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14. ABSTRACT

Severe congenital neutropenia (SCN) is an inherited bone marrow failure syndrome most often resulting from autosomal dominant or de novo transmission of heterozygous mutations in the gene, *ELANE*, encoding neutrophil elastase. Other causes of SCN include autosomal recessive inheritance of *HAX1* mutations. The purpose of this research is to understand how mutations in both *ELANE* and *HAX1* lead to neutropenia, in order to gain further understanding into normal homeostatic regulation of granulopoiesis and how it is disrupted in a variety of bone marrow failure syndromes. Based on identification of a new class of mutations in *ELANE* that disrupt the translational start site and recent findings that *HAX1* may be an RNA-binding protein, we originally hypothesized, and have now shown in patient-derived induced pluripotent stem cells (iPSC), that *ELANE* can encode amino-terminally truncated polypeptides initiating from internal translational start sites. In scope, we have proposed a series of experiments to be performed in cell culture systems to identify RNA sequences bound by HAX1, to determine if *ELANE* mutations influence HAX1 binding to *ELANE* mRNA and influence use of alternate translational start sites, and to biochemically characterize amino-terminally truncated neutrophil elastase. In the year since our last report, significant progress has been made in using new tools to model granulopoiesis, including generation of patient-derived iPSC and CRISPR-Cas9 genome-editing technology to introduce site specific repair or mutation into the *ELANE* locus, allowing for more relevant modeling of *ELANE* and HAX1 interactions in vitro.

15. SUBJECT TERMS
Neutropenia, bone marrow failure, neutrophil elastase, ELANE, HAX1, alternate translation, induced pluripotent stem cells (iPSC)

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1. INTRODUCTION

The overall goal of this research is to understand how mutations in the genes *ELANE*, encoding neutrophil elastase, and *HAX1*, encoding HCLS1 associated protein X-1 (HAX1), cause hereditary forms of neutropenia. Previously, competing hypotheses have posited that mutant forms of neutrophil elastase either subcellularly mislocalize or misfold, whereas the specific role of *HAX1* has been less clear. Our recent identification of a subgroup of patients with *ELANE* translational start site mutations has challenged both hypotheses because, at first glance, such mutations are not expected to result in translation of protein. We have offered a new, unified hypothesis in which we propose that *ELANE* may encode a set of polypeptides with variable in-frame amino-terminal initiation sites whose production is ordinarily suppressed by *HAX1* and that mutations in either gene may lead to their pathogenic production. To test this hypothesis, we proposed three aims designed to, first, determine HAX1 binding specificity to *ELANE* mRNA; second, identify the range of mutations within *ELANE* that may contribute to aberrant translation initiation; and, third, characterize the biochemical properties of amino-terminally truncated neutrophil elastase polypeptides.

During the first year of this project, we made considerable progress toward each of the aims, resulting in a publication (Tidwell et al., 2014. Blood 123:562-569). As part of that work, we developed a new patient-derived induced pluripotent stem cell (iPSC) model of *ELANE*-associated neutropenia.

During the second year of this project, in order to facilitate performance of the specific tasks, we have expanded upon initial studies and further developed the iPSC system, including recently, CRISPR-Cas9 mediated genome editing, which allows us to address experimental aims using a model system of granulopoiesis that more precisely mirrors *ELANE* and HAX1 pathology.

2. KEY WORDS

neutropenia
bone marrow failure
neutrophil elastase
ELANE
HAX1
alternate translation
induced pluripotent stem cells (iPSC)

3. ACCOMPLISHMENTS

►What were the major goals of the project?

The objective of the project is to determine how mutations in *ELANE* and/or *HAX1* produce neutropenia, toward the overall goal of obtaining a better understanding of the mechanisms of normal leukocyte homeostasis, identify final common pathways in different forms of hereditary neutropenia, and better understand how different types of mutations result in pathogenesis. Specific Aims/Tasks are delineated in the section below.

►What was accomplished under these goals?

