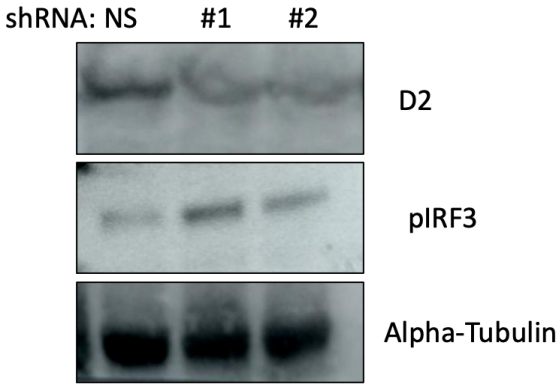

Fig. 7 CD34+ cells were transduced with GFP-shRNA directed against luciferase or FANCD2 and cells sorted for GFP expression. Lysates were immunoblotted as indicated.

2.2 Synthetic lethal screen.
Using the Yale Center for Molecular Discovery, we performed high throughput siRNA transfection of FA-D2 mutant cells, null for FANCD2 expression, and the same cells corrected with the FANCD2 wild type cDNA cultured in 384-well format plates. These plates were pre-aliquoted with an siRNA library directed against 20,000 expressed genes, and transfection was conducted in in situ. After 48 hours, cells were exposed to CELLGLO reagent as a test of viability, which was analyzed by fluorescence. Ratios of corrected cell:mutant cell viability were calculated, and positives were assessed by those ratios as greater than 2.0, with roughly 200 genes identified. In particular, groups of genes were identified, including subunits of the proteasome and cell cycle genes (PLK1 and WEE1). In order to validate such findings, we tested mutant and wild type cell survival in response to PLK1 knockdown using siRNA as well as in survival assays in the presence of the PLK1 inhibitor volasertib (**Fig**. **8** left panel). Both knockdown (**Fig. 8** middle panel) as well as pharmacologic inhibition of PLK1 (**Fig. 8** righ panel) resulted in increased cell death of mutant FA-D2 cells relative to wild type cells, suggesting that targeting PLK1 in cancer cells mutated in FA genes in patients or in non-FA patients is a viable therapeutic approach.

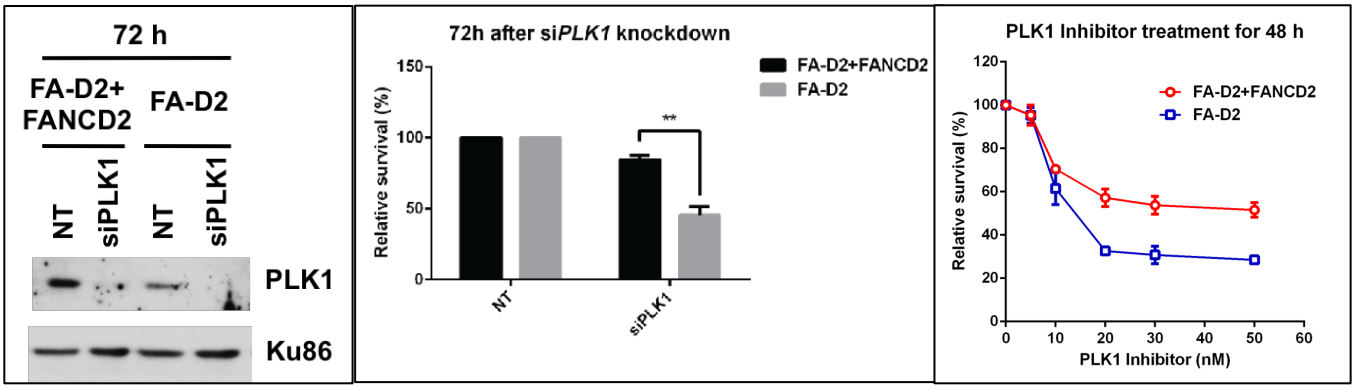

Figure 8. Decreased expression or pharmacologic inhibition of PLK1 is synthetically lethal in mutant FA-D2 cells

