Award Number: W81XWH-12-1-0586

TITLE: Bone Marrow Failure Secondary to Cytokinesis Failure

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REPORT DATE: December 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2015	Final	30Sep2012 - 29Sep2015

4. TITLE AND SUBTITLE

Bone Marrow Failure Secondary to Cytokinesis Failure

5a. CONTRACT NUMBER
W81XWH-12-1-0586

5b. GRANT NUMBER
BM110181

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S):

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Boston, Massachusetts 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Fanconi anemia (FA) is a human genetic disease characterized by a progressive bone marrow failure and heightened susceptibility to cancer. The main objective of our project was to understand the mechanisms of bone marrow failure in FA. The specific objective was to determine the role of cytokinesis failure of hematopoietic stem cells in the pathogenesis of bone marrow failure in FA. During the award period, we have assessed the role of FA pathway in mitosis and confirmed that murine FA-deficient hematopoietic stem cells exhibit p53-mediated growth defects, cytokinesis failure and apoptosis. In order to study the role of bone marrow microenvironment in bone marrow failure in FA, we have also evaluated primary FA-deficient bone marrow stromal cells for mitotic defects. Interestingly, these cells also exhibit cytokinesis defects. Collectively, our results suggest that bone marrow failure in FA may be caused, in part, by p53-mediated cellular defects and underscore the importance of therapeutically targeting p53 in FA.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	9	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:

Fanconi anemia (FA) is the most commonly inherited bone marrow failure syndrome. FA patients develop bone marrow failure during the first decade of life due to attrition of hematopoietic stem cells (HSCs). In addition, FA patients also develop other hematologic manifestations, including myeloid malignancies [1, 2]. Hematopoietic stem cell transplantation is the only long-term curative treatment option for the hematologic manifestations of FA patients. Therefore, novel alternative treatment options are needed. A greater understanding the mechanisms of bone marrow failure may allow better diagnosis and treatment for FA patients. FA is caused by biallelic mutation in one of eighteen FA genes, the products of which cooperate in the FA/BRCA DNA repair pathway and regulate cellular resistance to genotoxic DNA cross-linking agents [3-5]. Disruption of any of the 18 FA genes accounts for a variable degree of hematopoietic stem cell dysfunction and ultimately bone marrow failure in humans. How disruption of this pathway leads to bone marrow failure is a critical unanswered question. Recent studies suggest that FA pathway function is required for normal cell cycle events during mitosis [6, 7]. We have previously shown that disruption of FA pathway in hematopoietic stem cells results in cytokinesis failure, and as a consequence in an increase in binucleated cells leading to the apoptosis [6]. Whether cytokinesis failure accounts for a bone marrow failure in FA remained to be investigated. Accordingly, the goal of our proposal was to understand the importance of cytokinesis failure in bone marrow failure using FA mouse models. Our preliminary data had indicated that p53 knockdown rescues the FA-deficient hematopoietic cells from cytokinesis failure and apoptosis. We had therefore hypothesized that the HSCs from FA patients have underlying defect in cytokinesis and bone marrow failure in FA is caused by p53-mediated apoptosis of HSCs due to cytokinesis failure. The major goal of the project was to assess whether the p53-mediated apoptosis due to cytokinesis failure in FA-deficient bone marrow HSCs is the cause of bone marrow failure in FA using mouse models.

BODY (Research Accomplishments):

We determined that FA pathway deficient murine bone marrow HSCs undergo cytokinesis failure and apoptosis and that these defects can be rescued by knockdown of p53. Following tasks were proposed for the funding period. We had anticipated that it will take approximately 36 months to complete the experiments proposed in specific aims 1- 3 (Tasks 1-3).

Task 1: To determine whether HSCs from Fanconi anemia mouse models have increased cytokinesis failure.

Task 2. To determine whether increase in cytokinesis failure in FA mouse models is the cause of bone marrow failure.

Task 3. To test the anti-apoptotic agents and p53 inhibitors for their ability to rescue binucleated cells in FA mouse models from cell death.

Task 1: *To determine whether HSCs from Fanconi anemia mouse models have increased cytokinesis failure.*

Task 1a. The specific aim of this task was to test HSCs from FA mouse models, namely, Fancg-/- mice, Fancd2-/- mice, and Fancd2-/-p53-/- double knockout mice for binucleated cell formation and HSC defects. Accordingly, we evaluated bone marrow HSCs from the wild-type mice, Fancd2-/- mice, Fancg-/- mice and *Fancd2-/-p53-/- double knockout mice* for apoptosis, binucleated cells (a hallmark of cytokinesis failure) and hematopoietic activity using methods described in our publications [6, 8]. The results were compared between the bone marrow of different genotypes. Briefly, the bone marrow cells were stained with antibodies against Lineage markers, Sca-1 and c-Kit. The cells were then subjected to FACS (fluorescence activated cell scanning) analysis and Lin-Sca-1+c-Kit+ (LSK) cells enriched for HSC populations were sorted. The sorted LSK cells were grown in culture for 2-3 days and analyzed for binucleated cells. The cells were stained for microtubules, DNA and nuclear membrane Lap2 and analyzed microscopically for the presence of binucleated cells as described previously [6]. The HSC activity was determined by subjecting the total bone marrow cells to cobblestone area-forming cell (CAFC) assay as described [8]. The cells were also stained with Annexin V and apoptotic cells were analyzed by FACS analysis.

