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Fort Detrick, Maryland 21702-5012

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14. ABSTRACT
Shwachman-Diamond Syndrome (SDS) is an underdiagnosed and clinically-heterogeneous disorder resulting in bone marrow (BM) failure. SDS is caused by biallelic mutations in the SBDS gene, which normally functions in ribosomal subunit joining and mitotic spindle stabilization. Despite these insights, the molecular pathways leading to BM failure are unknown because the hematopoietic stem and progenitor cells (HSPC) affected by SBDS mutations are rare and heterogeneous. To investigate the mechanisms of SDS pathogenesis, we performed single cell RNA-sequencing on primary CD34+ HSPC from normal and SDS BM. We generated a single cell map of early lineage commitment, and found that SDS hematopoiesis was left-shifted with selective loss of granulocyte-monocyte progenitors (GMPs). Differential gene expression analysis revealed dysregulation of TGFß target genes in SDS hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs), but not in lineage-committed progenitors. Proteomic analysis of primary SDS patient plasma identified increased TGFß-family ligand production. Treatment of SDS patient BM with TGFß inhibitors increased hematopoietic colony formation, supporting a causative role for TGFß-signaling in SDS pathogenesis. These data establish TGFß as a therapeutic target in SDS and translate insights from single cell biology into a novel potential therapy.

15. SUBJECT TERMS
Single cell RNA-seq; bone marrow failure; TGFß

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Introduction

Patients with Shwachman-Diamond Syndrome (SDS) suffer from hematopoietic abnormalities including neutropenia, thrombocytopenia, pan-anemia and myelodysplasia (MDS) with progression to acute myeloid leukemia (AML). The affected cell types and altered genetic networks *in vivo* remain unknown, primarily due to the rarity and heterogeneity of bone marrow progenitors. Our overarching goal was to define transcriptional signatures of bone marrow failure in SDS by performing single cell RNA sequencing (RNA-seq) on freshly isolated patient cells. Indeed, we detected dysregulation of TGFß signaling in rare hematopoietic stem cells and multipotent progenitors, and found that inhibition of this pathway improves SDS hematopoiesis. These studies may lead to clinical use of TGFß inhibitors to treat bone marrow failure in SDS.

Keywords
Single cell RNA-seq; bone marrow failure; hematopoiesis; TGFß

Accomplishments

What were the major goals of the project?

Aim 1. Define the molecular basis for the SDS myelopoiesis defect at single cell resolution.

Sub-Task 1A: Consent 6 SDS patients under protocol 10-02-0057, and 6 normal donors under protocol 09-04-0167; obtain patient bone marrow samples, purify CD34+ cells. (Start date 9/30/2014; duration 24 months; protocol renewed 11/09/2015 and 8/21/2015, respectively, by local IACUC).
Milestone: Obtain 6 patient and 6 donor samples.
Percent completion: 100%; milestone was adjusted because we obtained a sufficient number of cells from 4 normal donors and 4 SDS patients to identify key pathways.

Sub-Task 1B: Load purified cells onto C1 chips; prepare ~96 single cell RNA-seq libraries per sample; run sequencing reactions. (Start date 9/30/2014; duration 24 months)
Milestone: Generate genome-wide transcriptional profiles of CD34+ cells form normal donors and SDS patients at single cell resolution.
Percent completion: 100%

Sub-Task 1C: Process data; perform bioinformatic analyses. (Start date 3/30/2015; duration 30 months)
Milestone: Identify SDS affected progenitor cells; Define SDS gene expression networks in single cells or subpopulations of CD34+ cells; Predict microRNA-targeted mRNAs that explain pathogenesis of SDS.
Percent completion: 90%; we continue to work on bioinformatic approaches to study microRNA function.

Aim 2. Functionally annotate SDS transcriptomes to myelopoiesis defects.

