

Award Number: W81XWH-14-1-0297

TITLE: Small Molecule Protection of Bone Marrow Hematopoietic Stem Cells

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CONTRACTING ORGANIZATION: University of Washington  
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E-Mail: monnat@u.washington.edu					
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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> During the above project period we have demonstrated the following progress on Aims 1 and 2, and have keyed the following results to specific major tasks under each Aim: 1. mitotic expansion of human mobilized peripheral blood CD34+ hematopoietic stem cells (HSCs) in culture in defined medium supplemented with hematopoietic growth factors and small molecule inhibitors of differentiation; 2. suppression of differentiation of CD34+ CD38- CD90+ during mitotic expansion in culture by addition of the small molecule inhibitors SR-1 or UM-171, alone or in concert; 3. dose-dependent killing of CD34+ CD38- CD90+ HSCs by the addition of formaldehyde; 4. development of sensitive and specific mass spec assays for DNA adducts and DNA-protein crosslinks (DPCs) generated by formaldehyde in human cells.					
<b>15. SUBJECT TERMS</b> CD34+ human hematopoietic stem cells (HSCs), bone marrow, formaldehyde toxicity					
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<b>a. REPORT</b>  Unclassified	<b>b. ABSTRACT</b>  Unclassified	<b>c. THIS PAGE</b>  Unclassified			<b>19b. TELEPHONE NUMBER</b> (include area code)

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## 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. **Note: in order to facilitate reporting and assessment we have structured this Progress Report to directly reflect the order of Revised Specific Aims/Major Tasks below.**

<b>Revised Specific Aim 1: Small molecule protection of human cells from aldehyde-induced killing (<i>in vitro</i> studies - no mice or human subjects)</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 + <i>FANCG</i> -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: <i>In vitro</i> 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 above project period, we have made the following progress on the Revised scope of Work Aims and Major Tasks above. We have also identified obstacles to progress, work-arounds where needed and identified priorities and next steps for the coming work period.

Given the results reported below and the above timetable, we are on or ahead of schedule on most of the major tasks that define this project, despite the late start.

**Revised Specific Aim 1: Small molecule protection of human cells from aldehyde-induced killing (*in vitro* studies - no mice or human subjects)**

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

**Accomplishments:**

**1. Identify and screen shRNAs specific for FANCG for ability to deplete the human FANCG protein.** The shRNA sequences listed below have been synthesized and successfully

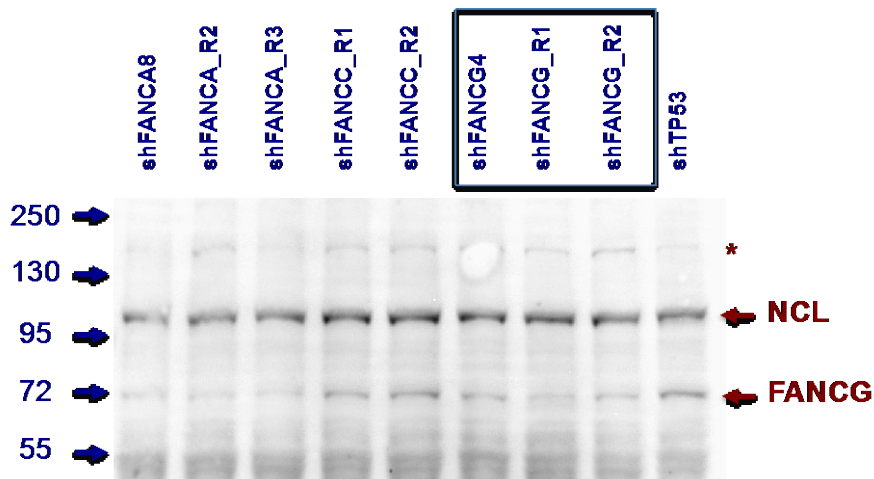
cloned into two lentiviral vectors, pLKO and pLKO-Tet-on lentiviral vectors, then sequence-verified for correct insertion and sequence.

shRNA	Ref. Seq.	Sequence	Location	siRNA/shRNA source
FANCG1	NM_004629	CCGCGGGAAGTAAGTCTTCAA	3'UTR	Hs_FANCG_1
FANCG4	NM_004629	CTCATTGAGGTAGAATTAATA	964-984(ORF)	Hs_FANCG_4
FANCG_R1	NM_004629	(GA)GTCTGGAGCTGCTAGTTGA	903-923(ORF)	Rosetta design 1
FANCG_R2	NM_004629	(CA)GAGTGGAGCCTCTAAGGAT	508-528(ORF)	Rosetta design 2
FANCG_R3	NM_004629	(AG)GAGTTGCTCAGCCGCACAT	1253-1273(ORF)	Rosetta design 3
FANCG_2858	NM_004629	CCTGTGAAATTTGCCCTAGTT	3'UTR	Broad RNAi Consortium
FANCG_2859	NM_004629	GCCAAAGTCTTGTGACCTGTA	1848-1868(ORF)	Broad RNAi Consortium
FANCG_2860	NM_004629	GCCTCTAAGGATCTGCTGTTA	517-537(ORF)	Broad RNAi Consortium

