

Award Number: W81XWH-14-1-0297

TITLE: Small Molecule Protection of Bone Marrow Hematopoietic Stem Cells

PRINCIPAL INVESTIGATOR: Raymond J. Monnat, Jr. M.D.

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REPORT DATE: October 2016

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
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| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |              |                          |                            | Form Approved<br>OMB No. 0704-0188        |                                           |
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| 1. REPORT DATE<br>October 2016                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |              | 2. REPORT TYPE<br>Annual |                            | 3. DATES COVERED<br>30Sep2015 - 29Sep2016 |                                           |
| 4. TITLE AND SUBTITLE<br>Small Molecule Protection of Bone Marrow Hematopoietic Stem Cells                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |              |                          |                            | 5a. CONTRACT NUMBER<br>W81XWH-14-1-0297   |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |              |                          |                            | 5b. GRANT NUMBER                          |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |              |                          |                            | 5c. PROGRAM ELEMENT NUMBER                |                                           |
| 6. AUTHOR(S)<br>Raymond J. Monnat, Jr. M.D. P.I.<br><br>E-Mail: monnat@u.washington.edu                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |              |                          |                            | 5d. PROJECT NUMBER                        |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |              |                          |                            | 5e. TASK NUMBER                           |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |              |                          |                            | 5f. WORK UNIT NUMBER                      |                                           |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br>University of Washington<br>4333 Brooklyn Avenue NE<br>Seattle. WA 98195-0001                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |              |                          |                            | 8. PERFORMING ORGANIZATION REPORT NUMBER  |                                           |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br><br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |              |                          |                            | 10. SPONSOR/MONITOR'S ACRONYM(S)          |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |              |                          |                            | 11. SPONSOR/MONITOR'S REPORT NUMBER(S)    |                                           |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br><br>Approved for Public Release; Distribution Unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |              |                          |                            |                                           |                                           |
| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |              |                          |                            |                                           |                                           |
| 14. ABSTRACT<br><br>The goal of the proposed research is to determine whether several recently identified small molecules can protect hematopoietic stem cells (HSCs) from damage or killing by endogenous aldehydes. Proof-of-concept for these experiments has been developed using isogenic (mutant/complemented) human cell line pairs from patients with Fanconi anemia (FA), a heritable human bone marrow failure (BMF) syndrome in which HSCs and other cell types are hypersensitive to selected types of DNA damage. The proposed research addresses a key question—the relationship between DNA damage and cell killing—and may identify small molecules that protect HSCs from an important endogenous source of DNA damage. These small molecules could be therapeutically useful in reducing the risk of BMF in diseases such as Fanconi anemia, and perhaps after radiation exposure. The proposed research thus has the potential to catalyze new basic and translational research focused on achieving the BMFRP goals of understanding and curing BMF diseases. |              |                          |                            |                                           |                                           |
| 15. SUBJECT TERMS<br>CD34+ human hematopoietic stem cells (HSCs), bone marrow, formaldehyde toxicity                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |              |                          |                            |                                           |                                           |
| 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               | 47                                        | 19b. TELEPHONE NUMBER (include area code) |

Standard Form 298 (Rev. 8-98)  
Prescribed by ANSI Std. Z39.18

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**Preprint:**

Zhang et al. (2016) Metformin improves defective hematopoiesis and delays tumor formation in Fanconi anemia mice. *Blood* Oct 18. [Epub ahead of print].  
DOI:10.1182/blood-2015-11-683490.

## 1. INTRODUCTION:

**Goal:** The goal of the proposed research is to determine whether several recently identified small molecules can protect hematopoietic stem cells (HSCs) from damage or killing by endogenous aldehydes. Proof-of-concept for these experiments has been developed using isogenic (mutant/complemented) human cell line pairs from patients with Fanconi anemia (FA), a heritable human bone marrow failure (BMF) syndrome in which HSCs and other cell types are hypersensitive to selected types of DNA damage. The proposed research addresses a key question—the relationship between DNA damage and cell killing—and may identify small molecules that protect HSCs from an important endogenous source of DNA damage. These small molecules could be therapeutically useful in reducing the risk of BMF in diseases such as Fanconi anemia, and perhaps after radiation exposure. The proposed research thus has the potential to catalyze new basic and translational research focused on achieving the BMFRP goals of understanding and curing BMF diseases.

## 2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Fanconi anemia  
bone marrow failure  
CD34+ hematopoietic stem cells  
aldehydes  
formaldehyde  
DNA damage  
DNA base adduct  
DNA-protein crosslink  
mass spectrometry

## 3. ACCOMPLISHMENTS:

Our stated goals and timetable for the scope of work are provided in our Revised Scope of Work Table below.

| <b><i>Revised Specific Aim 1: Small molecule protection of human cells from aldehyde-induced killing (in vitro studies - no mice or human subjects)</i></b> | <b>Timeline</b> | <b>Site(s)</b> |               |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|----------------|---------------|
| <b>Major Task 1: Generate and confirm FANCG protein-depleted human CD34+ cells</b>                                                                          | <b>Months</b>   | <b>Site 1</b>  | <b>Site 2</b> |
| Transduce CD34+ cells with GFP/RFP + FANCG-specific shRNA lentiviruses                                                                                      | 1 - 9           | Dr. Monnat     | —             |
| Quantify FANCG protein depletion extent and time course by Western blot analysis                                                                            | 9 - 12          | Dr. Monnat     | —             |
| Milestone Achieved: FANCG-depleted CD34+ cells                                                                                                              | 1 - 12          | Dr. Monnat     | —             |
| <b>Major Task 2: In vitro formaldehyde dose and small molecule protection of FANCG-depleted human CD34+ cells</b>                                           |                 |                |               |
| Determine formaldehyde dose-dependent survival on FANCG-deficient/control CD34+ cells in culture                                                            | 9 - 15          | Dr. Monnat     | —             |

|                                                                                                                                        |         |            |              |
|----------------------------------------------------------------------------------------------------------------------------------------|---------|------------|--------------|
| Determine small molecule dose-dependent protection from formaldehyde toxicity/cell killing                                             | 12 - 18 | Dr. Monnat | –            |
| Milestone(s) Achieved: validation of small molecule formaldehyde antagonism in human CD34+ cells                                       | 9 - 18  | Dr. Monnat | –            |
| <b>Revised Specific Aim 2: Does small molecule formaldehyde damage protection result from a reduction in DNA damage?</b>               |         |            |              |
| <b>Major Task 1: Develop/validate stable isotope mass spec assay of formaldehyde DNA damage</b>                                        |         |            |              |
| Develop adduct/crosslink assays on new UPLC-MS/MS device                                                                               | 1 - 9   | –          | Dr. Swenberg |
| Demonstrate detection of formaldehyde-induced DNA damage using DNA isolated from CD34+ cells                                           | 9 - 15  | Dr. Monnat | Dr. Swenberg |
| Milestone(s) Achieved: validated high-sensitivity detection of DNA damage in CD34+ progenitor cells                                    | 1 - 15  | Dr. Monnat | Dr. Swenberg |
| <b>Major Task 2: Apply mass spectrometric assay to DNA derived from treated CD34+ cells</b>                                            |         |            |              |
| Quantify DNA damage in formaldehyde-treated CD34+ cells using isotope-labeling/MS methods                                              | 15-24   | Dr. Monnat | Dr. Swenberg |
| Quantify DNA damage by MS in formaldehyde-treated FANCG-depleted CD34+ cells treated with the most effective small molecule antagonist | 18-36   | Dr. Monnat | Dr. Swenberg |
| Milestone(s) Achieved: Test of hypothesis that SM protection from aldehyde damage acts at the level of DNA adduct/damage reduction     | 15-36   | Dr. Monnat | Dr. Swenberg |

– **What was accomplished under these goals?**

During the preceding award period we have made the following progress on the Revised Scope of Work, Aims and Major Tasks outlined above. We have also identified obstacles to progress, work-arounds where needed and identified priorities and next steps for the coming work period.

**Revised Specific Aim 1:**

**Major Task 1: Generate and confirm FANCG protein-depleted human CD34+ cells**

**Accomplishments:** In the preceding award period we:

- developed Western blot assays to detect and quantify depletion in human cells;
- identified, after extensive search, 2 shRNAs that can substantially and reproducibly deplete FANCG protein from human cells (to ≥90% depletion) after 5 days of shRNA expression;
- generated inducible FANCG shRNA lentiviral expression vectors to use to transduce and inducibly deplete FANCG protein from human CD34+ cells.

**Next steps:** In the next award period we will:

- determine CD34+ transduction/depletion time course, on a scale large enough to feed both cell survival and mass spectrometry assays

**Major Task 2: *In vitro* formaldehyde dose and small molecule protection of FANCG-depleted human CD34+ cells.**

**Accomplishments:** In the preceding award period we:

- demonstrated substantial, reproducible mitotic expansion of human peripheral blood mobilized CD34+ cells in defined media;
- confirmed ability of small molecules to partially suppress differentiation with loss of cell surface antigen staining during CD34+ *in vitro* mitotic expansion;
- determined formaldehyde dose-survival curve using mitotically expanded CD34+ cells;
- demonstrated ability of two small molecule protectants, metformin and aminoguanidine, to improve the growth, suppress aldehyde-induced DNA damage and improve the aldehyde dose-dependent survival of FANCG-deficient human cells;
- used a combination of chemical determination and dose-response assays to provide mechanistic insight into likely mechanisms by which each small molecule provides aldehyde dose-dependent protection in human cells in culture.