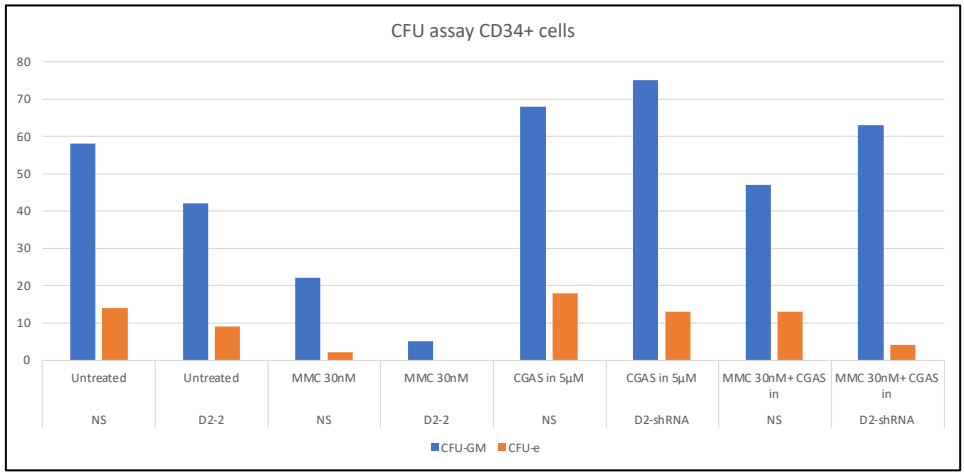

Fig 9. cGAS inhibition improves clonal growth in CD34+ cells knocked down for FANCD2

Contrastingly, we also observed specific genes that when knocked down resulted in survival improvement in FA-D2 mutant cells: cGAS-STING, phospho-IRF3, and phospho-IRF7. We blotted for phospho-IRF3 in the CD34+ cells knocked down for FANCD2 by shRNA and demonstrated increased signal, consistent with our synthetic lethal data (**Fig 9**). Such data may explain in part why FA stem cells have diminished proliferative capacity as a result of activation of pro-inflammatory pathways. The data also show an avenue that can be targeted for therapeutic intervention for bone marrow preservation in FA patients, as cGAS STING inhibitors are in clinical trials. We subjected CD34+ cells knocked down for FANCD2 expression to further simultaneous treatment with cGAS STING inhibition and to shRNA knockdown against cGAS. Resulting CFU-M and CFU-E assays revealed marked improvement in clonogencity (Fig 8), suggesting a means to test for improved stem cell function in our mouse model, where poor FA stem cell proliferation and survival has been a block. Testing of PLK1 inhibition in genetically engineered FANC gene deficient cells will be tested in the future with the goal to test PLK1 inhibition in FA-derived MDS/AML in vivo.

Task 3. In vivo studies of FA (mths 4-24):

3.1 Study of primary FA patient bone marrow into MISTRG-ckit^{MUT} mice.

i. Primary cells. We collected and cryopreserved 6 primary FA bone marrow samples over the study period (5 FA, 1 FA-sMDS/AML).

ii. Targeted exome sequencing. For each FA BM sample we have obtained clinical annotation (age, bone marrow biopsy studies, cytogenetics, FISH, flow-cytometry, clinical course) and performed

targeted exome sequencing.

iii. Humanized mice optimized for BMF. We have crossed MISTRG-kit W41 mice to homozygosity and successfully tested transplantation into these mice.

iv. Xenotransplantation. Based on our extensive experience with MDS-PDX models and our limited experience with FA we have transplanted FA patient BM-derived cells, enriched for CD34+ cells or depleted for CD3 cells intrahepatically into MISTRG-ckit^{W41} newborn mice.
We first engrafted a FA-MDS/sAML BM CD34+ cells into heterozygous MISTRG-ckit$^{MUT/WT}$ mice with very good engraftment results as shown in **Fig. 10**. Engrafted mice were analyzed at 30 weeks post primary transplant and assessed for engraftment of huCD45+ cells in PB and BM and CD34+ cells in BM (**10A,B**), validated by immune-histochemistry on BM (**10C**). We validated on two mice that FANCC mutant cells were engrafted as expected, as FA mutations are germline (**10D**). Next, we performed whole exome sequencing with spike in for 450 frequently mutated genes in cancer (**10E**) comparing mutational composition in the same two transplanted mice against the patient's mutations. These data suggest that mice engrafted with FA derived secondary myeloid malignancies replicate the clonal genotype of the primary patient sample. Next, we performed secondary transplantation from the same mice analyzed in Fig. 8D,E with successful, albeit low (0.1%) engraftment in secondary recipients (3 mice, data not shown), confirming that primary recipients were engrafted by hematopoietic stem cells. However, low secondary engraftment levels suggest a lack of expansion of FA MDS/sAML cells in MISTRG ckit mutant mice.