Task 1. Perform "RIP-Seq" next-generation DNA sequencing of transcripts co-immunoprecipitating with HAX1 (months 1-24).

The goals of this task are to identify a consensus RNA sequence or structure for HAX1 binding, determine if there is a set of genes coordinately regulated at the post-transcriptional level by HAX1, and identify potential new candidate genes for genetically unexplained cases of neutropenia. In order to identify mRNAs bound by HAX1, we have immunoprecipitated HAX1 from cell lysates and performed next-generation DNA sequence analysis of co-immunoprecipitating RNA species.

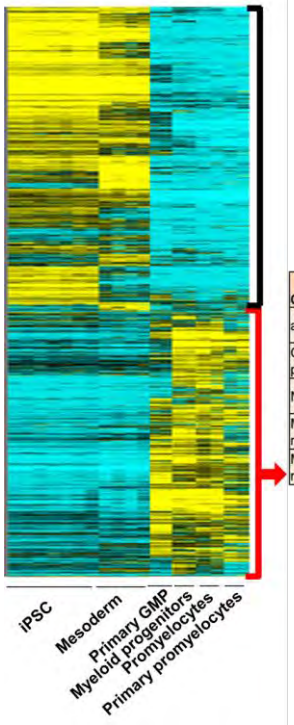
As documented in the prior reporting period, we developed a specific protocol for co-immunoprecipitation of HAX1-bound mRNA and performed sequencing of corresponding cDNA on the SOLiD platform. Sequencing identified specific binding of HAX1 to ~20-30 nucleotide segments for the following genes: *EPHB2* (Ephrin Receptor), *ASTN1* (Astrotactin), *CASP9* (Caspase 9), and *FAF1* (FAS associated factor 1). Unfortunately, there were too few genes to identify statistically significant enrichment for Gene Ontogeny pathway analysis or to extract primary or tertiary consensus sequences or structure, respectively. Although not a specific task, but consistent with long-term objectives of the task, in parallel work, we have continued to analyze unexplained cases of congenital neutropenia lacking mutations in *ELANE*, *HAX1*, or other known genes, but no pathogenic variants are detectable in *EPHB2, ASTN1, CASP9, or FAF1* genes to date. This task is now largely complete, but results at this stage are best characterized as indeterminate. A technical concern relates to insufficient read-depth in the experiments we performed on the ABI SOLiD sequencing platform. In the third year we intend to repeat sequencing on the Illumina platform. Given advances in sequencing technology, we anticipate that there will be more efficient recovery of co-immunoprecipitated cDNA, enabling sufficient power to complete a more conclusive bioinformatic analysis.

Task 2. Determine if ELANE mutations, particularly synonymous ELANE mutations, affect HAX1 binding and influence cryptic start site utilization (months 1-36).

The goal is to determine if different *ELANE* and/or *HAX1* sequence alterations actually converge on a common cellular effect, with respect to production of amino terminally-truncated polypeptides initiating from internal *ELANE* translational start sites.

As previously reported and now published in Tidwell *et al*. (Neutropenia-associated *ELANE* mutations disrupting translation initiation produce novel neutrophil elastase isoforms *Blood* 2014;123:562-569), we have shown, utilizing both cell culture and patient-derived induced pluripotent stem cells (iPSC) models, that mutations altering the translational start site, as well as internally within the coding sequence, lead to production of internally-translated polypeptides and disrupt potential internal ribosome entry site (IRES) activity. In general, this task has largely been completed.

However, development of iPSC models of neutrophil differentiation has significantly furthered our capacity to address the original objectives of this task, using a more physiologically relevant in vitro system. Working toward the original aims, in collaboration with Drs. H. Leighton Grimes, Carolyn Lutzko, and Jose Cancelas at Cincinnati Children's Hospital, we have continued to develop iPSC models of *ELANE* associated neutropenia, recently expanding our capabilities through the use of CRISPR-Cas9 genome editing technology. Using these assays, which we present below (in discussion of Task 3), we can assess various elements of Tasks 2 and 3 in a single experiment.