**Next steps:** In the next award period we will:

- extend above results to FANCG shRNA-depleted CD34+ cells to determine formaldehyde dose-dependent cell killing and small molecule protection as a function of genotype, small molecule type and dose and formaldehyde dose.

**Revised Specific Aim 2: Does small molecule formaldehyde damage protection result from a reduction in DNA damage?**

**Major Task 1: Develop/validate stable isotope mass spec assay of formaldehyde DNA damage** (in conjunction with collaborator James Swenberg/University of North Carolina).

**Accomplishments:** In the preceding award period we:

- developed and published new quantitative formaldehyde-DNA adduct/crosslink HPLC-MS/MS assays;
- demonstrated application of mass spectrometry-based methods to quantify DNA damage due to formaldehyde in Adh5(-/-)Fancd2(-/-) deficient mice and related control genotypes.

**Major Task 2: Apply mass spectrometric assay to DNA derived from treated CD34+ cells** (in conjunction with collaborator James Swenberg/University of North Carolina).

**Next steps:** In the next award period we will:

- use these newly developed and verified assays to quantify formaldehyde-DNA adducts and DNA-protein crosslinks in control and FANCG-depleted human CD34+ cells.

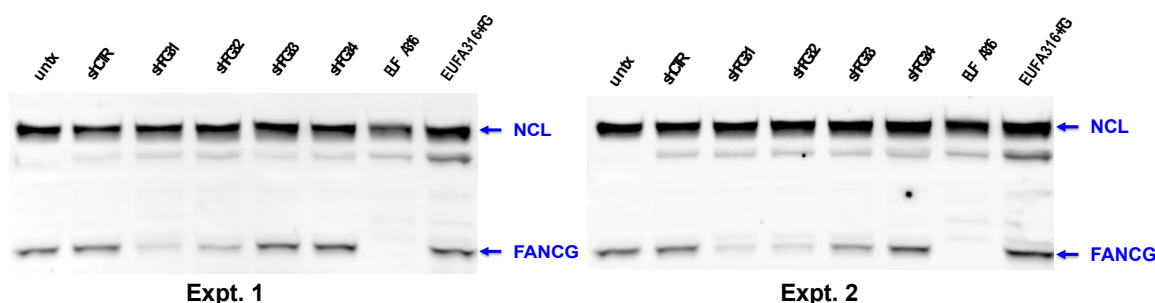
**The following sections provide additional technical detail and results for each of the above accomplishments.**

**Aim 1 - Major Task 1: Demonstrate of lentiviral depletion of FANCG proteins from human cells.**

**Screen to identify shRNAs for human FANCG protein depletion:** We screened a large number (>12) human FANCG-specific shRNAs from various sources to identify two that gave

consistent, reproducible and substantial ( $\geq 90\%$ ) depletion of FANCG protein from human cells. Two of these (shFG31 and shFG32, shown below), when transferred to and expressed from a lentiviral vector backbone, again were shown to reproducibly deplete to  $\geq 90\%$  FANCG protein from human cells.

**Western blot verification of FANCG depletion by shRNA:** Whole cell lysates from GM639 human SV40-transformed fibroblasts (Expt. 1) and U2-OS human osteosarcoma cells (Expt. 2) that were either untransduced (untx), transduced with and expressing a scrambled shRNA (shCTR), or transduced with and expressing one of 4 FANCG-targeting shRNAs (shFG31, shFG32, shFG33, and shFG34) were blotted with extracts from a control isogenic human FANCG-deficient/complemented lymphoblastoid cell line pair (EUFA316, negative control for FANCG protein expression) and its FANCG-complemented pair EUFA316+FG, a positive control for FANCG expression). Extracts were separated by SDS-PAGE electrophoresis, followed by immunoblotting with antibodies against FANCG and nucleolin (NCL, a loading control). Two representative Western blot experiments are shown below, demonstrating ability of shFG31 and shFG32 to deplete FANCG protein from human cells.



#### Detail of FANCG shRNAs:

|                         |                       |                       |
|-------------------------|-----------------------|-----------------------|
| <b>shRNA:</b>           | shFG31                | shFG32                |
| <b>target sequence:</b> | ggtagaattactactgccacc | ggtaatcgagacacttacttt |
| <b>target location:</b> | nt 972-995 (ORF)      | nt 1636-1657(ORF)     |

**Generation of inducible lentiviral FANCG shRNA expression vectors:** We have constructed inducible lentiviral shRNA expression vectors based on the shFG31 and shFG32 shRNA sequences shown above in the pLKO-tet-on lentiviral vector backbone to use to transduce and inducibly express shRNA for depletion of FANCG in human CD34+ HSC.

#### Aim 1 - Major Task 2: *In vitro* formaldehyde dose and small molecule protection of FANCG-depleted human CD34+ cells.

**Small molecule protection of human cells from aldehyde-mediated cell killing:** In conjunction with collaborators at Oregon Health Sciences University we demonstrated the ability of two small molecule protectants, metformin and aminoguanidine, to improve the growth, suppress aldehyde-induced DNA damage and improve the aldehyde dose-dependent survival of FANCG-deficient human cells.

**Small molecules protection in vivo:** We also showed that one of these small molecules, metformin, improves defective hematopoiesis and delays tumor formation in Fanconi anemia

mice. This work, just accepted for publication (see Zhang et al. (2016) Metformin improves defective hematopoiesis and delays tumor formation in Fanconi anemia mice. *Blood* Oct 18. [Epub ahead of print]. DOI:10.1182/blood-2015-11-683490), provides critical pre-clinical data to inform eventual Phase I trials of the use of metformin in Fanconi anemia patients. This manuscript identifies this award as one of the sources of research support. A copy has been provided in the Appendix.

**Aim 2 - Major Task 1: Develop/validate stable isotope mass spec assay of formaldehyde DNA damage** (in conjunction with collaborator James Swenberg/University of North Carolina).

Our collaborator Dr. James Swenberg completed the development of and published a new quantitative formaldehyde-DNA protein mass spectrometry-enabled assay in the past grant period: Lai et al. (2016) Measurement of endogenous versus exogenous formaldehyde-induced DNA-protein crosslinks in animal tissues by stable isotope labeling and ultrasensitive mass spectrometry. *Cancer Research* 76(9):2652-61. doi:10.1158/0008-5472.CAN-15-2527. PMCID: PMC4879886.

**Aim 2 - Major Task 2: Apply mass spectrometric assay to DNA derived from treated CD34+ cells** (in conjunction with collaborator James Swenberg/University of North Carolina).

Dr. Swenberg also demonstrated with collaborator KJ Patel at the MRC Cambridge Lab or Molecular Biology the use of a related mass spectrometry-based method to detect and quantify DNA base adducts due to formaldehyde in Adh5(-/-)Fancd2(-/-) deficient mice and related control genotypes. This work has been published (Pontel et al. (2015) Endogenous formaldehyde is a hematopoietic stem cell genotoxin and metabolic carcinogen. *Molecular Cell* 60(1):177-88. doi: 10.1016/j.molcel.2015.08.020. PMCID:PMC4595711).

**Note:** Both of the above-cited publications are directly related to, and provide proof-of-concept for, our Revised Aim 2. The original connection of Drs. Swenberg and Patel that fostered the above-noted successful research collaboration and publication was made by the P.I. of this award (Dr. Monnat).

**– What opportunities for training and professional development has the project provided?**

Development of the methods for the growth and depletion of FANCG protein from CD34+ cells provided a new opportunity for Dr. Tang in the Monnat Lab. In similar fashion the mass-spectrometry-based assay development providing training and development opportunities for members of the Swenberg Lab.

**– Describe how the results were disseminated to communities of interest.**

All three of the above publications were highlighted in platform talks at the 28th Annual Fanconi Anemia Research Fund Meeting that was held in Bellevue, WA on 15-18 September 2016. Dr. KJ Patel also gave a Keynote Address as part of this meeting entitled 'Aldehydes and the phenotype of Fanconi anemia: A complex illness with a simple explanation?' A full half-day session was also devoted to 'Aldehydes, Genotoxicity & Disease'.

**– What do you plan to do during the next reporting period to accomplish the goals?**

Please see our detailed plans for the next (final) award period above.

**IMPACT:**

As noted above, our results provide proof-of-concept of both biological as well as technical/methodology aspects of this project, and have provided key findings that provide the basis for early phase clinical trials of metformin in Fanconi anemia patients.

**– What was the impact on other disciplines?**

The fields of DNA damage-repair, DNA damage response, Fanconi anemia and associated bone marrow failure syndromes and environmental and molecular toxicology will all be interested in our newly generated data on the role of aldehydes, especially endogenous aldehydes generated as part of normal cellular metabolism, as an important source of DNA damage in human cells.

**– What was the impact on technology transfer?**

The mass spectrometry-based methods established in Dr. Swenberg's lab provide new tools for molecular assessment of aldehyde (and other types of) damage able to generate DNA base adducts or DNA-protein cross-links.

**– Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use.**

We anticipate the eventual results may have utility in preventing or attenuating bone marrow failure in patients with Fanconi anemia, and potentially in other heritable or acquired bone marrow failure syndromes

**CHANGES/PROBLEMS:**

No significant changes or problems to report.

**PRODUCTS:****- Publications, conference papers, and presentations**

Please see our notes above on three related manuscripts, one directly supported in part by this award, together with presentations at the recent Fanconi Anemia Research Fund Symposium.

**PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS****– What individuals have worked on the project?**

Name: Ray Monnat

Project Role: Principal Investigator

Researcher Identifier (e.g. ORCID ID): 0000-0001-7638-7393

Nearest person month worked: 0.24 CM

Contribution to Project: design and conduct of experiments, interpretation of data, manuscript writing.

Funding Support: this award supported the above work

Name: Weiliang Tang

Project Role: Research Scientist  
Researcher Identifier (e.g. ORCID ID): N/A  
Nearest person month worked: 1 CM  
Contribution to Project: design and conduct of experiments, interpretation of data  
Funding Support: this award supported the above work

**- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

No change in active support during the previous award period.

**– What other organizations were involved as partners?**

University of North Carolina  
Chapel Hill, NC

Collaborator James Swenberg and his lab are located at UNC-Chapel Hill. They have developed the mass spectrometric analytical methods to detect and quantify specific DNA base adducts and DNA-protein crosslinks, and have demonstrated the application of both methods in work now published (see above references).

**SPECIAL REPORTING REQUIREMENTS:**

Nothing to report.

**COLLABORATIVE AWARDS:**

Nothing to report.

**For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.**

**QUAD CHARTS:**

Nothing to report.

**APPENDICES:**

preprint of Zhang et al. (2016) Metformin improves defective hematopoiesis and delays tumor formation in Fanconi anemia mice. *Blood* Oct 18. [Epub ahead of print].  
DOI:10.1182/blood-2015-11-683490).

**Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. DO NOT RENUMBER PAGES IN THE APPENDICES.**

## **Metformin improves defective hematopoiesis and delays tumor formation in Fanconi anemia mice**

Qing-Shuo Zhang,<sup>1</sup> Weiliang Tang,<sup>2</sup> Matthew Deater,<sup>1</sup> Ngoc Phan,<sup>1</sup> Andrea N. Marcogliese,<sup>3</sup> Hui Li,<sup>2,4</sup> Muhsen Al-Dhalimy,<sup>5</sup> Angela Major,<sup>6</sup> Susan Olson,<sup>5</sup> Raymond J. Monnat Jr.,<sup>2,7</sup> and Markus Grompe<sup>1</sup>

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<sup>2</sup>Department of Pathology, University of Washington, Seattle, WA 98195, USA;

<sup>3</sup>Department of Pathology, Baylor College of Medicine, TX 77030, USA;

<sup>4</sup>Current address: Department of Anatomy and Neurobiology, Northeastern Ohio Medical University, Rootstown, OH 44272, USA;

<sup>5</sup>Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR 97239, USA;

<sup>6</sup>Department of Pathology, Texas Children's Hospital, Houston, TX 77030, USA;

<sup>7</sup>Department of Genome Sciences, University of Washington, Seattle, WA 98195, USA.

Address correspondence to: Qing-Shuo Zhang, Oregon Stem Cell Center, Department of Pediatrics, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA. Phone: (503) 494-6889; Fax: (503) 494-5044; Email: zhangqi@ohsu.edu.

**Keywords:** Bone Marrow Failure; Fanconi Anemia; Hematopoietic Stem Cell; Metformin; Aminoguanidine; Hematopoiesis

**Running title:** Metformin benefits Fanconi anemia mice.

**Word count:** 3987; **Abstract word count:** 211;

**Figure count:** 6; **Table count:** 1; **Reference count:** 56.

**Scientific category:** Hematopoiesis and Stem Cells

**Abbreviations:** FA, Fanconi anemia; HSPC, hematopoietic stem and progenitor cell; HSC, hematopoietic stem cell; KSL, c-Kit<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>-</sup>; CBC, complete blood count; AMPK, AMP-activated protein kinase; Poly(I:C), polyinosinic:polycytidylic acid; TGF- $\beta$ , transforming growth factor- $\beta$ .

### **Key Points:**

1. The widely used diabetes drug metformin improves hematopoiesis and delays tumor formation in a preclinical murine model of Fanconi anemia;
2. Metformin reduces DNA damage in human Fanconi anemia patient-derived cells.

## Abstract

Fanconi anemia is an inherited bone marrow failure disorder associated with a high incidence of leukemia and solid tumors. Bone marrow transplantation is currently the only curative therapy for the hematopoietic complications of this disorder. However, long-term morbidity and mortality remain very high and new therapeutics are badly needed. Here we show that the widely used diabetes drug metformin improves hematopoiesis and delays tumor formation in *Fancd2*<sup>-/-</sup> mice. Metformin is the first compound reported to improve both of these Fanconi anemia phenotypes. Importantly, the beneficial effects are specific to Fanconi anemia mice, and not seen in the wild-type controls. In this preclinical model of Fanconi anemia, metformin outperformed the current standard of care, oxymetholone, by improving peripheral blood counts in *Fancd2*<sup>-/-</sup> mice significantly faster. Metformin increased the size of the hematopoietic stem cell compartment and enhanced quiescence in hematopoietic stem and progenitor cells. In tumor-prone *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice, metformin delayed the onset of tumors and significantly extended the tumor-free survival time. In addition, we found that metformin and the structurally related compound aminoguanidine reduced DNA damage and ameliorated spontaneous chromosome breakage and radials in human Fanconi anemia patient-derived cells. Our results also indicate that aldehyde detoxification might be one of the mechanisms by which metformin reduces DNA damage in Fanconi anemia cells.

## Introduction

Fanconi anemia (FA) is an inherited bone marrow failure disorder associated with a high incidence of leukemia and solid tumors.<sup>1</sup> The disorder is caused by a disrupted FA-BRCA pathway, and is genetically heterogeneous, with at least 21 complementation groups and genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1/BRCA2*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCL*, *FANCM*, *FANCN/PALB2*, *FANCO/RAD51C*, *FANCP/SLX4*, *FANCQ/XPF/ERCC4*, *FANCR/RAD51*, *FANCS/BRCA1*, *FANCT/UBE2T*, *FANCU/XRCC2*, and *FANCV/MAD2L2-REV7*) identified thus far. The main role of this gene network is to repair DNA lesions such as interstrand crosslinks, which impede replication and transcription.<sup>2,3</sup>

The primary cause of early morbidity and mortality for FA patients is bone marrow failure.<sup>4</sup> Hematopoietic stem cells (HSCs) in FA patients are reduced in number, function poorly compared to healthy HSCs, and also suffer from progressive elimination due to the accumulation of unrepaired DNA damage.<sup>5,6</sup> Although most strains of FA mutant mice are poor models of the human disease, *Fancd2*<sup>-/-</sup> mice recapitulate the key human disease phenotypes well, including HSC defects and progressive bone marrow failure.<sup>7,8</sup> *Fancd2*<sup>-/-</sup> mice display thrombocytopenia by 3 to 6 months of age and eventually progress to peripheral pancytopenia by 18 months.<sup>9</sup> *Fancd2*<sup>-/-</sup> HSCs are less quiescent and show a severely reduced capacity to repopulate the hematopoietic system *in vivo*.<sup>8</sup>

The FA pathway plays a fundamental role in protecting cells against DNA damage-inducing aldehydes.<sup>10</sup> Disruption of key aldehyde detoxifying enzymes such as the aldehyde dehydrogenases *Aldh2* or *Adh5* in Fanconi mice induces phenotypes resembling clinical Fanconi anemia, and leads to spontaneous bone marrow failure.<sup>11,12</sup> Of note, human FA patients carrying a dominant-negative allele of *ALDH2*, demonstrate accelerated progression of bone marrow

failure.<sup>13</sup> These observations suggest that attenuating aldehyde toxicity may provide a novel therapeutic approach to FA. Metformin (N,N-dimethylbiguanide) is a widely used drug to treat diabetes with proven safety after decades of clinical use.<sup>14</sup> As a guanidine derivative, metformin has the potential to scavenge DNA damage-inducing aldehydes through the Mannich reaction.<sup>15</sup> Metformin also induces the activation of AMP-activated protein kinase (AMPK) and is thought to have its anti-diabetic effect via this mechanism.<sup>16,17</sup> We have reported that the plant polyphenol resveratrol helps to restore the quiescence of *Fancd2*<sup>-/-</sup> HSCs and improves the function of hematopoietic stem and progenitor cells (HSPC) in these mice.<sup>8,18</sup> Resveratrol has several bioactivities, including acting as an antioxidant, activating Sirt deacetylases (sirtuins), and activating AMPK.<sup>19</sup> However, we have recently demonstrated that a potent sirtuin activator, SRT3025, does not mimic the effects of resveratrol in FA mice.<sup>20</sup> Given that AMPK plays an important role in HSCs,<sup>21</sup> AMPK activation may be the primary mechanism by which resveratrol improves hematopoiesis. The ability of metformin to activate AMPK and act as a potential aldehyde scavenger makes metformin a potential candidate for the treatment of FA. In the current study, we tested the effects of chronic metformin therapy on hematopoiesis and cancer incidence in *Fancd2*<sup>-/-</sup> mice.

## **Materials and methods:**

### *Mice*

*Fancd2* mutant mice were maintained on the 129S4 background.<sup>22</sup> The metformin diet was made by milling metformin with standard rodent diet (Purina Chow 5001) at 3.75 g/kg diet (Bio-Serv, Flemington, NJ, USA) and was administered to mice upon weaning (3 - 4 weeks of age). The treatment lasted 6 months unless specified otherwise. All animals were treated in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Polyinosinic:polycytidylic acid (poly(I:C)) was purchased from GE Healthcare (Piscataway, NJ 08885, USA) and given to the mice at 8 mg/kg body weight via intraperitoneal injection. Control mice were injected with saline.