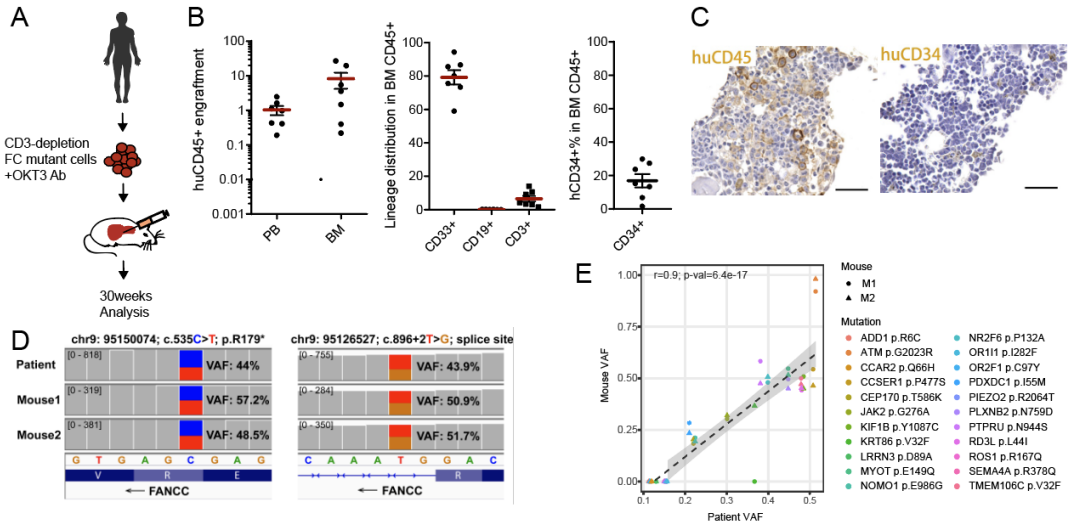

Fig. 10 Engraftment of FA-derived MDS/sAML

Next, we tested engraftment of primary FA bone marrow derived CD34+ cells into MISTRG-ckit^{W41} mice. Of note, one problem with FA samples is the low cell number available due to the bone marrow failure nature of the disease as well as the age of the patient, precluding larger volume aspirations as part of our research protocol. Mice were engrafted either via the intrahepatic route into newborn mice or intrafemorally into 10-12 week-old mice.

Both FANCG (**Fig. 11** top) and FANCA (**Fig. 11** bottom) CD34+ cells engrafted. However, engraftment was highly variable and dependent on CD34+ cell number available, greatly limiting down-stream application. For the FANCG mutant samples (top) 200,000 CD34+ cells were engrafted into each mouse. For sample 3108 15,000 and for sample 3109 35,000 CD34+ cells were injected, resulting in very low engraftment levels (BM huCD45+ < 1%, data not shown).