GO analysis	p(B-H FDR)	#genes in list
adaptive immunity	3.11E-68	225
Granulocyte physiology	5.99E-50	87
Neutrophil physiology	3.03E-38	64
Mononuclear cell morphology	1.82E-33	180
Myeloid leukocyte morphology	6.63E-29	116

Fig. 1 Comparative transcriptome of BM promyelocytes and relevant hematopoietic populations derived from iPSC. A total of 1,028 genes representing iPSC signature (black line cluster) are downregulated while 828 hematopoietic genes (red line cluster, GO analysis adjacent) are upregulated in sorted myeloid progenitors (day 10) and promyelocytes (day 15) obtained from iPSC cultures compared with their counterparts in primary human BM. Each lane represents an independent specimen and lanes are clustered according to their cell type.

Task 3. Characterize the biochemical properties of internally-translated neutrophil elastase polypeptides (months 14-36).

In order to determine how internally-translated neutrophil elastase polypeptides contribute to pathogenesis, it is important to characterize their biochemical properties. To a large degree, the proposed experiments have been accomplished and published in *Tidwell et al.*, utilizing a combination of in vitro assays, cell culture systems, and limited application of patient-derived iPSC. In order to further characterize the properties of these shorter polypeptides, we have continued to pursue development of patient-derived iPSC models, in conjunction with CRISPR-Cas9 genome editing.

We have performed an experiment to first assess the validity of iPSC as models for granulopoiesis. We used gene expression profiling to compare the iPSC-derived mesodermal and myeloid progenitors and promyelocytes to primary human bone marrow (BM) derived cells (**Fig. 1**). Comparison of 1,028 genes representing an undifferentiated iPSC signature (black line cluster) are downregulated in differentiated myeloid cells from iPSC and BM. We also identified 828 hematopoietic genes (red line cluster) that are upregulated in myeloid progenitors (day 10) and promyelocytes (day 15) obtained from differentiated iPSC cultures and that are similar to BM promyelocytes. The hematopoietic genes were not expressed in undifferentiated iPSC or mesodermal progenitors. Evaluation of the genes that are overexpressed in hematopoietic cells from bone marrow or iPSC are shown (right side) with Gene Ontology (GO) analysis indicating roles in adaptive immunity, granulocyte physiology, and myeloid functions. Transcriptional profiling therefore demonstrates that iPSC derived myeloid progenitors are largely indistinguishable from their genuine equivalents found in human bone marrow.

We have also derived iPSC from two additional unrelated SCN patients with 'conventional' missense mutations, Q68P and I89N, using lentiviral transduction of peripheral blood mononuclear cells with Yamanka factors and analyzed their granulopoietic capacity. Compared to control iPSC, they similarly exhibit promyelocytic arrest and failure to form neutrophils (**Fig. 2A**). As expected, they also activate the unfolded protein response, as demonstrated by upregulation of *BIP* and *ATF6* by TaqMan qRT-PCR (**Fig. 2B**). Neutrophil elastase exhibits mislocalization, compared to granular localization in control iPSC lacking *ELANE* mutations (**Fig. 2C**). We are now in a position to use this system to determine whether other types of *ELANE* mutations, as well as deletion of *HAX1*, force translational expression of internally translated neutrophil elastase polypeptides (Task 2) and, if so, to characterize their properties in a physiologically relevant model of granulopoiesis (Task 3).