### *Complete blood count (CBC)*

Blood samples were collected in EDTA-coated capillary tubes and complete blood counts were measured on a Hemavet 950FS Multi-species Hematology System (Drew Scientific Inc., Dallas, TX, USA).

### *Flow cytometry*

Bone marrow cells were isolated from the femora and tibiae of mice and stained as described previously.<sup>8</sup> The KSL antibody cocktail was comprised of anti-mouse c-Kit, Sca-1, and lineage markers (CD3e, CD4, CD5, CD8a, B220, Ter119, NK1.1, Mac1, Gr1). For analysis of CD34<sup>+</sup> KSL cells, nucleated bone marrow cells were stained with anti-mouse CD34 along with the KSL antibody cocktail. All the antibodies were from eBioscience (San Diego, CA, USA). Flow cytometry analysis was performed on a Cytopenia Influx cell sorter.

### *Colony-forming unit-spleen (CFU-S) assay*

CFU-S assay was performed as described previously<sup>8</sup> (See also Supplemental Methods).

### *Cells and reagents*

PD259i fibroblast cells, provided by the OHSU FA Cell Repository (<http://www.ohsu.edu/research/fanconi-anemia/celllines.cfm>), were originally derived from a human FA-A patient. EUFA316 lymphoblastoid cells were originally derived from a human FA-G patient.<sup>23</sup> EUFA316+FANCG cells were modified EUFA316 cells that stably express the wild-type FANCG.<sup>24</sup>

Metformin and aminoguanidine were purchased from MP Biomedicals (Santa Ana, CA, USA) and Tokyo Chemical Industry (Tokyo, Japan), respectively. The Adh5 inhibitor C3 compound was obtained from ChemDiv (San Diego, CA, USA).

### *Radial and chromosomal breakage assay*

PD259i cells were treated with either metformin, aminoguanidine, or placebo. In the case when the C3 compound was used for the assay, C3 was added 1 hour after the addition of metformin. Forty-eight hours later, metaphase spreads were made and scored for radial contents and chromosomal breakage on a Zeiss Axioskop photoscope.

### *Statistical analysis*

Unless specified otherwise, the two-tailed, unpaired student's *t*-test was used for statistical analysis. A *P* value of less than 0.05 was considered significant.

## Results

### *Dietary metformin administration enhances hematopoiesis.*

To determine whether metformin can influence hematopoiesis, cohorts of *Fancd2*<sup>-/-</sup> and wild-type mice were given either metformin-supplemented rodent chow or placebo for 6 months beginning at 1 month of age. The food intake was measured and the effective dose via ingestion was calculated to be 300 mg/kg/day. On the basis of the body surface area conversion,<sup>25</sup> this dose was equivalent to only ~65% of the maximum dose used routinely in humans (~1,300 mg/m<sup>2</sup>). After 6 months, complete blood counts were examined. *Fancd2*<sup>-/-</sup> mice on the placebo diet showed mild pancytopenia in multiple lineages, including lower platelet counts, lower white and red blood cell counts, and lower hemoglobin levels than their wild-type gender-matched littermate placebo controls (Table 1). A complete blood count analysis revealed that chronic metformin treatment significantly increased platelet counts ( $P < 0.05$ , Figure 1A) in *Fancd2*<sup>-/-</sup> mice, but not in wild-type controls. In contrast, white blood cell counts ( $P < 0.05$ , Figure 1A) increased in both metformin-treated *Fancd2*<sup>-/-</sup> and wild-type mice. Although metformin-treated *Fancd2*<sup>-/-</sup> mice showed only a mild and non-significant increase in red blood cell number ( $P = 0.08$ , Figure 1A), the hemoglobin levels in metformin-treated *Fancd2*<sup>-/-</sup> mice were significantly higher than those in placebo-treated *Fancd2*<sup>-/-</sup> mice ( $P < 0.005$ , Figure 1A), nearly comparable to those observed in the placebo-treated wild-type controls. These multilineage improvements in hematologic parameters took place significantly faster with metformin treatment as opposed to oxymetholone treatment, the current standard androgen treatment for FA patients, on the same *Fancd2*<sup>-/-</sup> murine model tested in our previous studies.<sup>9,20</sup>

We next focused on characterizing the bone marrow of mice in this cohort. The marrow cellularity in either *Fancd2*<sup>-/-</sup> or wild-type mice was not different between metformin-treated

animals and placebo-treated controls (data not shown). Interestingly, flow cytometry analysis demonstrated that the size of the CD34<sup>+</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>-</sup> (CD34<sup>+</sup>KSL) cell compartment, an immunophenotypically-defined HSC population in *Fancd2*<sup>-/-</sup> mice (Figure 1B), was significantly increased by 48% after 6 months of chronic metformin administration (Figure 1C). The size of the CD34<sup>+</sup>KSL cell compartment in wild-type mice, in contrast, was unchanged (Figure 1C), indicating that the effect of metformin on HSC population size was specific to *Fancd2*<sup>-/-</sup> mice.

Since our previous study showed that the AMPK activator resveratrol could help maintain stem cell quiescence in *Fancd2*<sup>-/-</sup> mice<sup>8</sup>, we measured the impact of metformin on the cell cycle status of HSPCs. The cell cycle profiles of KSL cells from metformin-treated mice were examined using Hoechst 33342 staining (for DNA content) and intracellular Ki67 staining (to discriminate cycling G1 cells from non-cycling G0 cells).<sup>26</sup> As shown in Figure 2A & 2B, the average frequency of quiescent G0 KSL cells in metformin-treated *Fancd2*<sup>-/-</sup> mice was 27.4%, which was substantially higher ( $P < 0.05$ ) than the average G0 fraction of 20.3% observed in placebo-treated gender-matched *Fancd2*<sup>-/-</sup> mice. Correspondingly, the average S-G2-M proportion of KSL cells in metformin-treated *Fancd2*<sup>-/-</sup> mice was 21.2%, which was significantly lower ( $P < 0.05$ ) than the average S-G2-M percentage of 25.7% observed in placebo-treated controls. These results indicate that metformin treatment increased the quiescence of HSPCs in *Fancd2*<sup>-/-</sup> mice. In contrast, the cell cycle status of KSL cells in wild-type mice was unchanged after metformin treatment (Figure 2A & 2B).

Next, we performed CFU-S assay, a short-term quantitative *in vivo* functional assay for HSPCs, using bone marrow from metformin-treated mice (Figure 3A). Metformin-treated *Fancd2*<sup>-/-</sup> bone marrow cells formed almost twice as many macroscopic splenic colonies ( $P < 0.03$ ) as

placebo-treated *Fancd2*<sup>-/-</sup> controls (Figure 3B), suggesting a marked improvement of HSPC function in metformin-treated *Fancd2*<sup>-/-</sup> bone marrow. In contrast, the CFU-S forming capacity in wild-type bone marrow was unchanged after metformin treatment ( $P = 0.80$ , Figure 3B).

*Metformin administration protects FA cells from poly(I:C)-induced hematologic abnormalities.*

To further evaluate metformin's effects on hematopoiesis, we took advantage of the recent finding that HSC cycling induced by poly(I:C) in *Fancc*<sup>-/-</sup> mice caused aplastic anemia.<sup>27</sup> As depicted in Figure 3C, cohorts of 3-month old *Fancd2*<sup>-/-</sup> mice and wild-type controls on metformin or placebo diet were given three consecutive high doses of poly(I:C) spaced three days apart. The mice were harvested 2 weeks after the completion of poly(I:C) treatment followed by analyses of bone marrow function. The CFU-S forming capacity of the bone marrow in *Fancd2*<sup>-/-</sup> mice was dramatically reduced after poly(I:C) administration ( $P = 0.0001$ ; Figure 3D). Importantly, *Fancd2*<sup>-/-</sup> mice fed with a metformin diet while being given poly(I:C) displayed a complete protection from poly(I:C)-induced loss of HSPC activity, as evidenced by their maintenance of normal levels of CFU-S forming potential ( $P < 0.01$ ; Figure 3D). This protection may reflect the ability of metformin to re-enforce HSPC quiescence and hence counteract the deleterious effects of poly(I:C)-induced cycling on HSCs.

One characteristic FA patient phenotype, red cell deficiency, only becomes apparent in very old (18 months) *Fancd2*<sup>-/-</sup> mice.<sup>9</sup> However, only three weeks after poly(I:C) administration in 3-month old *Fancd2*<sup>-/-</sup> mice, CBC analysis revealed a red cell deficiency and low hemoglobin levels ( $P < 0.01$ ) (Figure 3E). This effect was not observed in comparable control wild-type mice ( $P = 0.35$ ). In a parallel study where *Fancd2*<sup>-/-</sup> mice were simultaneously fed with metformin while being given poly(I:C), metformin-fed mice displayed a clear protection from poly(I:C)-

induced red cell deficiency ( $P < 0.01$ ; Figure 3E). Collectively, these results indicate that metformin protects *Fancd2*<sup>-/-</sup> mice from poly(I:C)-induced hematologic abnormalities.