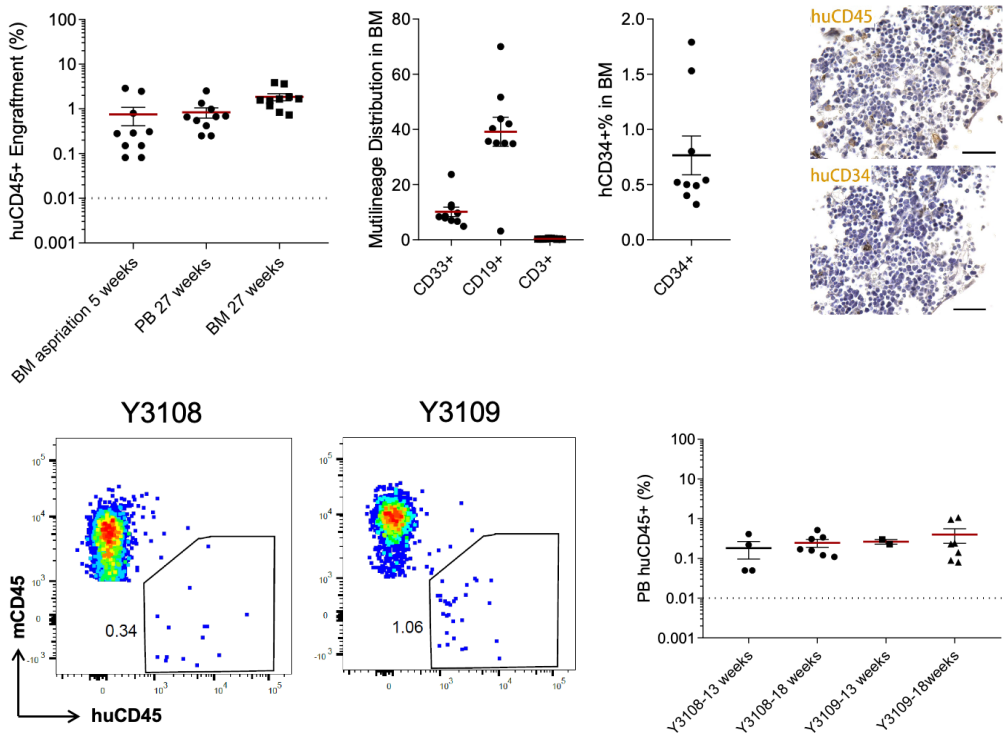

Fig. 11 Engraftment of FANCG (top) and **FANCA** (bottom) **mutant primary FA samples into MISTRG ckit^{W41} mutant mice.**

3.2 Transplantion and study of genetically engineered FA CD34+ cells into MISTRG-ckit^{MUT} mice.

For results see under **2.1 Fig. 6**.

Future plans beyond this funding period:
Engraftment of engineered CD34+ cells that are CRISPR modified is hampered by the growth disadvantage of modified compared to non-modified cells. Inducible, transient expression of FANC genes was tested to mitigate this growth disadvantage at initial engraftment. With the silencing of the dox-inducible CMV-based promoter we are working on an alternate strategy using a non-CMV based inducible promoter. Our initial work in CD34+ cells demonstrates effective knockdown of FANCD2. At the same time, with Dr. Kupfer's recent move to Georgetown University, we will continue to acquire primary FA samples at the time of bone marrow aspiration performed for routine care. We will pay specific attention to obtaining larger volume samples, as permissible by the local IRB to allow engraftment of a larger number of mice and higher engraftment levels. We will also prioritize CD34+ cell number per mouse over number of mice engrafted.
Future studies will also seek to take advantage of cell expansion methods as reported for murine HSCs by several groups, with the caveat that the inherent defects in FA cells may preclude expansion.
In studies independent of this funded project, we have developed a novel humanized mouse model with successful rescue of circulating human red blood cells via humanization of the murine host's liver. This liver-humanized mouse model also resulted in overall higher engraftment levels, suggesting that this model may be more permissive to engraftment by FA CD34+ HSPCs. In parallel to our synthetic lethal screen we have identified a subset of genes that when knocked down result in rescue of the FA mutant phenotype, including cGAS-STING, phospho-IRF3, and phospho-IRF7. Given our initial data showing improvement of colony forming activity in CD34+ cells in the presence of cGAS inhibition, we anticipate such treatment will improve engraftment and will be pursuing such experiments presently.

In conclusion, we have advanced in the difficult task to engraft primary FA cells into humanized mice, but significant improvements are necessary that are subject of ongoing studies.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project? We have generated tools to model FA in vitro and in vivo. Modeling primary patient derived and genetically engineered human CD34+ derived FA in humanized mice will be very valuable in the field to discover novel approaches to the treatment of FA. In continued studies we seek to advance xenotransplantation of FA to allow the study of FA in vivo and to make this model available to other researchers.
What was the impact on other disciplines? N/A
What was the impact on technology transfer? N/A
What was the impact on society beyond science and technology? N/A

CHANGES/PROBLEMS:
Changes in approach and reasons for change: nothing to report
Actual or anticipated problems or delays and actions or plans to resolve them: nothing to report
Changes that had a significant impact on expenditures: nothing to report
Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: nothing to report
Significant changes in use or care of human subjects: nothing to report
Significant changes in use or care of vertebrate animals: nothing to report
Significant changes in use of biohazards and/or select agents: nothing to report

PRODUCTS:
Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Name:	*Wei Liu*
Project Role:	*Postdoctoral Associate*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Liu has designed CRISPR/Cas9 and FANC gene constructs; he has transduced and transfected cell lines and primary cells and tested engraftment of genetically engineered cells and primary FA cells into MISTRG ckit mutant mice.*
Funding Support:	*N/A*
Name:	*Caroline Tang*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	*N/A*

Nearest person month worked:	*6*
Contribution to Project:	*Caroline Tang contributed to the shRNA screens for FA knockdown in CD34+ cells and in the procurement of primary FA samples.*
Funding Support:	*N/A*

SPECIAL REPORTING REQUIREMENTS: N/A