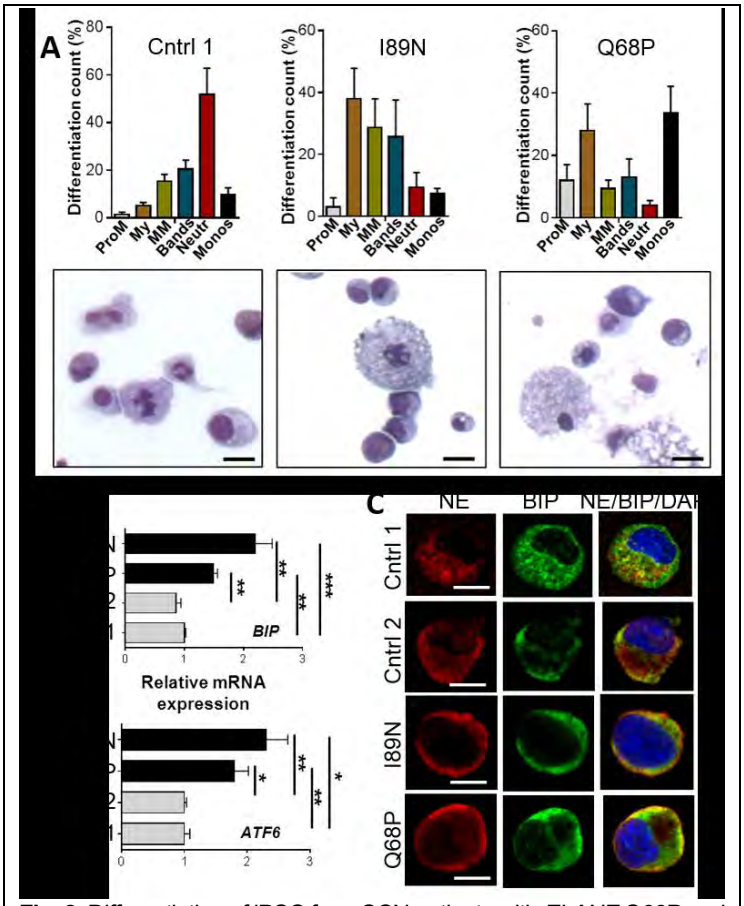


Fig. 2. Differentiation of iPSC from SCN patients with *ELANE* Q68P and I89N mutations. **A.** Promyelocytic arrest and absence of neutropenia. **B.** Induction of UPR. **C.** NE mislocalization using counterstaining to BIP.

To take advantage of this superior in vitro model of granulopoiesis, which avoids the use of transfection or transduction of site-specifically mutated cDNAs, we have also adapted methods for targeting mutations in these two iPSC lines. We developed CRISPR/Cas9 gene targeting of *ELANE* exon 3 using the Guide RNA Sequence Design Platform for optimal gene targeting to design guide RNAs to cut at the flanks of *ELANE* exon 3 (**Fig. 3A**). We designed and synthesized two donor constructs for homology-directed repair (HDR) of *ELANE* exon 3 with either mRUBY2 or EGFP selectable marker genes (**Fig. 3B**). A diagram of the *ELANE* locus and screening primers to determine integration are shown (**Fig. 3C**). *ELANE* exon 3 gene-targeting was validated in HEK-293 cells following triple transfection of the *ELANE* exon 3 CRISPR guide RNAs, Cas9 nickase plasmid, and either mRUBY2 or GFP donor constructs. Transfection resulted in a 3% stable mRUBY2 expression after two weeks demonstrating successful integration (**Fig. 3D**). These cells were FACS sorted into bulk culture resulting in 53% purity of mRUBY2 positive cells, and a second FACS sort with single cell isolation generated clones. Twelve clones were evaluated, and ten were positive for mRUBY2 expression (**Fig. 3D**). Molecular analysis using a forward primer (FP) in exon 1 within the genomic location (and outside of the HDR donor) and the reverse primer (RP) in the EFS promoter of the expression cassette confirmed integration into the expected location (**Fig. 3E**). Sequencing of PCR amplicons from the genomic to donor PCR confirmed