*Metformin reduces DNA damage in FA cells.*

Due to a deficiency in interstrand crosslink repair, FA cells display high levels of radial chromosome formation and chromosomal breaks. These chromosomal changes are characteristic features of FA cells and are widely used to help confirm a clinical diagnosis of FA (Figure 4A).<sup>28</sup> Treatment with DNA cross-linking agents strongly induces this phenotype, but some FA cells also display spontaneously elevated chromosome breakage levels. We thus used this classic radial and breakage assay to determine whether metformin could protect FA cells from spontaneous DNA damage. FA-A patient derived fibroblast cells (PD259i) that displayed spontaneous radials and breakage were treated with metformin (1 $\mu$ M or 10 $\mu$ M) for 48 hours before cytogenetic analysis. As shown in Figure 4B, metformin significantly reduced the levels of both radials and chromosomal breaks in PD259i cells, indicating that metformin can protect FA cells from developing DNA damage.

To better understand the mechanism behind this protective effect, we also tested another guanidine derivative, aminoguanidine, in the same assay. As shown in Figure 4C, aminoguanidine also significantly suppressed the formation of radials in PD259i cells, consistent with the chemical similarity and inferred mode of action of these two compounds.

*Metformin may act by aldehyde detoxification.*

Increased sensitivity to DNA crosslinking agents such as formaldehyde and MMC is a characteristic hallmark of FA cells. Recent research has emphasized the role of endogenously

produced aldehydes in producing DNA interstrand crosslinks and contributing to the pathogenesis of bone marrow failure in FA.<sup>10,11,13,29</sup> In particular, endogenous formaldehyde, a highly reactive and abundant aldehyde generated by normal cellular processes such as DNA demethylation, has recently been shown to be a HSC genotoxin.<sup>12</sup> It is known that FA cells are sensitive to formaldehyde.<sup>30</sup> Consistent with these observations, we were able to demonstrate that cultured FA-G patient-derived lymphoblastoid cells (EUFA316) were sensitive to both the classic DNA crosslinking agent mitomycin C (MMC) and to formaldehyde (Figure 5A and 5C). Guanidine derivatives such as metformin and aminoguanidine have the ability to react with aldehydes through the Mannich reaction,<sup>15,31</sup> and could potentially serve as aldehyde scavengers in FA cells to prevent DNA damage. Indeed, we found that aminoguanidine protected EUFA316 cells from dose-dependent, formaldehyde-induced growth arrest (Figure 5B). Surprisingly, we also observed a mild protection of aminoguanidine from MMC-induced growth arrest (Figure 5D).

To further assess whether metformin or aminoguanidine could protect FA-deficient cells, we devised a more sensitive co-cultivation experiment in which equal numbers of EUFA316 cells and EUFA316+FANCG cells (stably complemented with a wild-type *FANCG* cDNA-encoding retrovirus) were labeled with different fluorescent proteins and allowed to compete in the presence of media only, 40 $\mu$ M formaldehyde or 6.25 nM MMC together with metformin or aminoguanidine (0.01, 0.1 or 1 mM). The competitive growth of these cells was monitored by flow cytometry. Both aminoguanidine and, to a lesser degree, metformin was able to provide dose-dependent protection against exogenous formaldehyde (Supplemental Figure 1). We also observed protection against MMC by both aminoguanidine and metformin, though only at the highest dose tested (1 mM; Supplemental Figure 1), probably reflecting the non-specific

protective effects of suppressed cell cycling by aminoguanidine or metformin. These results demonstrate that metformin and aminoguanidine protect FA-deficient cells from formaldehyde-induced and, to a much lesser extent, MMC-induced toxicity.

Formaldehyde is detoxified principally by formaldehyde dehydrogenase, encoded by the *Adh5/Gsnor* gene. *Adh5*<sup>-/-</sup> mice accumulate formaldehyde adducts in DNA.<sup>12</sup> To determine whether metformin was able to suppress formaldehyde-induced DNA damage, we utilized a recently discovered small molecule inhibitor of Adh5, the C3 compound, to induce the accumulation of endogenous formaldehyde in FA cells.<sup>32,33</sup> After human FA-A patient derived fibroblast cells PD259i were treated with 100μM C3 for 48 hours, the levels of spontaneous radials and chromosomal breaks were significantly increased by 2-fold and 3-fold, respectively ( $P < 0.0001$  in both cases, Figure 5E and 5F). Importantly, concurrent treatment with metformin significantly suppressed C3-induced radials and chromosomal breaks ( $P < 0.0001$  and  $P < 0.001$ , respectively; Figure 5E and 5F), consistent with a role for metformin in detoxifying formaldehyde.

#### *Metformin delays tumor formation in *Fancd2*<sup>-/-</sup> mice.*

The *in vitro* experiments above showed that metformin could reduce spontaneous DNA damage in FA cells. In addition, metformin is well known to reduce the incidence of several human cancers.<sup>34,35</sup> For these reasons, metformin was tested as a cancer chemopreventive agent. A cohort of tumor prone *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice along with *Fancd2*<sup>+/+</sup>*Trp53*<sup>+/-</sup> littermate controls were divided into two groups and treated with either metformin or placebo diet. The tumor spectrum in metformin-treated *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice was similar to that in placebo-treated controls. The most common type of tumors was ovarian in origin, consistent with our previous

observation on tumor types in *Fancd2*<sup>-/-</sup> mice<sup>9,22</sup> and an earlier study reporting that over 18% of human primary ovarian epithelial cancers have a disrupted FA/BRCA pathway.<sup>36</sup> Specifically, 61 *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice under placebo treatment developed 91 tumors, among which 37 (41%) were ovarian tumors; 31 *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice under metformin treatment developed 34 tumors, among which 13 (38%) were ovarian tumors. These spectra were similar to what we have reported before.<sup>37</sup> However, as shown in Figure 6, *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice on metformin diet showed a significantly longer ( $P < 0.05$ ) mean tumor-free survival time (mean survival of 405 days) than the *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice on placebo diet (mean survival of 368 days). The first tumor was seen at 142 days in the *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice on placebo diet, whereas the earliest tumor in the *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice on metformin appeared much later at 244 days of age. Overall, these results indicate that metformin administration significantly delays tumor formation in *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice.

In contrast, as shown in Figure 6, the *Fancd2*<sup>+/+</sup>*Trp53*<sup>+/-</sup> mice on placebo diet had a mean tumor-free survival of 510 days, and those on metformin diet survived an average of 535 days. There was no significant difference between metformin and placebo treatment in these p53 heterozygotes ( $P = 0.86$ ), indicating that the tumor-delaying effect of metformin was specific to only FA mutant mice.

## Discussion

The majority of genes responsible for FA have now been found and many of their biochemical functions are being uncovered.<sup>2,3</sup> The FA network consists of at least 21 proteins that serve to maintain genome stability, enhance stem cell survival and suppress cancer, and are functionally integrated with genes involved in inherited breast and ovarian cancers (e.g., *BRCA1* and *BRCA2*). Despite these scientific insights, there has been little progress in treating human FA patients or preventing bone marrow failure. Bone marrow transplantation is currently the only curative therapy for the hematopoietic complications of the disorder, but when performed without a matched sibling donor, it is often accompanied by both short term and long term morbidities.<sup>1</sup> Among these complications is a very high risk of secondary cancer.<sup>38</sup> Synthetic androgens have been used for many years to support marrow function and improve cytopenias for a subset of FA patients.<sup>39,40</sup> However, these outcomes are limited by incomplete or transient responses, together with unacceptable side effects and toxicities. Gene therapy remains a promising approach for FA<sup>41</sup>, but to date there have been no reports of clinical success despite the selective advantage for gene corrected stem cells in this disorder. Furthermore, as noted above, successful treatment of bone marrow failure does not diminish the severity or risk of non-hematopoietic consequences of FA, most notably the high risk of solid tumors.<sup>38</sup> New therapeutic approaches that have the ability to treat or prevent bone marrow failure and cancer are thus clearly needed for FA.<sup>8,9,20,37,42</sup>

Here we show that the widely used diabetes drug metformin improves hematopoiesis and delays tumor formation in *Fancd2*<sup>-/-</sup> mutant mice. Of note, metformin is the first compound to improve both of these FA phenotypes: oxymetholone,<sup>9</sup> resveratrol,<sup>8</sup> sirtuin activator,<sup>20</sup> and *N*-acetylcysteine<sup>42</sup> all improve hematopoiesis in *Fancd2*<sup>-/-</sup> mice, but none has been shown to

diminish tumor incidence. In contrast, the antioxidant tempol delays cancer in FA, but does not benefit hematopoiesis.<sup>37</sup> Our results indicate that metformin can ameliorate both of these key phenotypes of FA, and that its beneficial action was specific to FA mutant mice. In contrast, oxymetholone and the sirtuin activator SRT3025 affect both wild-type and mutant stem cells equally,<sup>9,20</sup> indicating that their mechanisms of action do not target the pathophysiology of FA bone marrow failure. Furthermore, it is particularly intriguing that the chronic administration of metformin significantly increases the frequency of HSCs in the adult *Fancd2*<sup>-/-</sup> mice. The loss of HSCs in FA lies at the root of bone marrow failure and is a progressive process that extends from adolescence into adulthood.<sup>5,6,9</sup> There is an emerging body of evidence supporting that this progressive HSC loss may begin in utero.<sup>8,43,44</sup> Although the magnitude of HSC rescue by metformin is relatively small, this drug demonstrates a unique ability to restore the HSC numbers in post-natal life in FA mice.

The specificity of action of metformin and the structurally related compound aminoguanidine in the protection of FA mutant cells may be explained by our observation that both compounds appear to selectively reduce DNA damage in FA cells. This is demonstrated by the dose-dependent reduction of spontaneous radial chromosome formation and chromosome breaks in a human FA cell line treated with either compound.