Sub-Task 2A: Obtain 2-4 frozen mononuclear cells from SDS patient repository under protocol 10-02-0057 and 2-4 normal donors under protocol 09-04-0167. (Start date 9/30/2015; duration 24 months; protocol renewed 11/09/2015 and 8/21/2015, respectively, by local IACUC).
Milestone: Obtain 2-4 SDS patient and 2-4 donor samples.
Percent completion: 100%

Sub-Task 2B: Culture CD34+ cells; over-express or knockdown key central and peripheral node genes in SDS networks; assess myeloid/neutrophil phenotypes in myelopoiesis culture ex vivo. (Start date 9/30/2015; duration 24 months

Milestone: Identify SDS myeloid phenotypes ex vivo; define genes responsible for myeloid phenotypes; functionally annotate microRNA-dependent and -independent pathways explaining SDS pathogenesis.

Percent completion: 70%; rather than manipulating individual genes, we have treated SDS CD34+ cells with pharmacological inhbitors of TGFß to rescue hematopoietic phenotypes.

Sub-Task 2C: Quantify microRNA activity in SDS myeloid progenitor cells; perform microarray Expression profiling of microRNAs in SDS myeloid progenitor cells; re-evaluate microRNA target predictions accounting for microRNA expression changes in affected SDS progenitor cells. (Start date 9/30/2016; duration 12 months)

Milestones: Quantify microRNA activity in SDS myeloid progenitors; discern SDS pathways that are affected by reduced microRNA activity and/or altered microRNA expression

Percent completion: 20%

What was accomplished under these goals?

Patients with SDS suffer from complex and unstable hematopoietic defects. Neutropenia is most common in early stages of the disease, with some patients progressing to pan-anemia, thrombocytopenia or MDS/AML. Due to the clinical complexity and the rarity and heterogeneity of bone marrow progenitors, the affected cell types and altered genetic networks *in vivo* remain unknown. To uncover pathogenic mechanisms in SDS, we analyzed bone marrow progenitors from SDS patients by single cell RNA-seq.

Aim 1. We performed single cell RNA-seq on CD34+ hematopoietic stem and progenitor cells (HSPC) from four normal donors (n=256 cells) and four SDS patients (n=283 cells). CD34+ cells comprise several stages of hematopoietic development, which we reasoned could be distinguished by transcriptional signatures. To derive these signatures, we performed bulk RNA-seq of FACS-purified hematopoietic stem cells (HSC), multipotent progenitors (MPP), common myeloid progenitors (CMP), granulocyte-monocyte progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP)[1]. We then applied tSNE based on these bulk signatures to generate single cell maps of early hematopoietic lineage commitment (Fig. 1). Single cell mRNA expression patterns and immunophenotypes validated that our lineage map is consistent with known markers of hematopoietic development. Comparative analysis of normal and SDS maps revealed modest accumulation of HSC/MPP and a drastic reduction of GMP in SDS bone marrow, which was rescued by G-CSF therapy (Fig 2).

We compared gene expression between normal and SDS cells for each cell type except for GMP, which was excluded due to the low number of GMP in untreated SDS patients. Overall, 1680 genes were differentially expressed in at least one cell type. Among these genes, 94% were also significant in a comparative analysis across all cell types, but the fold changes were uninterpretable due to HSPC heterogeneity and altered cellular architecture in SDS. For example, fold reductions in GMP-enriched genes were exacerbated due to the reduced frequency of this cell type in SDS. Remarkably, 81.5% of all differentially expressed genes were specific to either HSC/MPP or CMP, indicating