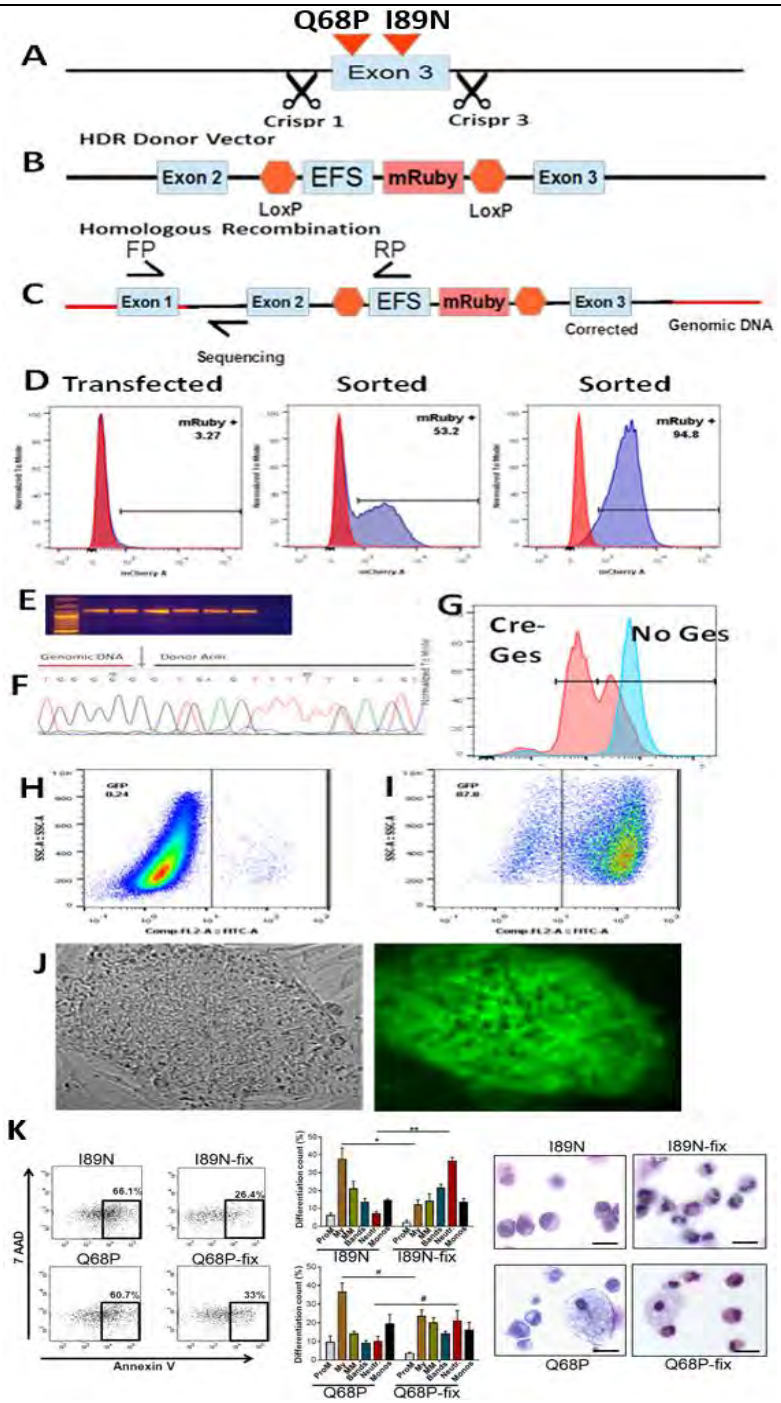


Fig 3. CRISPR/Cas9 editing of *ELANE*. A. CRISPR guide RNAs flank exon 3. **B**. Schematic of HDR donor cassette with either mRUBY2 or EGFP markers. **C**. *ELANE* locus after CRISPR/Cas9 gene with HDR. Forward primer (FP), reverse primer (RP), and sequencing primer used to confirm integration. **D**. HEK-293 cells before and after transfection using mRUBY2 donor, sorting and cloning. **E**. PCR using FP in exon 1 genomic region (outside of the donor) and the RP in EFS promoter in the donor confirming cassette integration occurred in *ELANE*. Note 6 of 7 clones shown are positive. **F**. Sequence confirmation that PCR product spans genomic HDR boundary and that donor integrated into targeted region. **G**. Cre-removal of mRUBY2 selection cassette 48h after Cre-gessicle administration (red) compared to no gessicle treatment (blue). **H**. CRISPR/Cas9 gene editing in I89N HDR EGFP donor. FACS analysis of iPSC 2 weeks after transfection and **I**. 1 month after sorting. Note post-sort cells are cultured on GFP-neg feeders. **J**. Phase and fluorescence image of sorted gene-edited line. **K.** Corrected iPSC lines Q68P and I89N ('fix') are functionally corrected, showing (L→R), normalization of apoptosis, reversal of promyelocytic arrest, and ability to form neutrophils.