The precise mechanism by which metformin and aminoguanidine reduce DNA damage in FA cells remains unclear, though here we present evidence that aldehyde detoxification may be an important part of the protective effect conferred by both metformin and aminoguanidine. Endogenously produced aldehydes, including acetaldehyde and formaldehyde, are clearly genotoxic in FA mutant cells.<sup>11,12,29,45</sup> We found that the pharmacological inhibition of Adh5, the main enzyme responsible for cellular formaldehyde detoxification, induced chromosome

breakage and radials in FA cells. Importantly, metformin rescued this defect at physiologically relevant concentrations. Given that the chemically related guanidine derivative aminoguanidine was also able to block formaldehyde toxicity, presumably through the well-described Mannich reaction,<sup>15,31</sup> our data are consistent with the hypothesis that metformin acts at least in part through an aldehyde scavenging mechanism. It is surprising that metformin also mildly protected FA patient cells from MMC-induced growth arrest. This could be due to the release of lipid peroxidation-derived aldehyde 4-hydroxy-2-nonenal associated with MMC treatment<sup>46</sup> and/or methanol (which could be oxidized to formaldehyde) during the activation of MMC.<sup>47</sup> However, it is also possible that metformin may act via other mechanisms to attenuate the FA phenotype. For example, metformin potently activates AMPK, a kinase known to be important in protecting HSCs from genomic instability.<sup>21</sup> Of note, in our previous transcriptome analysis of HSPCs,<sup>9</sup> the mRNA encoding *SLC22A3*, one of the membrane transporters important for metformin uptake,<sup>34,48</sup> is preferentially enriched by 12.3 fold in HSPCs, as compared with whole bone marrow cells. It is thus possible that metformin can exert its effects directly on HSPCs. Metformin's effects on the *Fancd2*<sup>-/-</sup> hematopoietic system, including re-enforcing quiescence in HSPCs and increasing CFU-S forming capacity of bone marrow cells, resemble the effects of resveratrol,<sup>8</sup> another known AMPK activator.<sup>19</sup> It is, therefore, tempting to suggest that metformin and resveratrol might exert their hematopoietic benefits through the AMPK signaling pathway. However, this does not explain all the effects from metformin because metformin delays tumor formation in *Fancd2*<sup>-/-</sup> mice, whereas resveratrol does not. We recently discovered that transforming growth factor- $\beta$  (TGF- $\beta$ ) inhibitors can protect HSCs in FA mice and patients by altering the balance of non-homologous end-joining and homologous recombination.<sup>49</sup> Although metformin is not known to directly interfere with TGF- $\beta$  signaling, several reports

indicate interactions between the LKB1/AMPK pathways and TGF- $\beta$ .<sup>50</sup> Other mechanisms by which metformin may act to protect FA mice include: reducing the activity of mitochondrial complex 1 activity,<sup>14</sup> thus potentially reducing oxidative DNA damage;<sup>51,52</sup> supporting the expansion of HSCs *in vitro* by switching the metabolic balance between oxidative phosphorylation and anaerobic glycolysis;<sup>53</sup> downregulating inflammatory pathways,<sup>54</sup> which are thought to contribute to bone marrow failure in FA.<sup>55</sup> Future studies will be needed to determine whether one or more of these known mechanisms in addition to aldehyde quenching are responsible for the beneficial effects of metformin in FA.

Despite these uncertainties as to the exact mechanism of protection by metformin, a compelling argument can be made for a clinical trial of metformin to protect FA patients from bone marrow failure and tumorigenesis. Metformin has a superb safety record in light of its wide clinical use to treat diabetes mellitus over more than two decades. In our preclinical model, metformin outperforms the current standard of care, oxymetholone. Oxymetholone therapy had no significant effect on peripheral blood counts of FA mice at 6 months.<sup>20</sup> Its benefits only became apparent after 17 months of treatment.<sup>9</sup> In contrast, metformin improved peripheral blood counts after only 6 months of therapy.

Several important questions remain to be answered. The optimal dose of metformin for FA therapy and disease prevention is not known. If metformin acts predominantly as an aldehyde scavenger, higher doses may be optimal, and a well-tolerated high dose could be readily determined from use and toxicity data. It is also not known when beginning metformin treatment would be most beneficial: prior to the onset of bone marrow failure, or only after the development of anemia. Finally, potential synergies between metformin and anabolic androgens, the current gold standard of therapy, have not been studied. These interactions are difficult to

predict directly, as androgens accelerate the cell cycle of stem cells,<sup>9</sup> whereas metformin increases quiescence.

Our results may also have relevance in regards to the use of metformin in the general population as an anti-aging and cancer chemoprevention drug.<sup>56</sup> Metformin has not previously been reported to protect the genome from DNA damage and mutation. However, such activity would go a long way towards explaining why it can.

## **Acknowledgements**

We thank Pamela Canady and Mandy Boyd at the Oregon Health & Science University flow cytometry core for fluorescence activated cell sorting, William H. Fleming and Devorah C. Goldman for valuable advice, and Laura Marquez-Loza for help with animal care. This work was supported by NIH grant P01 HL048546 and Fanconi Anemia Research Foundation (to MG), and an award from the Office of the Assistant Secretary of Defense for Health Affairs, through the Bone Marrow Failure Research Program under Award No. W81XWH-14-1-0297 (to RJMJr).

## **Authorship**

Contributions: Q.S.Z. designed the study, performed research, analyzed and interpreted the data, and wrote the manuscript; M.D., N.P., W.T., H.L., M.A., and A.M. performed research; R.J.M. Jr. and S.O. designed the *in vitro* studies, interpreted the data, and wrote the manuscript; A.N.M. examined the histological slides and wrote the manuscript; and M.G. designed the study, analyzed and interpreted the data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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**Table 1:****Complete blood counts in metformin-treated *Fancd2*<sup>-/-</sup> and *Fancd2*<sup>+/-</sup> mice**

|                            | <i>Fancd2</i> <sup>-/-</sup><br>Placebo | <i>Fancd2</i> <sup>-/-</sup><br>MET | <i>P</i> | <i>Fancd2</i> <sup>+/-</sup><br>Placebo | <i>Fancd2</i> <sup>+/-</sup><br>MET | <i>P</i> | <i>P</i> (Mut placebo<br>vs Wt placebo) |
|----------------------------|-----------------------------------------|-------------------------------------|----------|-----------------------------------------|-------------------------------------|----------|-----------------------------------------|
| WBCs, ×10 <sup>3</sup> /μL | 4.1 ± 0.2                               | 5.1 ± 0.4                           | <0.05    | 5.5 ± 0.4                               | 6.8 ± 0.5                           | <0.05    | <0.003                                  |
| RBCs, ×10 <sup>6</sup> /μL | 8.9 ± 0.1                               | 9.3 ± 0.2                           | 0.08     | 9.5 ± 0.1                               | 9.7 ± 0.2                           | 0.24     | <0.01                                   |
| HGB, g/dL                  | 12.8 ± 0.1                              | 13.5 ± 0.2                          | <0.005   | 13.5 ± 0.2                              | 13.6 ± 0.2                          | 0.80     | <0.003                                  |
| HCT, %                     | 49.5 ± 0.5                              | 51.2 ± 0.8                          | 0.07     | 51.3 ± 0.8                              | 51.6 ± 0.8                          | 0.76     | 0.08                                    |
| MCV, fL                    | 55.6 ± 0.5                              | 55.3 ± 0.3                          | 0.59     | 53.0 ± 0.3                              | 53.4 ± 0.8                          | 0.49     | <0.0001                                 |
| MCH, pg                    | 14.4 ± 0.2                              | 14.6 ± 0.1                          | 0.40     | 14.1 ± 0.2                              | 14.0 ± 0.1                          | 0.52     | 0.24                                    |
| MCHC, g/dL                 | 25.9 ± 0.2                              | 26.3 ± 0.2                          | 0.16     | 26.6 ± 0.2                              | 26.2 ± 0.1                          | 0.11     | <0.05                                   |
| PLT, ×10 <sup>3</sup> /μL  | 404 ± 17                                | 465 ± 20                            | <0.05    | 530 ± 19                                | 562 ± 28                            | 0.37     | <0.0001                                 |

Data were pooled results from multiple mice (17 – 19 mice each group) and presented as mean value ± SEM. WBCs denotes white blood cells; RBCs, red blood cells; HGB, Hemoglobin; HCT, Hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, Platelet; MET, metformin.

## Figure Legends:

### Figure 1: Metformin administration enhances hematopoiesis.

A). Complete blood counts after 6 months of treatment with metformin. D2, WBC, HCT, RBC, HGB, NS, and MET denote *Fancd2*, white blood cells, hematocrit, red blood cells, hemoglobin, not significant, and metformin, respectively. The data are pooled results from 17 to 19 individual mice in each group. B). Representative flow cytometry profiles for placebo and metformin-treated *Fancd2*<sup>-/-</sup> mice. The percentages on the profiles indicate the mean value for each group. PI denotes propidium iodide. C). Quantification of CD34<sup>+</sup>KSL frequency in bone marrow. The data represent the percentage of CD34<sup>+</sup>KSL cells in all nucleated bone marrow cells from 15 mice in each group.

### Figure 2: Metformin administration helps FA HSPCs maintain quiescence.

A). Representative flow cytometry profiles of the cell cycle analysis for KSL cells. The percentages on the profiles indicate the mean value for each group. B). Statistical analysis of the cell cycle status. Data are pooled results from 10 to 15 mice. NS denotes not significant.