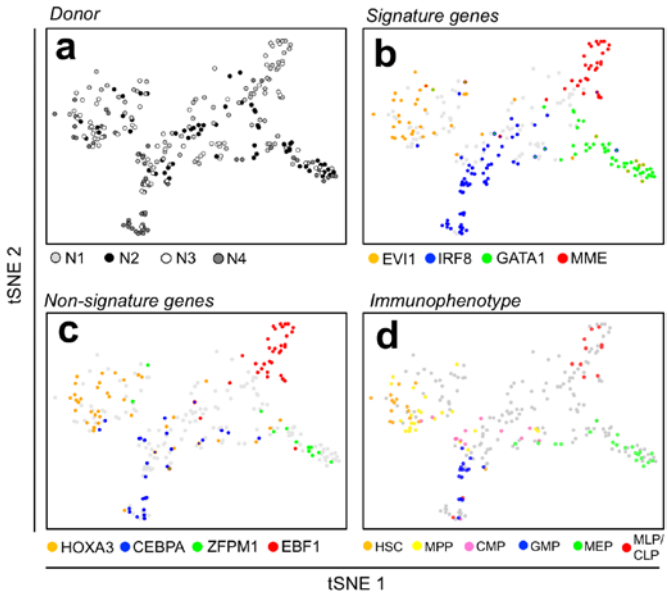

Figure 1. Supervised dimensionality reduction maps lineage commitment of CD34+ cells. tSNE plot of hematopoietic lineage commitment was derived from an empirically-defined gene expression signature. Shown here are cells from four normal donors (n^{N1}=70, n^{N2}=58, n^{N1}=69, n^{N1}=59, n^{total}=256). Cells are colored based on (a) donor identity, (b) mRNA expression of selected signature genes, (c) mRNA expression of lineage-restricted genes reported elsewhere[12], and (d) immunophenotypes. Numerical axes derived from tSNE are arbitrary, and therefore not shown.

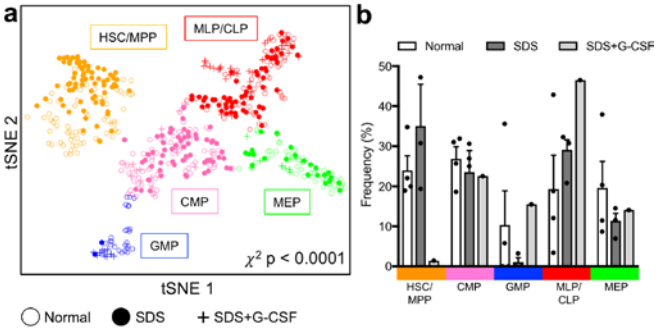

Figure 2. The cellular architecture of early hematopoiesis is altered in SDS. (a) tSNE plot of hematopoietic lineage commitment showing cells from normal donors as in Figure 1, untreated SDS patients ($n^{SDS1.1}$=72, $n^{SDS1.2}$=62, $n^{SDS2.1}$=78, n^{total}=212), and an SDS patient who was being treated with 4.2ug/kg/day G-CSF ($n^{SDS2.2}$=71). Clusters were determined using 'partitioning around medoids' version of *k*-means clustering (*k*=5), and labeled based on the enrichment of index sorted HSC, MPP, MLP, CMP, GMP and MEP as shown in Figure 1d. The sum of normal cells and SDS cells in each cluster is significantly changed using the χ^2 test. (b) Mean relative frequencies of normal, untreated SDS, and G-CSF-treated SDS cells in each cluster. No significant differences were detected from averaged data due to high interindividual variability. Error bars=SEM.

that these are the primarily affected HSPC in SDS (Figure 3a). These data demonstrate that despite the general biochemical functions of the SBDS protein in ribosomal subunit joining and mitotic spindle stabilization[2-6], *SBDS* mutations have cell type-dependent consequences.

The Inflammatory Response was enriched among differentially expressed genes in HSC/MPP and CMP (maximum *p*-value 4.98×10^{-5} and 1.18×10^{-3}, respectively). However, the genes contributing to the enrichment differed between the clusters (Figure 3b). TGFß was the top regulator predicted for the HSC/MPP inflammatory response ($p=4.03 \times 10^{-15}$), and dysregulation of this subset of TGFß targets was restricted to HSC/MPP (Figure 3c). Together, these data implicate dysregulated TGFß signaling in HSC/MPP as a mechanism of BM failure in SDS patients.

