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TITLE: Mechanisms of Bone Marrow Failure and Leukemia Progression in Primary Human Fanconi Anemia Stem Cells in a Novel FA PDX Model

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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

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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 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.
Note that in the 4 months our work was significantly impacted by COVID. We are now in Phase 2 of reopening our laboratory and work has resumed within limitations of scheduling lab work around social distancing requirements.
We have encountered 2 difficulties which we are still addressing: 1) inefficiency of deleting FA genes via CRISPR/Cas9 and selection against deleted cells; 2) silencing of rescue lentiviral vectors in primary hematopoietic cells.
We are addressing 1) by using AAV vectors to provide the repair template and by using 2 reporter genes of distinct color to sort double mutant cells to assure that both alleles are mutated. We are addressing 2) in two ways: 2.1) at the time of transplantation of FANC gene deleted cells we will transiently express the respective FANC to rescue cells at time of engraftment; 2.2) as our mouse model has no need for irradiation and with use of the stringently sorted AAV/CRISPR/Cas9 modified cells we may not need interim rescue; and 2.3) we will clone the respective FANC genes with mutated PAM sites into a retro- or lentiviral vector that does not get silenced in hematopoietic stem cells.
We are actively expanding our immunodeficient humanized mouse colonies and actively transplanting primary FA samples with the goal to further optimize engineering of FA samples and transplantation in MISTRG mice.

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 continues to learn about the biology of FA and xenotransplantation into humanized mice. He continues to enhance 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. **Planned experiments** are outlined under each Task.

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. This protocol has recently changed PI from Dr. Kupfer to Dr. Halene as Dr. Kupfer has left Yale University to Georgetown. DoD regulatory review of the submitted HRPO protocol is complete. Our goal was to recruit an estimated total of 10 human subjects for the studies proposed in this award during the funding period. We have successfully obtained 5 samples. We continue to emphasize genetically engineered FA cells. De-identified peripheral blood mobilized and umbilical cord blood CD34+ are being obtained as fee for service through the core facility at the Yale Cooperative Center for Experimental Hematology.

1.2 Local IACUC and DoD regulatory review and approval for the use of animals (mice) have been obtained.
We have successfully bred MISTRG-kit^{MUT} mice to homozygosity; we are re-expanding the colony to serve for transplantation studies for this grant.
We have optimized transplantation into MISTRG- kit^{MUT} mice.
We have transplanted FA primary samples into kit^{MUT} mice that are ongoing post COVID.

Task 2. In vitro studies of FA
2.1 Production of shRNA knockout and CRISPR-altered FANCD2 knockout cells

CRISPR/Cas9 deletion of FANC genes:
Deletion of FANC genes will confer a growth disadvantage to deleted cells. CRISPR/Cas9 mediated gene deletion is highly efficient >>50%, but does not occur in all cells. Non-modified cells are highly likely to outgrow deleted cells even if present at a very low ratio.
We have chosen FANCC and FANCG as FANCA is a very large gene and difficult to work with; two of our patient samples are FANCG mutant.

In year 1 we designed a series of guide (g)RNAs against FANCC and FANCG; transfected these into 293T cells and human leukemia cell lines and tested the resulting populations of cells for (a) decrease in FANCC and FANCG transcript (b) knockdown of FANCC and FANCG protein expression and (c) decrease of FANCD2 monoubiquitination, which depends on an intact FA core complex, which contains 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 and data was presented in year 1 progress report. However, deletion of FANCC and FANCG could not be guaranteed in all cells despite sorting for 1) fluorescently labelled gRNAs and 2) the CRISPR/Cas9 expressing vector.
To increase efficiency and to sort for gene corrected cells with biallelic mutation of FANCC or FANCG we will use AAV mediated infection to introduce the repair template as per Dever et al., Nature 2016 and Bak et al., eLIFE 2017 with the option to sort BFP-GFP-double positive cells that carry biallelic mutations. We will use CRISPR/Cas9 protein+gRNA RNP concurrent with AAV transfection (**Fig.1**).

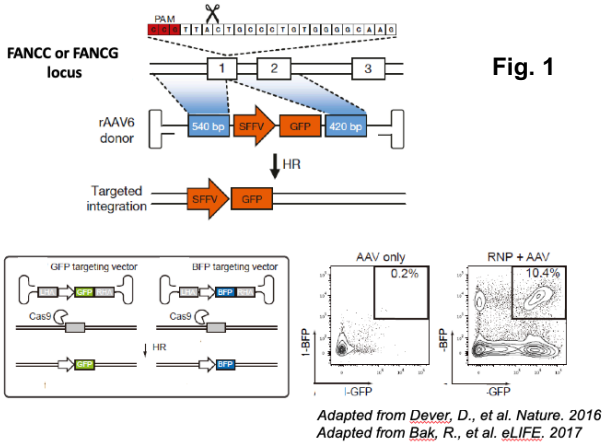


Fig. 1
Adapted from Dever, D., et al. Nature. 2016
Adapted from Bak, R., et al. eLIFE. 2017