### Figure 3: Metformin administration improves the function of FA bone marrow cells.

A). Representative pictures of spleens analyzed in the CFU-S assay. B). Statistical analysis of CFU-S assays. Forty thousand donor bone marrow cells were injected intravenously into each recipient mouse. Data represent 3 or 4 donors in each group of mice, with 2 - 4 recipients for each donor. D2 denotes *Fancd2*; NS, not significant. C). Schematic chart to show the procedures of poly(I:C) experiments. Three month old mice were injected intraperitoneally with either poly(I:C) or saline at 8 mg/kg body weight. The mice were harvested either 2 weeks (for CFU-S

assay) or 3 weeks (for complete blood count analysis) after the completion of poly(I:C) treatment. D). Statistical analysis of CFU-S assays after poly(I:C) administration. Data represent 8 or 9 donors in each group of mice, with 2 - 4 recipients for each donor. Total recipients in each group ranged from 23 to 28 mice. E). Statistical analysis of CBC tests after poly(I:C) administration. Data are pooled results from 11 to 17 mice each group for wild-type mice and 18 to 19 mice each group for *Fancd2*<sup>-/-</sup> mice.

**Figure 4: Metformin prevents FA patient-derived cells from developing radials and chromosomal breaks.**

A). Representative pictures of spontaneous radials and breaks in PD259i human FA-A fibroblasts. The arrows indicate a chromosomal break (left) or a radial (right). B, C). Statistical quantitation of radials and breaks in PD259i human FA-A fibroblasts after aminoguanidine or metformin treatment. PD259i cells were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin /streptomycin. Cells were cultured with metformin or aminoguanidine for 48 hours before metaphase spreads were made. Fifty metaphases for each sample were scored for radial contents and chromosomal breakage. Data are combined results from 6 independent experiments. AG and MET denote aminoguanidine and metformin, respectively.

**Figure 5: Aldehyde sensitivity of human FA cells and the detoxification of aldehydes by aminoguanidine.**

A). Formaldehyde dose-dependent survival of EUFA316 human FA-G mutant lymphoblastoid cells compared with an isogenic, FANCG-complemented EUFA316 control. Complementation

of patient cells was performed by stably transducing FANCG-mutant EUFA316 cells with a retrovirus expressing a wild-type human *FANCG* cDNA. EUFA316 and EUFA316+FANCG cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. B). Aminoguanidine shows dose-dependent rescue of EUFA316 FANCG-mutant cells from formaldehyde-induced cell death. AG denotes aminoguanidine. C). MMC dose-dependent survival of EUFA316 and wild-type controls. D). Aminoguanidine provided a mild protection on EUFA316 cells from MMC-induced cell death. E, F). Statistical quantitation of radials and chromosomal breaks in 259i human FA-A fibroblasts treated with C3, the ADH5 inhibitor. Metformin was added to the cell culture at 10  $\mu$ M and maintained at the same concentration throughout the experiment. One hour later, C3 was added at 100  $\mu$ M. Forty-eight hours later, cells were harvested for breakage and radial analysis. MET denotes metformin. Data are combined results from 4 independent experiments.

**Figure 6: Metformin protects *Fancd2*<sup>-/-</sup> mice from tumor development.**

Kaplan-Meier survival curves of the *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice and *Fancd2*<sup>+/+</sup>*Trp53*<sup>+/-</sup> mice. For *Fancd2*<sup>-/-</sup>*Trp53*<sup>+/-</sup> mice, the data represent 31 mice for metformin treatment and 60 mice for placebo treatment; For *Fancd2*<sup>+/+</sup>*Trp53*<sup>+/-</sup> mice, the data represent 30 mice for metformin treatment and 60 mice for placebo treatment. Tumor samples and selected tissues were fixed in 10% phosphate-buffered formalin, stained with H&E, and examined under a microscope. The Kaplan-Meier survival curves were generated by Prism 6.0c Software (GraphPad Software, Inc.) and *P* values were calculated using the Log-rank (Mantel-Cox) test. NS denotes not significant.

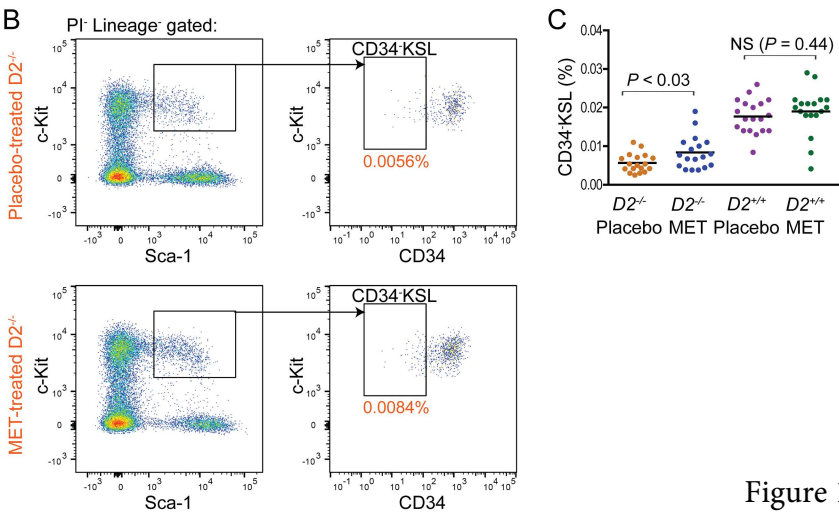
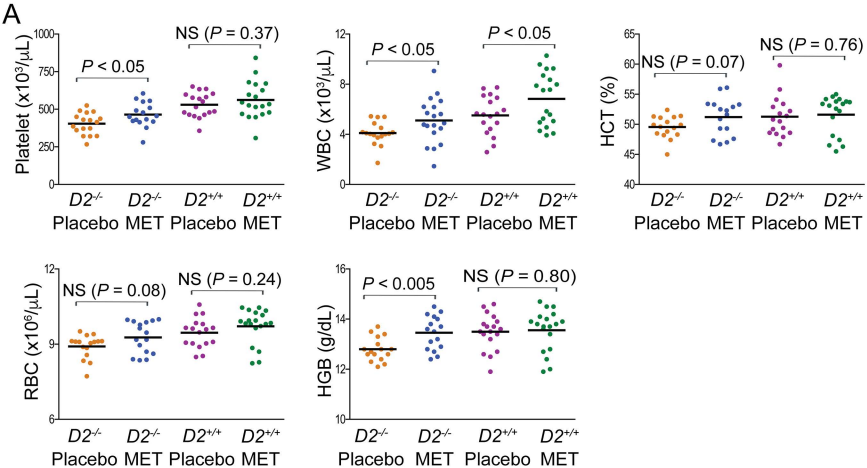
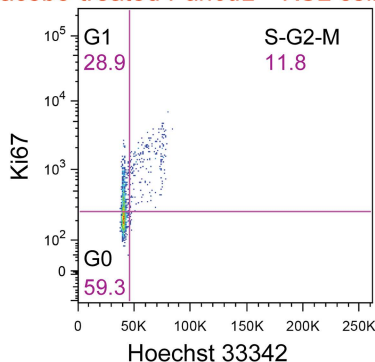
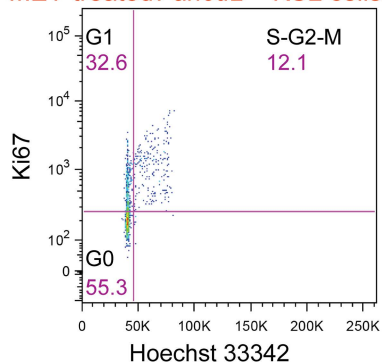
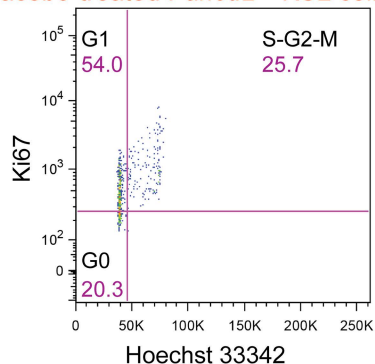
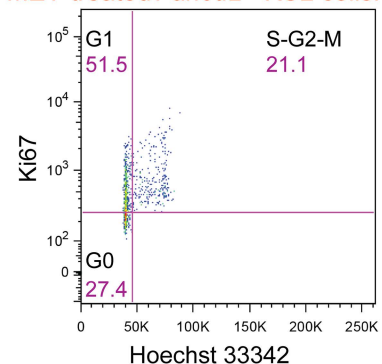


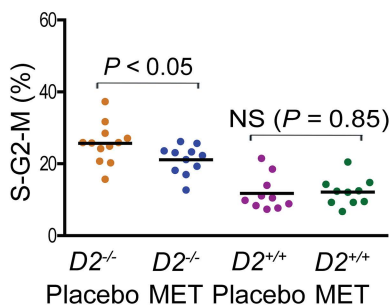
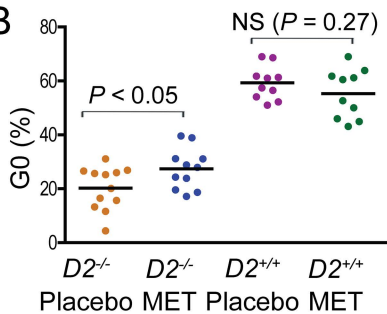
Figure 1