genome targeting to *ELANE* (**Fig. 3F**). The selection cassette in the HDR was flanked with loxP sites for easy removal to ensure that functional studies of myeloid differentiation were not compromised by expression of the selectable marker. To confirm that the expression cassette could be removed, we evaluated Cre-gesicles, which are fusogenic particles to facilitate protein delivery3. Forty-eight hours after Cre-gesicle addition into a GFP-positive HEK-293 clone, there was a reduction in GFP expression (**Fig. 3G**), demonstrating that this approach can be used to remove the selection cassette. We next evaluated exon 3 CRISPR/Cas9 gene-editing using the GFP donor construct in order to correct mutations in two different patient-derived iPSC lines, containing *ELANE* Q68P or I89N mutations. As expected, the overall frequency of GFP expression was lower than seen in HEK-293 cells, with a stable ~0.2% GFP expression for at least three weeks, confirming integration (**Fig. 3H**). After transfection, cells were expanded and sorted for GFP expression (**Fig. 3H**). Post-sort bulk and clonal cultures were recovered on murine embryonic feeders to increase (**Fig. 3I**). Both flow cytometry and fluorescence microscopy (**Fig. 3J**) confirm the presence of EGFP positive colonies, suggesting successful targeting of *ELANE* in iPSC. We performed *ELANE* RT-PCR analysis in myeloid differentiated cells from the CRISPR/Cas9 line to determine whether the mutant transcript was expressed. Only the wild type *ELANE* transcript was expressed (not shown), suggesting successful integration of the HDR into the mutant *ELANE* allele. Finally, we subjected each of the starting and corrected iPSC lines to differentiation conditions (as described in Tidwell *et al*.), where they exhibit reversal of elevated apoptosis and promyelocytic arrest, and ability to generate neutrophils (**Fig. 10K**). Further molecular characterization is ongoing to evaluate for off-target events.

We now plan in the remaining year of this project to perform western blots and neutrophil elastase catalytic assays on the patient-derived iPSC corresponding to the different amino acid missense substitutions, in order to determine if they, too, produce shorter polypeptides commencing from internal translation start sites. This system will also afford an opportunity to regulate HAX1 expression, either through RNAi knockdown or directly via gene-editing to introduce precise HAX1 loss of function mutations (i.e., W44X) corresponding to actual human mutations.

Task 4. Data will be analyzed and a report shall be prepared, concurrent with the overall aim to report findings in the peer-revised biomedical literature, in order to allow accessibility to physicians, scientists, patients, and the general public.

As noted previously, some of the tasks have been completed and are now published in Tidwell *et al*. (PubMed PMID 24184683), which received an accompanying commentary (Borregaard. Severe congenital neutropenia: new lane for ELANE. *Blood*. 2014;123:462-3).

A manuscript describing new findings with gene correction of *ELANE* mutations and characterization of neutrophil elastase properties has been submitted.

We plan to prepare abstracts for submission to the American Society for Hematology (ASH) annual meetings.

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

Donovan Anderson, a research scientist who receives partial salary support for this project and who primarily generated data for Task 1 further developed bioinformatic skills by participating in seminars and classes on the R programming language and Bioconductor at the Fred Hutchinson Cancer Research Center.

Timothy Tidwell, a graduate student who has recently obtained his Ph.D. and has worked on this project (but whose support came through other means), travelled to Cincinnati Children's Hospital to learn iPSC and gene editing technologies, skills which he has introduced to our group in Seattle.