**2. Generated lentiviruses from cloned shRNA vectors.** The above vectors were prepared for use to transduce human cells by transfecting the vector DNAs separately into packing cells and then harvesting the viral supernatants to use to express CD34+ cells.

**3. Western blot verification of shRNAs to deplete the FANCG protein.** In order to test the function of the above shRNAs as expressed from their lentiviral vectors we have developed Western blot assays to detect and quantify protein depletion of the FANCA, C and G proteins from human cells. An example is provided below.

To test ability of lentiviral expressed shRNAs to deplete FANCG, U2-OS cells were transduced with pLKO-derived lentivirus vectors expressing shRNA targeting FANCG (shFANCG4, shFANCG\_R1, and shFANCG\_R2 at MOI of 10. Whole cell lysates were extracted 12 days after shRNA expression and probed with an anti-FANCG antibody (Abcam). Nucleolin (NCL), was used as protein loading control.



**Next steps:**

1. identify better shRNAs for depletion of FANCG: We were not happy with the extent of depletion of the 8 shRNAs described above upon transfection into human cells. In order to rectify this we will test additional commercial sources of human FANCG lentiviral shRNA

vectors that are guaranteed to produce 70% or more gene expression knock-down. These are available from and will be ordered from: 1) Gene ID 2189, OriGene, Rockville, MD, and 2) TLHSU2300-2189, transOMIC, Huntsville, AL.

2. determine the transduction/depletion timecourse: demonstrate transduction and depletion timecourse, on a scale large enough to feed viability and MS assays (through using inducible lentiviral shRNA expression vector and mitotic expansion of CD34+ cells)
3. demonstrate inducible shRNA expression to deplete FANCG protein during the mitotic expansion of CD34+ cells: see Revised Specific Aim 2 below for rationale and additional detail.

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

**Accomplishments:**

**1. Mitotic expansion in defined medium of human PBM CD34+ cells**

**Rationale:** Development of the mass spectrometry-based assays to measure DNA base and DNA-protein crosslinks (DPCs) generated by formaldehyde require substantial numbers of cells, especially for the DPC assays. The number required will range from 1 - 10E+06 cells with the DPC assays requiring the larger number. The only practical way to get these numbers from readily available CD34+ cells is by mitotic expansion.

**Results: Mitotic expansion of mobilized human CD34+ cells**

Mobilized human CD34+ cells, obtained from Fred Hutchinson Cancer Research Center (Seattle, WA), were seeded at 1.0E+04/ml in Stemline II medium (Sigma, St Louis, MO) supplemented with SCF, TPO and G-CSF (Pepro Tech, Rocky Hill, NJ, all at final concentration of 100 ng/ml) and incubated overnight in a 37°C/5% CO<sub>2</sub> humidified incubator. Next day, cells were counted and analysed by flow for cell surface marker expression. Small molecule agonists of HSC self-renewal, SR1 (1 mM) or UM171 (34 nM), were added individually or in combination followed by continued culture for another 11 days. Cells were counted and analyzed by flow for cell surface marker expression at Day 8 and Day 12.

**Cell counts (cells/ml)**

	<i>Day 1</i>	<i>Day 8</i>	<i>Day 12</i>	<i>fold expansion</i>
medium only	1.04E+04	7.40E+05	4.25E+06	425X
SR1	1.04E+04	6.00E+05	3.72E+06	372X
UM171	1.04E+04	2.70E+05	2.84E+06	284X
SR1+UM171	1.04E+04	2.50E+05	2.04E+06	204X

We have also identified in the past month a source of CD34+ human cord blood T-cells to use in these experiments as an alternative, perhaps more physiologically relevant, cell source where the numbers of cells available from individual donors is substantially higher (>10E+7/donor), thus facilitating technically robust mass spec analyses with the addition of replicates and variants.

## 2. Quantified the effect of 2 small molecule antagonists of CD34+ differentiation (SR1 and UM171) on differentiation of CD34+ cells during mitotic expansion.

**Rationale:** CD34+ cells remain the best choice of a hematopoietic stem cell surrogate for these assays, and will remain our focus. Thus we tested two recently identified small molecules that antagonize CD34+ differentiation during mitotic expansion as described above.