Figure 2

A

Placebo-treated *Fancd2*<sup>+/+</sup> KSL cells:MET-treated *Fancd2*<sup>+/+</sup> KSL cells:Placebo-treated *Fancd2*<sup>-/-</sup> KSL cells:MET-treated *Fancd2*<sup>-/-</sup> KSL cells:

B



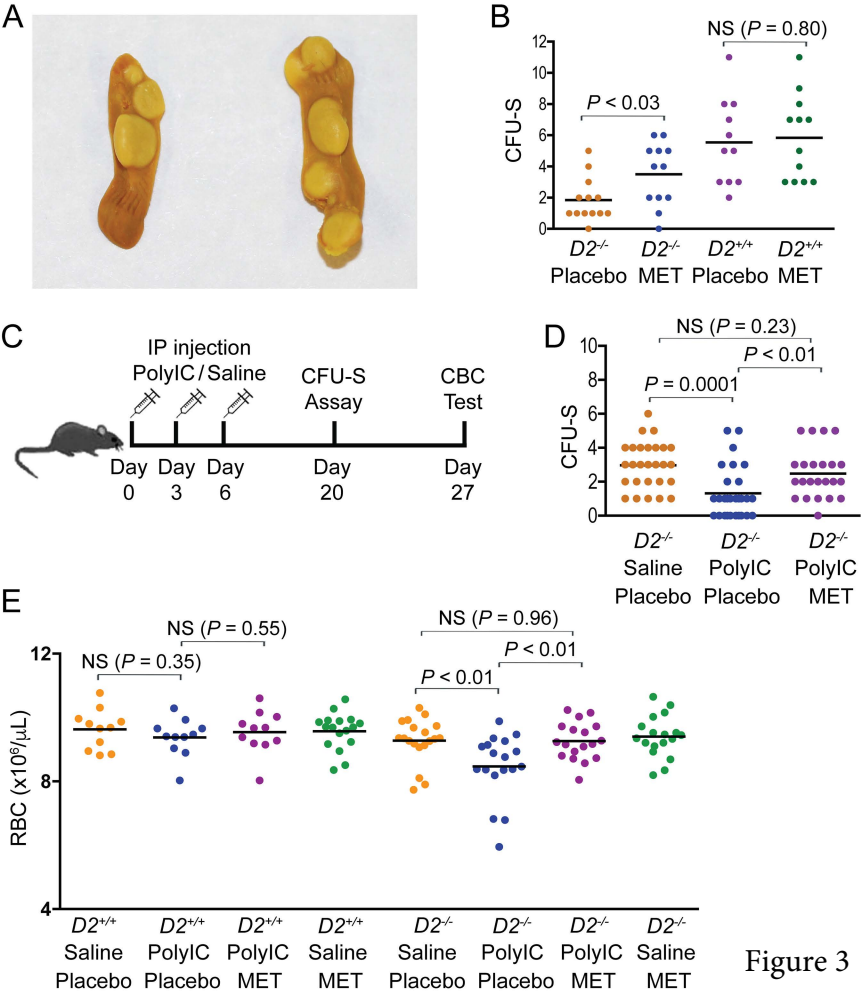


Figure 3

Figure 4

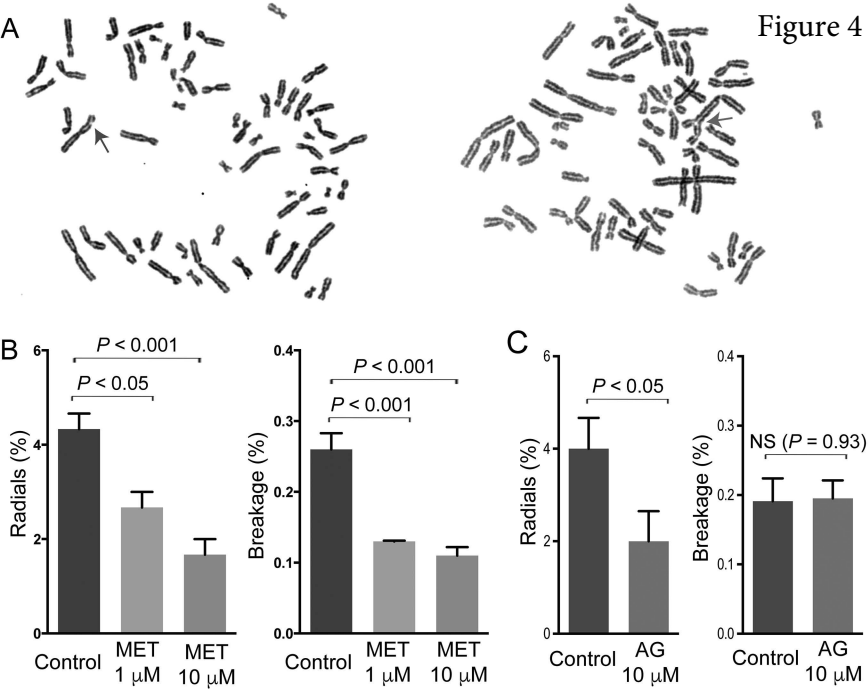
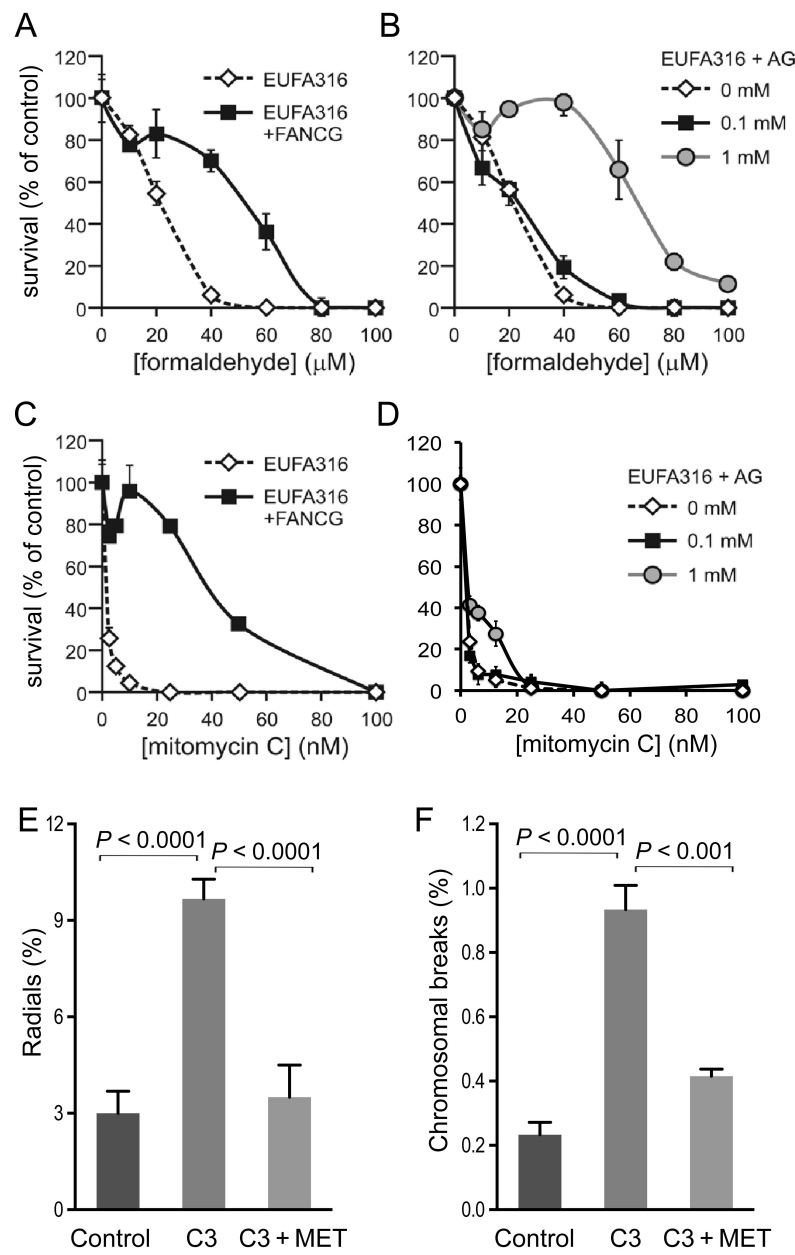
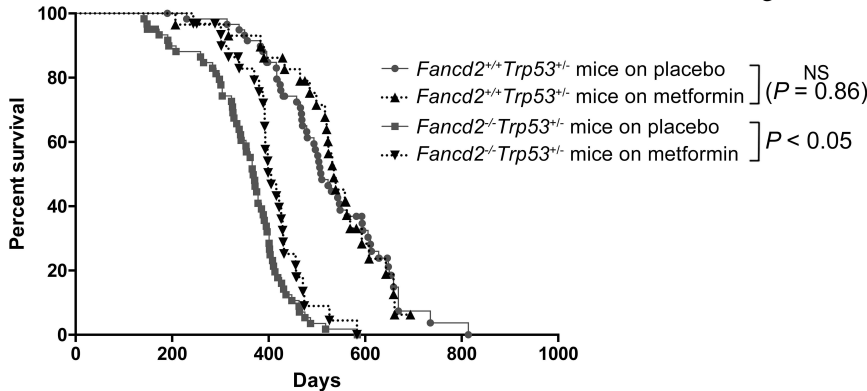


Figure 5



# Tumor-free Survival Curve

## Figure 6





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Prepublished online October 18, 2016;  
doi:10.1182/blood-2015-11-683490

## **Metformin improves defective hematopoiesis and delays tumor formation in Fanconi anemia mice**

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