►How were the results disseminated to communities of interest?

Scientific results are being communicated through one published manuscript and a second recently submitted paper.

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

Please see the plans corresponding to each task above.

4. IMPACT

►What was the impact on the development of the principal discipline(s) of the project?

Our studies have informed understanding of hereditary forms of neutropenia and other bone marrow failure syndromes, including myelodysplasia (which is a complication of hereditary neutropenia) by addressing how the most abundant protein of leukocytes, neutrophil elastase, contributes to the normal homeostatic regulation of blood cells. We have developed new patient-derived iPSC models of myelopoiesis and have validated that they faithfully recapitulate molecular genetic and cellular features occurring in the bone marrow, thus extending their use for analysis of other forms of hereditary blood disorders.

Beyond the scope of this proposal, and still in the future, gene-correction of *ELANE* mutations, as demonstrated here using CRISPR-Cas9 technology, may ultimately prove to be of use as a therapeutic tool for treating congenital neutropenia and other hereditary blood disorders.

Additionally, acquired forms of neutropenia, such as from chemotherapy, autoimmune disorders, or premalignant conditions, are not uncommon and likely involve common denominators that molecular studies of congenital disorders may help to reveal.

►What was the impact on other disciplines?

More generally, our studies have illuminated unconventional ways through which primary coding sequence mutations in genes may have unexpected effects on translation, including by disrupting translational start sites, activating internal ribosome entry sites, or affecting mRNA folding and interactions with RNA binding proteins. Given the large volume of 'variants of undetermined significance' now pouring in from whole genome and whole exome sequencing studies, our results may have bearing on the interpretation of rare variants that either do not directly alter protein coding or that are otherwise predicted to have benign effects on protein structure and function.

►What was the impact on technology transfer?

Nothing to report.

►What was the impact on society beyond science and technology?

Our studies are focused at the level of basic science of myelopoiesis. As such, we have nothing to

report with respect to technology transfer or impact on society beyond science and technology.

5. CHANGES/PROBLEMS

►Changes in approach and reasons for change

A minor, but not significant change, involves the unexpectedly rapid pace of advance in iPSC and genome editing technologies. We now propose to substitute use of these tools in place of less sophisticated approaches involving transfection of cultured cells and other in vitro methods, which we had proposed in the original submission. Specific subtasks listed in the third year of Tasks 2 and 3 can now be directly performed using the more relevant model system we describe. It should be noted that following initial submission of the Tidwell *et al.* manuscript, now published in *Blood* and which precisely parallels proposed tasks, reviewers requested validation using iPSC models. We feel that performing studies in iPSC will be needed in order to assure high impact dissemination to the scientific community.

►Actual or anticipated problems or delays and actions or plans to resolve them

None

►Changes that had a significant impact on expenditures

None

►Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

N/A

6. PRODUCTS

►Publications, conference papers, and presentations

Tidwell, T., Wechsler, J., Nayak, R.C., Trump, L., Salipante, S.J., Cheng J.C., Donadieu, J., Glaubach, T., Corey, S.J., Grimes, H.L., Lutzko, C., Cancelas, J.A., and Horwitz, M.S. (2014) Neutropenia-associated *ELANE* mutations disrupting translation initiation produce novel neutrophil elastase isoforms. **Blood** 123:562-569.

►Website(s) or other Internet site(s)

None

►Technologies or techniques

Generation of CRISPR-Cas9 *ELANE* gene correction tools.

►Inventions, patent applications, and/or licenses

None

►Other products

Generated *ELANE*-mutant induced pluripotent stem cells (iPSC).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

►What individuals have worked on the project?