Consistent with our previous study [8], bone marrow from Fancd2-/- mice and Fancg-/- mice had significantly decreased number of LSK cells and reduced frequencies of day 28 CAFCs compared to the bone marrow from wild-type mice. Moreover, LSK cells exhibited proliferation defects when grown in culture *in vitro* with cytokines. These results confirmed that FA-deficient bone marrow exhibit HSC defects. Strikingly, these HSC defects were rescued in Fancd2-/-p53-/- bone marrow. As expected [6], HSCs from murine Fancd2-/- bone marrow or Fancg-/- bone marrow exhibited a high rate of binucleated cells and apoptosis. These abnormalities were also rescued in Fancd2-/-p53-/- bone marrow indicating that cytokinesis failure leading to the apoptosis is p53 dependent.

Collectively, our results suggested that HSCs from FA-deficient bone marrow have defective hematopoietic activity and increased cytokinesis failure leading to apoptosis and that these defects can be rescued by p53 knockdown.

In addition to HSCs, we also used primary FA-deficient bone marrow stromal cells to investigate the role of FA pathway in maintaining bone marrow microenvironment. We observed that indeed primary FA-deficient bone marrow stromal cells display growth defects as well as cytokinesis defects. Specifically, these cells exhibit high basal level of micronuclei that increases upon replication stress.

Task 1b. *The induce bone marrow failure in FA mice (Fancd2-/- and Fancg-/- mice) by treatment with VX-680, CytocalasinB and Mitomycin C.*

FA mice do not exhibit spontaneous bone marrow failure [9]. However, bone marrow failure can be induced in these mice upon treatment with Mitomycin C. We had proposed to determine if treatment of FA mice with cytokinesis inhibitors causes bone marrow failure due to increased cytokinesis failure. We had proposed to use Mitomycin C as a positive control for these experiments. We had established a Mitomycin C-induced bone marrow failure mouse model of FA. Administration of two weekly doses of Mitomycin C at 0.6 mg/kg dose caused a severe bone marrow failure and lethality in Fancd2-/- mice but not in wild-type mice. During the past two years of the funding period, we have also generated sufficient number of FA mice (e.g. Fancd2 mutant mice, Fancg mutant mice). In future, we plan to test cytokinesis inhibitors in these mouse models.

Task 2. *To determine whether increase in cytokinesis failure in FA mouse models is the cause of bone marrow failure.*

We did rigorous analysis to systematically quantify apoptosis in steady-state as well as proliferating HSCs from murine FA bone marrow using AnnexinV staining. Briefly, freshly isolated bone marrow cells from wild-type mice, Fancd2-/- mice or Fancg-/- mice were stained with antibodies against lineage markers, Sca-1 and c-Kit, followed by staining with Annexin V and apoptotic HSCs (Lin-Sca-1+c-Kit+, LSK cells) were analyzed by FACS. In specific experiments, bone marrow was stained for LSK markers and HSCs were isolated by FACS. Apoptosis was then evaluated by Annexin V staining after culturing them for 48 hrs. We have consistently determined that HSCs from Fancd2-/- bone marrow and Fancg-/- bone marrow exhibit increased apoptosis compared to the HSCs from wild-type mice. The live cell imaging of the apoptotic cells showed that binucleated cells are more prone to apoptosis.

Task 3. *To test the anti-apoptotic agents and p53 inhibitors for their ability to rescue binucleated cells in FA mouse models from cell death.*

We had proposed to determine whether p53 inhibitors or apoptosis inhibitors can rescue the HSC defects in FA mouse models. We needed a large number of animals to perform these experiments. We had therefore expanded our breeding colonies of Fancd2 mutant mice and Fancg mutant mice and we have now generated enough number of Fancd2-/- mice and Fancg-/- mice along with wild-type sibling controls. In future, we plan to test the p53 inhibitors and apoptosis inhibitors in these mouse models.

During the course of our DOD funded project, we had interestingly discovered that the inhibitors of TGF-β (transforming growth factor -β) pathway also inhibit p53 and rescue the

growth defects of FA cells. We therefore next plan to test the TGF-β inhibitors in future in FA mouse models. The new data will be incorporated in future in a manuscript in which DOD funding will be acknowledged.