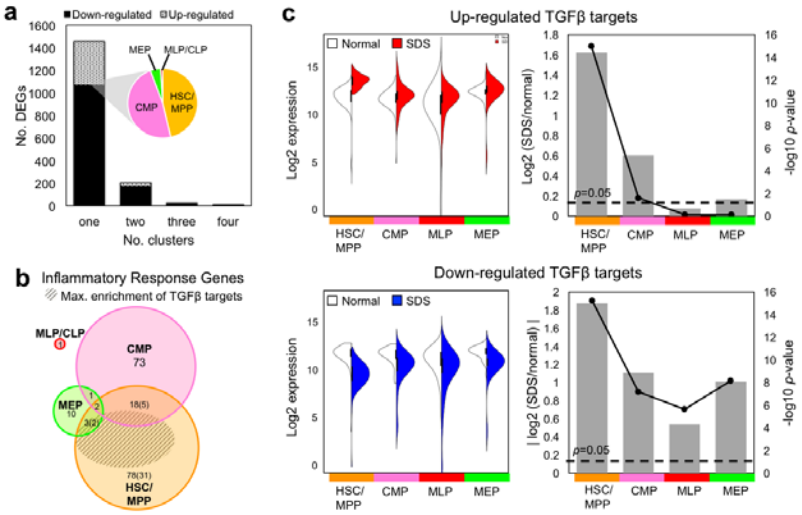

Figure 3. TGFb signaling is selectively dysregulated in SDS stem and multipotent progenitors. (a) Differentially expressed genes were identified among all SDS versus normal cells and within each cluster. To aid biological interpretation, this gene set was filtered to focus on genes with FDR adjusted p-value < .05 and log2(fold change) >|1| in at least one cluster. Plotted are the number of genes that were either up- or down-regulated in one, two, three or four clusters. GMP was excluded due to the paucity of SDS GMP. Inset pie chart shows the proportion of differentially expressed genes in each cluster. (b) Venn diagram of differentially expressed genes in each cluster that were annotated to the "Inflammatory Response" function in Ingenuity Pathway Analysis. The shaded region shows the area of maximal enrichment of TGFb targets ($p=4.03 \times 10^{-15}$). (c) Left: split violin for the summed expression of 25 upregulated TGFb targets and 52 down-regulated TGFb targets in SDS HSC/MPP. Right: Log2 fold changes (primary axis, bars) and *p*-values (secondary axis, lines) for the gene sets plotted in 'b'. Significance was determined by Holm-Sidak's multiple comparisons test.

Aim 2. TGFß induces context-dependent effects on cell growth, survival, inflammation, and extracellular matrix, among others. TGFß1 and TGFß3 have potent growth inhibitory effects on HSC[7,8], although this activity may vary among functional subsets[9]. To determine whether hyperactivation of TGFß signaling contributed to the functional impairment of HSC/MPP in SDS, we treated primary bone marrow mononuclear cells from normal donors and SDS patients with TGFß inhibitors. SD208 is an intracellular inhibitor that blocks signal transmission from TGFßR1[10]. AVID200 is a recently-developed extracellular inhibitor that functions as a TGFß1/3 receptor trap,; it is a promising therapeutic agent since it avoids cardiac toxicities associated with TGFß2 inhibition. Both SD208 and AVID200 improved hematopoietic colony formation in SDS patient samples, but not in normal donor controls (Figure 4a, b). Moreover, significant effects were observed for myeloid and erythroid colonies, suggesting that TGFß inhibition targets multipotent progenitors. These data support our computational prediction that hyperactivation of TGFß in HSC/MPP promotes bone marrow failure in SDS.

Hyperactivation of TGFß signaling may occur via increased expression of TGFß ligand(s), increased activation of latent TGFß ligands, or upregulation of the receptor. To begin to investigate these possibilities, we screened blood plasma from six SDS patients and six normal controls using SOMAscan; a highly-sensitive, aptamer-based proteomic platform[11]. The TGFß family ligands TGFß3 and GDF15 were significantly upregulated in SDS patient plasma, whereas TGFß1 or TGFß2 were unchanged (Figure 4c). BMP1, which activates latent TGFß in the extracellular matrix[12], was also upregulated as part of a larger network of dysregulated TGFß–associated factors in SDS patient plasma (Figure 4c). These data suggest that enhanced expression and activation of TGFß ligands could be a mechanism of HSC/MPP dysfunction in SDS. We are currently investigating the local source of TGFß in SDS bone marrow, which is likely to be a CD34- cell type given that