As an alternative to CRISPR/Cas9 mediated FANCC/G disruption we have also devised a shRNA mediated strategy. The shRNA approach allows flow storing of stably transduced cells. 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. We have devised

two shRNA system given the propensity of hematopoietic stem cells to silence retro- and lentiviral vectors. We are using the commercially available Sigma MissionBio System and the "Lego" vectors [1] that both are efficient for transduction of hematopoietic stem cells and escape silencing.
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. We have tested combined transduction of FANCG shRNA targeted against FANCG 3'UTR and inducible FANCG cDNA (PAM site mutated and lacking the 3'UTR) in colony forming unit assays with primary human CD34+ HSPCs. As expected knockdown of FANCG results in impaired colony formation that is rescued by FANCG re-expression by addition of doxycycline to CFU assay. Interestingly, overexpression of FANCG alone without knockdown of FANCG via shRNA also impairs colony formation (**Fig. 2**).

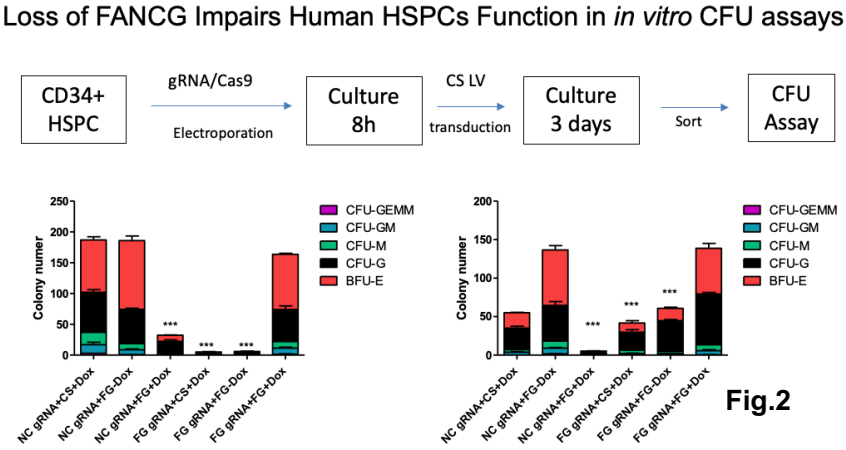


Fig.2

2.2 Synthetic lethal screen.

Test of screen in FA-D2 mutant cells.
In addition to studies reported for Year 1 (identification of subunits of the proteasome and cell cycle genes, PLK1 and WEE1, that result in synthetic lethality versus rescue, respectively, with FANC D2 knockout we tested the effect of FANCD2 loss in CD34+ cells to further understand the mechanism of FA induced BMF. We knocked down FANCD2 in CD34+ cells using siRNA and induced cytokine induced erythrocyte differentiation in these cells.

siRNA transfections were performed on the first days of culture in differentiation media I, II and III (**Fig. 3A**). This transfection protocol produced sufficient *FANCD2* knockdown (**Fig. 3B**). Cells were collected and analyzed by FACS. Loss of FANCD2 increased cell death in FACS analysis (**Fig. 3C**). We tested commercial lentiviral shRNA against FANCD2 and identified at least 2 different shRNAs with greater than 80% knockdown of FANC D2 protein expression (**Fig. 3D**). These data indicate that sustained RNAi mediated FANCD2