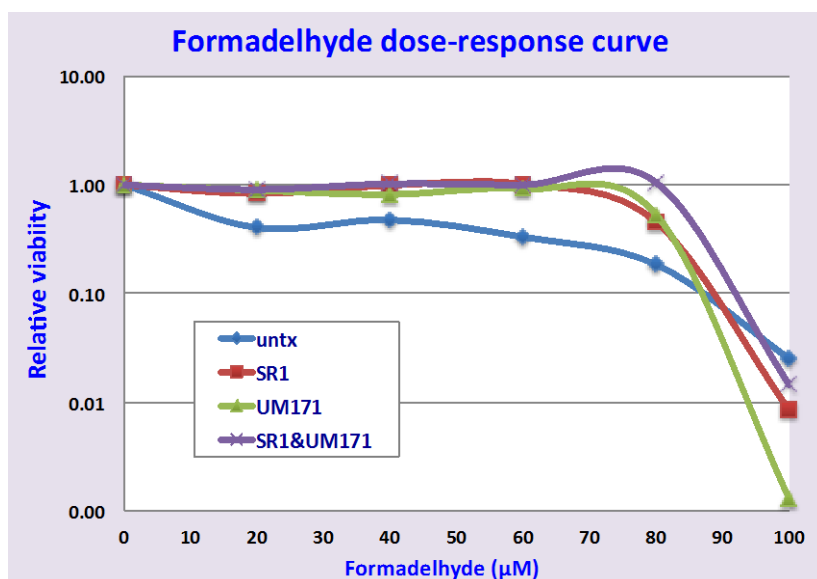
### Cell surface CD34 expression (% of total cells)

	Day 1	Day 8	Day 12
medium only	99.76	22.85	2.05
SR1	99.76	31.34	3.56
UM171	99.76	41.62	5.61
SR1&UM171	99.76	49.83	8.23

These results indicate that SR1 and UM171 each suppress CD34+ differentiation, and that their effects are additive with only a modest ( $\leq 2$ -fold) reduction in cell number even when used in combination.

## 3. Determined formaldehyde dose-dependent kill curve using mitotically expanded CD34+ cells

**Results:** Day 12 expanded human CD34+ cells from above were seeded at  $5 \times 10^4$  cells /well in 400  $\mu$ l medium in 48-well plate. Cells were treated with various doses of formaldehyde (0, 20, 40, 60, 80, and 100  $\mu$ M) for 4 days, then cell numbers and viability were assessed using the WST-1 cell proliferation assay (Roche Diagnostics, Indianapolis, IN).



These results indicate effective CD34+ cell killing in a physiologic formaldehyde concentration range that we will use in subsequent experiments.

**Next steps:**

1. Repeat formaldehyde dose-dependent cell killing using shRNA-depleted CD34+ cells.
2. Determine ability of two small molecule formaldehyde antagonists (metformin and aminoguanidine) to protect FANCG-depleted CD34+ cells from formaldehyde dose-dependent killing in culture.

**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.**

**Accomplishments:**

**1. Developed formaldehyde-DNA adduct/crosslink HPLC-MS/MS assays:** this was accomplished by collaborators James Swenberg and Rui Yu at UNC. This new analytical capacity was reported by Dr. Swenberg as a poster at the Fanconi Anemia Research Fund Scientific Symposium in Toronto in Sept 2015, and has been reported in part in the following publication: Pontel LB, Rosado IV, Burgos-Barragan G, Garaycoechea JI, Yu R, Arends MJ, Chandrasekaran G, Broecker V, Wei W, Liu L, Swenberg JA, Crossan GP, Patel KJ. (2015) Endogenous Formaldehyde Is a Hematopoietic Stem Cell Genotoxin and Metabolic Carcinogen. *Mol Cell*. 60:177-88. doi: 10.1016/j.molcel.2015.08.020. Epub 2015 Sep 24.

**2. Wrote and have had accepted a second manuscript further detailing these results as an 'in press' article in Cancer Research.** This ms. was not available on-line at the time of submission of this progress report (12 Jan 2016).

**Next steps:**

1. Determine cell numbers needed to reliably detect, quantify and replicate results for formaldehyde-DNA adduct/crosslinks in control and FANCG-depleted human cells.

**Revised Aim 2 - Major Task 2: Apply mass spectrometric assay to DNA derived from treated CD34+ cells.**

**Next steps:**

1. Demonstrate detection of formaldehyde-induced DNA damage using DNA isolated from CD34+ cells.
2. Determine cell numbers needed to reliably detect, quantify and replicate results for formaldehyde-DNA adduct/crosslinks in control and FANCG-depleted CD34+ cells
3. quantify DNA damage in formaldehyde-treated CD34+ cells using isotope-labeling/MS methods as a function of formaldehyde dose and concentration of small molecule antagonist.