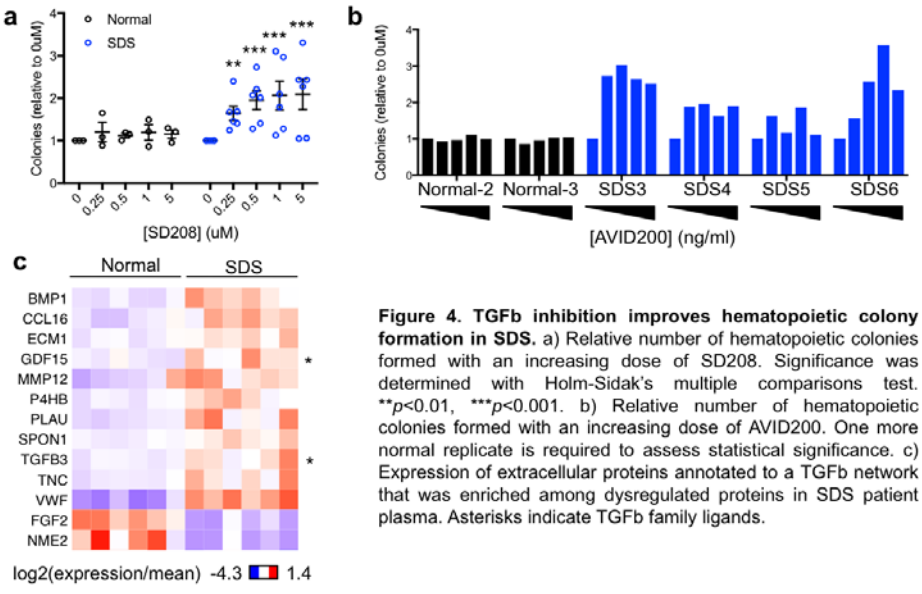

Figure 4. TGFb inhibition improves hematopoietic colony formation in SDS. a) Relative number of hematopoietic colonies formed with an increasing dose of SD208. Significance was determined with Holm-Sidak's multiple comparisons test. $^{**}p<0.01$, $^{***}p<0.001$. b) Relative number of hematopoietic colonies formed with an increasing dose of AVID200. One more normal replicate is required to assess statistical significance. c) Expression of extracellular proteins annotated to a TGFb network that was enriched among dysregulated proteins in SDS patient plasma. Asterisks indicate TGFb family ligands.

CD34+ HSPC did not express appreciable mRNA levels of TGFß ligands. Regardless, our data demonstrate that TGFß pathway components could be reliable diagnostic biomarkers for SDS.

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

Dr. Cailin Joyce, an Instructor in my lab, has attended three meetings related to this project: Single Cell Genomics (Utrecht, Netherlands, September 2015), the Harvard School of Public Health Program in Quantitative Genetics Annual Symposium (Boston, MA, November 2015), and the American Society of Hematology Annual Meeting (San Diego, CA, December 2016). For the latter, she was selected for an Abstract Achievement Award and oral presentation, and attended several trainee career development events. Dr. Joyce's contributions to this project and others as a postdoctoral fellow resulted in her promotion to Instructor as a first step in her transition to independence. She is currently revising and resubmitting her proposal to extend this work for a K01 through NIDDK.

How were the results disseminated to communities of interest?

Dr. Joyce has presented posters and given talks at national and international meetings, and has also given talks locally for the Department of Cancer Immunology and Virology (DFCI), the Division of Hematology/Oncology (Boston Children's Hospital), the Department of Microbiology and Immunobiology (HMS), and the Division of Immunology (HMS).

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

In the next reporting period, we will identify the key effectors downstream of TGFß signaling that promote bone marrow failure in SDS. The majority of this work will be done in primary CD34+ cells with SBDS knockdown. However, primary hematopoietic cells have a brief lifespan and yield little biomass in culture. If these factors are limiting, we will employ iPSC and mouse model systems that we have already established in collaboration with Dr. Akiko Shimamura (Children's Hospital Boston) and Dr. David Scadden (Massachusetts General Hospital). SDS iPSC are an especially promising model system in which to dissect the TGFß mechanism because the Shimamura lab has already demonstrated enhanced SMAD2/3 activation - the key signaling event downstream of TGFß - in these cells. Finally, we will assess whether miRNA regulation of the TGF pathway is perturbed in SDS, and if so, whether manipulation of miRNA expression and activity modulates SDS phenotypes.