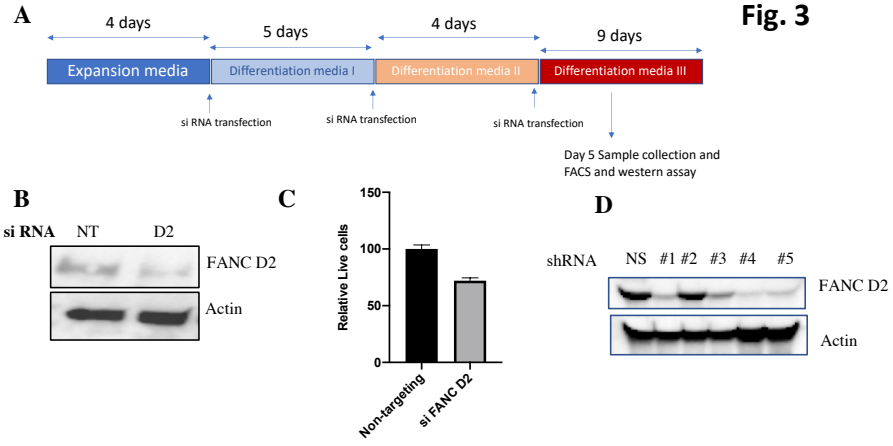


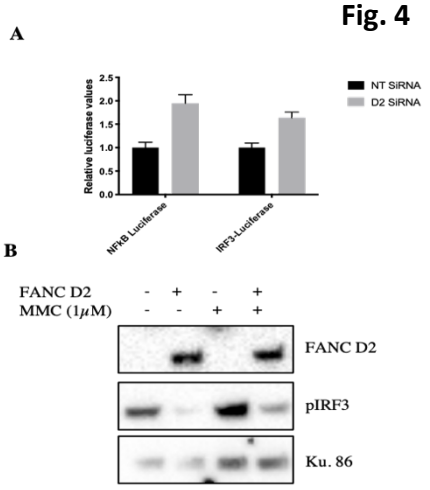
Fig. 3

Loss of FANC D2 in CD34+ cells leads to increased cell death and premature erythroid differentiation
A. Schematic of siRNA transfection in CD34+ cells **B**. Immunoblot assay of indicated proteins in CD34+ cells transfected with *NT* or *FANC D2* siRNA, on day 18 of erythroid differentiation. **C.** FACS analysis showing relative percentage of dead cells on day 18 of erythroid differentiation. **D.** Immunoblot assay of indicated proteins in HeLa cells stably expressing Non-silencing (NS) or indicated shRNA against FANC D2 (#1 to #5).

knockdown with stably integrated shRNA against FANCD2 in CD34+ cells can serve as effective tools in developing humanized mouse model of Fanconi anemia.

We hypothesize that FANCD2 loss-induced cell death can lead to poor engraftment of CD34+ cells carrying shRNA against FANCD2. We therefore examined ways to improve survival of cells with loss of FANCD2. Towards this end we analyzed data from an siRNA screen performed on isogenic patient-derived fibroblasts with genetic loss of FANCD2 or expressing exogenous FANCD2. Inhibition of IRF7, a key mediator of interferon pathway leads to increased survival of FANCD2 deficient cells. We decided to investigate the role of interferon signaling in Fanconi anemia. IRF3 and IRF7 are downstream adapters of interferon signaling pathway, and they are phosphorylated in response to activation of various upstream sensors. Luciferase reporter constructs with NFkB response elements and IRF3 response elements showed higher luciferase activity following RNAi knockdown of FANCD2 in 293T cells (**Fig. 4A**). Immunoblot analysis of PD20 fibroblasts derived from FANCD2 null human FA patients had high levels of pIRF3 which were reduced when complemented with WT FANCD2. Also, MMC-induced DNA damage increased phosphorylation of IRF3, which was reduced by FANCD2 complementation (**Fig. 4B**).

Fig. 4

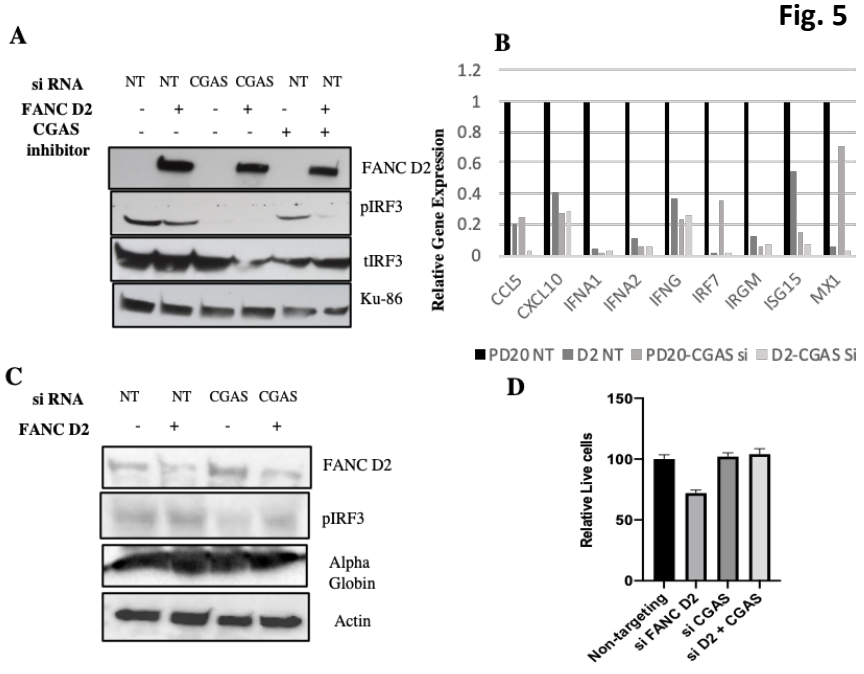


Loss of FANC D2 induces increased interferon signaling
A. Luciferase assay in 293T cells treated with either Non-targeting (NT) or *FANC D2* (D2) SiRNA for 72 hrs.
B. Immunoblot assay for indicated proteins in PD20 cells without or with FANC D2, left untreated or treated with MMC for 24 hrs.