Name:	Marshall S. Horwitz
Project Role:	PI
Research Identifier:	44913107 (NCBI My Bibliography)
Nearest person month worked:	2
Contribution to Project:	Responsible for overall design, planning of experiments and logistic details for executing the research plan, interpretation of data, and manuscript preparation and other forms of dissemination
Funding Support:	

Name:	Donovan Anderson
Project Role:	Research Scientist
Research Identifier:	
Nearest person month worked:	5
Contribution to Project:	Responsible for day-to-day design of experiments (in consultation with PI), laboratory bench work and bioinformatic analysis for Task 1. Has contributed to technical aspects of Tasks 2 and Tasks 3.
Funding Support:	

Name:	Timothy Tidwell
Project Role:	Graduate student
Research Identifier:	44913107 (NCBI My Bibliography)
Nearest person month worked:	12
Contribution to Project:	Responsible for day-to-day design of experiments (in consultation with PI), laboratory bench work, and preliminary interpretation of results for Tasks 2 and 3. Responsible for first drafts of manuscripts.
Funding Support:	NIH

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

Yes, please see “other support page” in appendix.

►What other organizations were involved as partners?

Cincinnati Children's Hospital Medical Center – As noted, investigators there are collaborators on generation of iPSC, ELANE gene targeting using CRISPR-Cas9, and have provided training at in this technology at their institution to Timothy Tidwell.

8. SPECIAL REPORTING REQUIREMENTS

None.

9. APPENDICES

Other Support follows

HORWITZ, Marshall

Active

LSDF Program Grant 4553677 (P.I.-M. Schwartz)
Life Science Discovery Fund Program 09/01/11 – 08/30/16 .72 Cal months
Pancreatic Stem Cells (Co-P.I. - Horwitz) Subproj.
The overarching goal of this proposal is to treat diabetes with stem cells. The subproject employs lineage tracing methods to track teratoma formation in mouse transplanted with stem cell derived insulin-producing cells. No overlap.

R01 CA160674 (Multi P.I.-L. Loeb, T Brentnall) 4/1/12 – 3/31/17 1.2 Cal months
NIH Subproj.
Clonal Field Expansion in Ulcerative Colitis (Co-P.I. - Horwitz)
Our proposed studies will further characterize and validate the association between mutations in polyguanine tracts in DNA with the development of colon cancer in UC. No overlap.

W81XWH-13-1-0066 (P.I.-Horwitz) 4/1/13 – 3/31/16 2.04 Cal Months
DOD
Translational Control in Bone Marrow Failure
We propose to identify the messenger RNAs produced from other genes that are also bound by HAX1and determine whether they share similar functions in regulating cell growth. We aim to determine if the many different mutations found in ELANE and HAX1 among different patients with SCN all have the common effect of producing this shorter form of neutrophil elastase. We then plan to characterize the biochemical properties of this short form of neutrophil elastase and determine how it is toxic to the cell. No overlap.

* 11732 (P.I.-Horwitz/Shendure) 12/1/13-11/30/16 2.0 Cal Months
Paul Allen Family Foundation
Cell lineage extracted from the transcriptome
This is an invited proposal to use transcriptome data to infer cell lineage history. No overlap.

* new support since last support period

Projects completed since last reporting period

R01 DK078340 (P.I.-Horwitz) 2/01/08-1/31/15 NCE 1.3 Cal months NIH
Genomic Fate Maps
This proposal is responsive to PA-06-231 “Developmental Biology and Regeneration of the Liver (R01)” and is intended to employ somatic mutation to resolve related to liver growth and development.

R01 DK58161 (P.I.-Horwitz) 8/01/00-7/31/14 NCE 1.3 Cal months
NIH
Molecular Genetic Basis of Cyclic Hematopoiesis
The goals of this project are to determine the molecular mechanisms of hereditary neutropenia.

DP1 OD003278 (P.I.-Horwitz) 9/30/07-7/31/14 NCE 3.0 Cal months
NIH 2007
NIH Director's Pioneer Award Program
“The NDPA is designed to support individual scientists of exceptional creativity who propose pioneering approaches to major challenges in biomedical and behavioral research. The term “pioneering” is used to describe highly innovative – potentially transformative – approaches that have the potential to produce an unusually high impact.” I will use the Pioneer Award to chart cell lineages by tracking mutations, in order to better understand how stem cells contribute to development and cancer.