Impact

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

Changes/Problems

Changes in approach and reasons for change
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
On 08-14-2017, we were granted a 12 month No Cost Extension to extend this project through 08-14-2018. This project was delayed due to a staffing issue. The original bioinformaticist (Dr. Lan Jiang) left partway through this project, and we had to transfer data and knowledge to his replacement (Dr. Assieh Saadatpour) last fall. Our final plans are to interrogate the mechanism(s) of action of TGFß inhibitors using CD34+ cells with SBDS knockdown and/or patient-derived iPSC. We expect that these additional data will strengthen our rationale for a clinical trial with TGFß inhibitors in SDS patients.

Changes that had a significant impact on expenditures
None of the changes described above will result in >25% change in budget allocation.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

Products

Publications, conference papers, and presentations

Joyce CE, Saadatpour A, Jiang L, Ruiz-Gutierrez M, Vargel Bolukbasi O, Hofmann I, Sieff CA, Nusbaum C, Shimamura A, Yuan GC, **Novina CD**. Single Cell Transcriptional Profiling Reveals Activation of TNF-alpha Signaling in Hematopoietic Stem and Progenitor Cells from Shwachman-Diamond Syndrome Patients, American Society of Hematology. Oral presentation, American Society for Hematology Annual Meeting, San Diego, CA, 2016.

Joyce CE, Li S, Hofmann I, Nusbaum C, Sieff C, Mason CE, **Novina CD**. "Single cell transcriptomic analysis of hematopoietic dysfunction in Shwachman-Diamond Syndrome". Poster, Keystone Hematopoiesis, Keystone, CO, 2015.

Joyce CE, Jiang L, Hofmann I, Nusbaum C, Sieff C, Yuan GC, **Novina CD**. "Lineage-restricted signatures of bone marrow failure in Shwachman-Diamond Syndrome revealed by single cell RNA sequencing of patient cells". Poster, Program in Quantitative Genetics Symposium, Harvard School of Public Health, 2015.

Journal publications
Nothing to report.

Books or other non-periodical, one-time publications
Nothing to report.

Other publications, conference papers, and presentations
Nothing to report.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
Nothing to report.

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
Nothing to report.

Participants and Other Collaborating Organizations

What individuals have worked on the project?

Name:	Carl Novina, M.D., Ph.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.0

Contribution to Project:	Dr. Novina is responsible for the conception and design of all the studies. He supervised their execution, analyzed data, prepared publications related to this work, and presented the findings to the scientific community.
Funding Support:	N/A

Name:	Cailin Joyce, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9.0
Contribution to Project:	Dr. Joyce has worked with Dr. Novina on the conception and design of all the studies. She obtained clinical samples, performed single cell RNA-sequencing, and worked collaboratively with computational biologists to analyze the data. She has prepared publications related to this work, and presented the findings to the scientific community.
Funding Support:	N/A

Name:	Dolly Thomas, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6.0
Contribution to Project:	Dr. Thomas performed colony formation assays, and worked on methods to purify and analyze hematopoietic progenitors from bone marrow.
Funding Support:	N/A

Name:	Elaine Yee
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.0
Contribution to Project:	Ms. Yee worked on methods to purify and characterize RNA.
Funding Support:	N/A

Name:	Dustin Griesemer
Project Role:	Student

Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6.0
Contribution to Project:	Mr. Griesemer worked on methods to purify and characterize RNA.
Funding Support:	N/A

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

Nothing to report.

What other organizations were involved as partners?

Organization Name: Boston Children's Hospital
Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Organization Name: Broad Institute of Harvard and MIT
Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Organization Name: Joslin Diabetes Center
Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Organization Name: Massachusetts General Hospital

Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
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- Other

Special Reporting Requirements

None