These two results show that DNA damage activate the interferon pathway, and the FA pathway downregulates it.

IRF3 and IRF7 are activated by several upstream sensors which respond to several pathogen derived ligands. Cytosolic double stranded DNA and DNA:RNA hybrids can be sensed by CGAS. We therefore decided to investigate the role of CGAS in FA-induced interferon activation. Both siRNA against CGAS and small molecule inhibitor against CGAS reduced IRF3 phosphorylation in PD20 cells in FANCD2-dependent manner (**Fig. 5A**). Also, several transcriptional targets of interferon signaling pathway that were activated in the absence of

Fig. 5



Loss of FANC D2 induces interferon signaling in a CGAS dependent manner.
A. Immunoblot assay in PD20 cells without or with FANC D2 treated with NT or CGAS siRNA or with CGAS inhibitor for 48 hrs.
B. Quantitaive RT-PCR assay of well known interferon regulated genes in PD20 cells without or with FANC D2 treated with NT or CGAS siRNA for 48 hrs. **C**. Immunoblot assay of indicated proteins in CD34+ cells transfected with indicated siRNA, on day 18 of erythroid differentiation. **D.** FACS analysis showing relative percentage of dead cells on day 18 of erythroid differentiation in CD34+ cells

FANCD2 were downregulated when CGAS was inhibited using siRNA. (**Fig. 5B**). We therefore investigated if CGAS inhibition can attenuate the increased cell death in FANCD2 inhibited primary human CD34+ cells. Using a similar siRNA transfection strategy as before we observed that double knockdown of FANCD2 and CGAS reduced IRF3 phosphorylation (**Fig. 5C**) and FANCD2 loss mediated cell death (**Fig. 5D**) These results show that CGAS inhibition can reduce FANCD2 loss-mediated activation of interferon pathway and resultant cell death.

ii. Targeted exome sequencing. For each FA BM sample we will obtain clinical annotation (age, bone marrow biopsy studies, cytogenetics, FISH, flow-cytometry, clinical course) and perform targeted exome sequencing. We will proceed with these studies in the second year of our proposal. Libraries and sequencing are available to us and ready to be used. We have extensive sequencing and bioinformatic expertise.
These studies are now in progress given delay due to COVID. Given the rarity of samples and the small cell number, DNA for sequencing has to be obtained at time of transplantation into MISTRG.

iii. Humanized mice optimized for BMF. Transplantation studies are ongoing.

iv. Xenotransplantation. Based on our extensive experience with MDS-PDX models and our limited experience with FA we will transplant FA patient BM-derived CD34+ selected cells intrahepatically into MISTRG-ckit^{MUT} newborn mice. In select cases where we have < 100,000CD34+ cells/recipient (with goal of transplanting minimum 3 recipients /sample) we will transplant intrafemorally into 8 week-old MISTRG-ckit^{MUT} mice.

These studies are ongoing. We will specifically sequence both patient samples and human cells isolated from transplant recipient mice. Depending on mutations identified in bulk sequencing we will pursue single cell DNA sequencing using the MissionBio platform.

3.2 Transplantion and study of genetically engineered FA CD34+ cells into MISTRG-ckit^{MUT} mice.
We have engineered CD34+ HSPCs with CRISPR/Cas9 to delete FANCC and FANCG genes and we have transplanted cells with co-transduction of a dox-inducible vector to transiently express FANCC and FANCG respectively. We have to optimize these tools, as described above for efficiency and persistence.

In conclusion, our genetic engineering studies and our xenotransplantation studies are ongoing. We have requested a NCE until July 2021 to complete the proposed studies.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project? We have generated the necessary 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. If we are successful we will share our experience and data to establish collaborations and contribute to the broader advancement of the field.
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; technical adjustments have been made as per above.
Actual or anticipated problems or delays and actions or plans to resolve them: the COVID19 pandemic has halted our laboratory operations for ~ 3months and continues to limit our ability to fully experiment, but studies have resumed.
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: Change of protocol PI from Dr. Kupfer to Dr. Halene has been reviewed and approved.
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 and FANG gene constructs, transduced and transfected cell lines and primary cells and transplanted MISTRG 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 synthetic screen, westernblotting, shRNA and siRNA experiments.*
Funding Support:	*N/A*

Dr. Kupfer has moved to Georgetown University as of 7/1/2020.
Approval of a subcontract will be requested.
No other institutions were involved in the research; going forward research will be performed at Georgetown University only after required permissions have been obtained.

SPECIAL REPORTING REQUIREMENTS: N/A

1. Weber K, Bartsch U, Stocking C, Fehse B. A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. *Mol Ther*. 2008;16(4):698-706.