KEY RESEARCH ACCOMPLISHMENTS:

- Using two different FA mouse models, we confirmed that FA-deficient bone marrow HSCs are defective in hematopoietic activity and they undergo cytokinesis failure leading to the apoptosis.
- Importantly, we determined that depletion of p53 rescues the HSC defects including the cytokinesis defects of the FA-deficient bone marrow.
- We determined that primary FA-deficient bone marrow stromal cells also display growth defects as well as cytokinesis defects.

REPORTABLE OUTCOMES:

None

CONCLUSION:

We confirmed that p53 knockdown rescues the hematopoietic stem cell defects including the cytokinesis defects and apoptotic defects of the FA-deficient murine bone marrow. Importantly, we also observed cytokinesis defects in primary FA bone marrow stromal cells. In addition, we established mouse models for further assessing the importance of cytokinesis failure in bone marrow failure. Collectively, we have confirmed that bone marrow failure in FA may occur, at least in part, due to cellular p53-mediated mitotic defects. Future studies will be carried out to determine if p53 inhibitors or apoptosis inhibitors can improve the bone marrow in FA.

REFERENCES:

1. Kee, Y. and A.D. D'Andrea, *Molecular pathogenesis and clinical management of Fanconi anemia.* J Clin Invest, 2012. **122**(11): p. 3799-806.
2. Shimamura, A. and B.P. Alter, *Pathophysiology and management of inherited bone marrow failure syndromes.* Blood Rev, 2010. **24**(3): p. 101-22.
3. Kim, H. and A.D. D'Andrea, *Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway.* Genes Dev, 2012. **26**(13): p. 1393-408.
4. Kottemann, M.C. and A. Smogorzewska, *Fanconi anaemia and the repair of Watson and Crick DNA crosslinks.* Nature, 2013. **493**(7432): p. 356-63.
5. Sawyer, S.L., L. Tian, M. Kahkonen, J. Schwartzentruber, M. Kircher, G. University of Washington Centre for Mendelian, F.C. Consortium, J. Majewski, D.A. Dyment, A.M.

Innes, K.M. Boycott, L.A. Moreau, J.S. Moilanen, and R.A. Greenberg, *Biallelic mutations in BRCA1 cause a new Fanconi anemia subtype.* Cancer Discov, 2015. **5**(2): p. 135-42.
6. Vinciguerra, P., S.A. Godinho, K. Parmar, D. Pellman, and A.D. D'Andrea, *Cytokinesis failure occurs in Fanconi anemia pathway-deficient murine and human bone marrow hematopoietic cells.* J Clin Invest, 2010. **120**(11): p. 3834-42.
7. Nalepa, G., R. Enzor, Z. Sun, C. Marchal, S.J. Park, Y. Yang, L. Tedeschi, S. Kelich, H. Hanenberg, and D.W. Clapp, *Fanconi anemia signaling network regulates the spindle assembly checkpoint.* J Clin Invest, 2013.
8. Parmar, K., J. Kim, S.M. Sykes, A. Shimamura, P. Stuckert, K. Zhu, A. Hamilton, M.K. Deloach, J.L. Kutok, K. Akashi, D.G. Gilliland, and A. D'Andrea, *Hematopoietic stem cell defects in mice with deficiency of Fancd2 or Usp1.* Stem Cells, 2010. **28**(7): p. 1186-95.
9. Parmar, K., A. D'Andrea, and L.J. Niedernhofer, *Mouse models of Fanconi anemia.* Mutat Res, 2009. **668**(1-2): p. 133-40.

APPENDIX: None

April 8th 2016

Shui-Lin Niu
Scientific Officer

Reference: Final progress report for grant number BM110181, Contract number W81XWH-12-1-0586, PI: Alan D'Andrea

Dear Shui-Lin,

Following is the response to the reviewer's comments sent to us on 2/9/16.

1. We have changed the distribution statement to "unlimited distribution".

2. We apologize that we had cited a publication that was published when the project officially started. We have accordingly removed the reference to this publication from the revised progress report.

3. The objective of Task 3 was to test the anti-apoptotic agents and p53 inhibitors for their ability to rescue binucleated cells in FA mouse models from cell death. The task 3 of the project was indeed initiated. We needed a large number of FA mutant mice to perform the experiments proposed in task 3 and therefore the required number of FA mutant mice were generated during the course of the funding period. However, the task 3 was not completed during the grant funding period. Importantly, during the course of the DOD funding period, we had interestingly discovered that the inhibitors of TGF-β (transforming growth factor -β) pathway also inhibit p53 and rescue the growth defects of FA cells. We therefore next plan to test the TGF-β inhibitors in future in FA mouse models. The new data will be incorporated in future in a manuscript in which DOD funding will be acknowledged. We have now added this information in the revised progress report. I would like to add that the discovery that TGFβ inhibitors improve the growth defects of FA cells and improve hematopoiesis is a very important finding, and a possible treatment for FA patients and patients with other inherited defects of hematopoiesis. Accordingly, we are especially grateful to the DOD for supporting this important work.

Thank you

Alan D'Andrea