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

Nothing to report.

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

Provisional results on the ability of a small molecule, aminoguanidine, to protect human Fanconi-deficient lymphoblastoid cell lines to killing by formaldehyde were orally presented at the 27th Annual Fanconi Anemia Research Fund Meeting in Toronto, Ontario CANADA 17-20 September 2015. These results were presented by a collaborator who is not participating directly in this project

A poster detailing the development of a mass spectrometry-based assay for aldehyde base adducts and DNA-protein crosslinks was presented by Dr. Jim Swenberg, a collaborating investigator, as a poster at the 27th Annual Fanconi Anemia Research Fund Meeting in Toronto, Ontario CANADA 17-20 September 2015. Dr. Swenberg is a collaborator who is participating directly in this project.

Two manuscripts detailing mass spec-based analytical aspects of this project have been published as noted above.

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

1. Demonstrate lentiviral depletion of FANC proteins from human CD34+ HSCs
2. Determine baseline values for DNA base adducts and DNA-protein crosslinks in human CD34+ HSCs growing in culture
3. Demonstrate formaldehyde dose-dependent killing of FANC protein-depleted versus control-transduced human CD34+ HSCs growing in culture
4. Determine DNA base adduct and DNA-protein crosslink levels in human CD34+ HSCs growing in culture as a function of formaldehyde dose and FANC protein content

**IMPACT:**

The results above helped further establish proof-of-concept for this project, and have provided practical ways to focus our experimental priorities in the coming 12 months.

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

Nothing to report yet.

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

Nothing to report yet.

**– 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 in scope of work.

**PRODUCTS:**

Nothing to report.

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

Provisional results were presented as part of an oral presentation and a poster at the 27th Annual Fanconi Anemia Research Fund Meeting held in Toronto, Ontario CANADA 17-20 September 2015.

**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  
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?**

**Project Ended:**

Butterfly Guild Monnat (PI)	4/1/14-3/31/15	0.15CM
	\$35,000	

Targeted Correction of the Shwachman-Diamond Syndrome Defect in Patient-Derived CD34+ and iPS Cells

The goal of this project is to use genome-editing TALEN and CRISPR/Cas9 nucleases to correct the two most common disease-causing mutations in iPS cells from Shwachman-Diamond syndrome patients. This work is one of three jointly funded projects by the Butterfly Guild, a private charitable organization founded to catalyze research on the treatment and cure of Shwachman-Diamond syndrome.

Role: PI

**This project had been submitted for a competitive renewal and was awarded:**

P01 CA077852 Monnat (PI) 7/8/15-6/30/20 4.2 CM  
NIH/NCI \$233,732

Genetic Instability in Werner Syndrome - Project 2: Werner Syndrome Cell Function  
The aims of this project, one of 4 in the Program Project are: 1) to identify WRN functional pathways in human cells; 2) identify in vivo substrates for WRN function in human cells; and 3) determine DNA damage response pathway activation and responsiveness in human somatic cells.

Role: PI

P01 CA077852 Monnat (PI) 7/8/15-6/30/20 0.6 CM  
NIH/NCI \$51,440

Genetic Instability in Werner Syndrome - Core C: Administration

The aims of this Core are to: 1) provide consistent planning, decision making and review progress for the Program Project as a whole; 2) identify and facilitate collaboration within and outside the Program Project; 3) co-ordinate activities of the Internal and External Advisory Boards, including the annual retreat and scientific review; and 4) provide centralized administrative, secretarial and fiscal support for the Program Project.

Role: PI

**This project was awarded:**

R01 CA181445 (Folch/Rostomily/Monnat) 07/01/14-06/30/19 1.05 CM  
NIH/NCI \$372,494

Microfluidic Device to Profile Chemosensitivity in Glioma Slice Cultures

This multi-investigator R01 is focused on developing microfluidic platform for chemotherapeutic sensitivity profiling of human glioma primary and xenograft specimens. The focus is on device design, fabrication and prototyping and developing endpoint analysis methods.

Role: one of three Co-PI's in a multiple-PI R01

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

University of North Carolina  
Chapel Hill, NC

Collaborators James Swenberg and Rui Yu are located at UNC-Chapel Hill. They have developed the mass spectrometric analytical methods to detect and quantify specific DNA base adducts and SDNA-protein crosslinks in CD34+ HSCs.

**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:**

Nothing to report.

**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.**