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Understanding the Delay in Onset of Paget's Disease of Bone

PRINCIPAL INVESTIGATOR:
Marc F. Hansen, Ph.D.

CONTRACTING ORGANIZATION: University of Connecticut
Farmington, CT 06032-1956

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6. AUTHOR(S)
Marc F. Hansen, Ph.D.

E-Mail:mhansen@uchc.edu

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263 Farmington Avenue
Farmington, CT 06032-1956

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14. ABSTRACT

One of the key questions in Paget's disease of bone (PDB) is the nature of the "trigger" for initiation of the disease. Inheritance of a predisposing mutation in the Sequestosome 1 (SQSTM1) gene and childhood infection with Measles virus are critical, but how this leads to initiation of the disease only after a significant period of time has passed, is unclear. Understanding the genetics underlying this disease process and how the predisposing mutation interacts with the measles virus genome is the goal of this proposal. We have developed a model to compare the gene sets from significantly regulated genes affected by the measles virus genome expression in the context of the SQSTM1 mutation in human osteoblasts and carried out RNA-seq analysis on this model.

15. SUBJECT TERMS
Paget's disease of bone, Measles virus, osteoclast, osteoblast, disease initiation, latent viral infection, *Sequestosome 1,* Measles V gene, Measles Nucleocapsid gene

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Table of Contents

1. Introduction

The long-term goal of this project is to understand how Paget's Disease of Bone (PDB) begins and the role of measles virus in that initiation. PDB can be inherited in families and several genes have been linked to PDB including *Sequestosome 1* (*SQSTM1*), which has been linked to 40% of familial PDB[1]. Measles virus infection has also been linked to PDB in a substantial fraction of cases[2, 3]. One of the key questions in PDB is the nature of the "trigger" for initiation of the disease[4]. As an illustrative example, individuals in a family with familial PDB inherit a germline mutation in the *SQSTM1* gene that predisposes those individuals to PDB. A predisposed individual contracts measles at age 8, which is the only other known factor for PDB. However, the disease does not reveal itself until that individual reaches age 50. What has occurred in the intervening time period that was not present at age 8 is the basis for this proposal. Our model involves the genes of the measles virus genome. The measles virus genome contains a number of genes. Two of these genes, the MVNP protein and the MVV gene appear to act in opposing fashion to control gene expression in an infected cell[2, 5-9]. The MVV gene suppresses gene expression as part of the virus' ability to evade immunological surveillance and promote persistent infection[5, 6]. In contrast, the MVNP gene appears to activate gene expression to promote acute infection[10-12]. Our hypothesis is that when the measles virus infects a bone cell, the MVV gene creates a latent infection state by suppressing gene expression in the cell. Then, over the years, a chance genetic event in a single bone cell containing the measles virus results in the loss of the MVV gene. At this time, the MVNP gene is unmasked and cooperates with the mutated *SQSTM1* to initiate the exaggerated pattern of bone cell growth characteristic of PDB.

2. Keywords

Paget's disease of bone, Measles virus, osteoclast, osteoblast, disease initiation, latent viral infection, *Sequestosome 1,* Measles *V* gene, Measles *Nucleocapsid* gene

3. Accomplishments

What were the major goals of the project?

To test our model, we proposed two specific aims.

Specific Aim 1: Determine whether loss of expression of MVV in the presence of MVNP leads to a pagetic phenotype.

Specific Aim 2: Screen matched normal and pagetic bone samples for evidence of MVV and MVNP and demonstrate that the MVV present in the pagetic samples is mutated in a way that causes inactivation of the MVV gene product

In the first aim, we proposed to introduce both the MVV and MVNP genes into bone cells in culture and then use siRNA technology to turn off the MVV gene and observe the change in the growing bone cells to see if it mimics PDB. Our Statement of Work proposed that we would clone the MVV gene from the measles' genome and co-transfect it together with the MVNP gene into pre-osteoclasts. We would then selectively inhibit MVV and MVNP by siRNA and examine the phenotype of the cells.

In the second aim, we proposed to examine affected bone tissue from patients with PDB to see if the affected bone has evidence of mutation of the MVV gene while maintaining the MVNP gene. Our Statement of Work proposed that we would use laser capture microdissection (LCM) to capture pagetic cells and then analyze RNA from these cells for expression of MVV and MVNP and mutation in MVV.

What was accomplished under these goals?

As noted in our 2014 Annual Summary Report, almost immediately, we ran into problems with the MVV gene. As is shown in Figure 1, the MVV gene and the MVP genes are encoded by a common sequence in the measles virus genome in which both use the ATG start site at

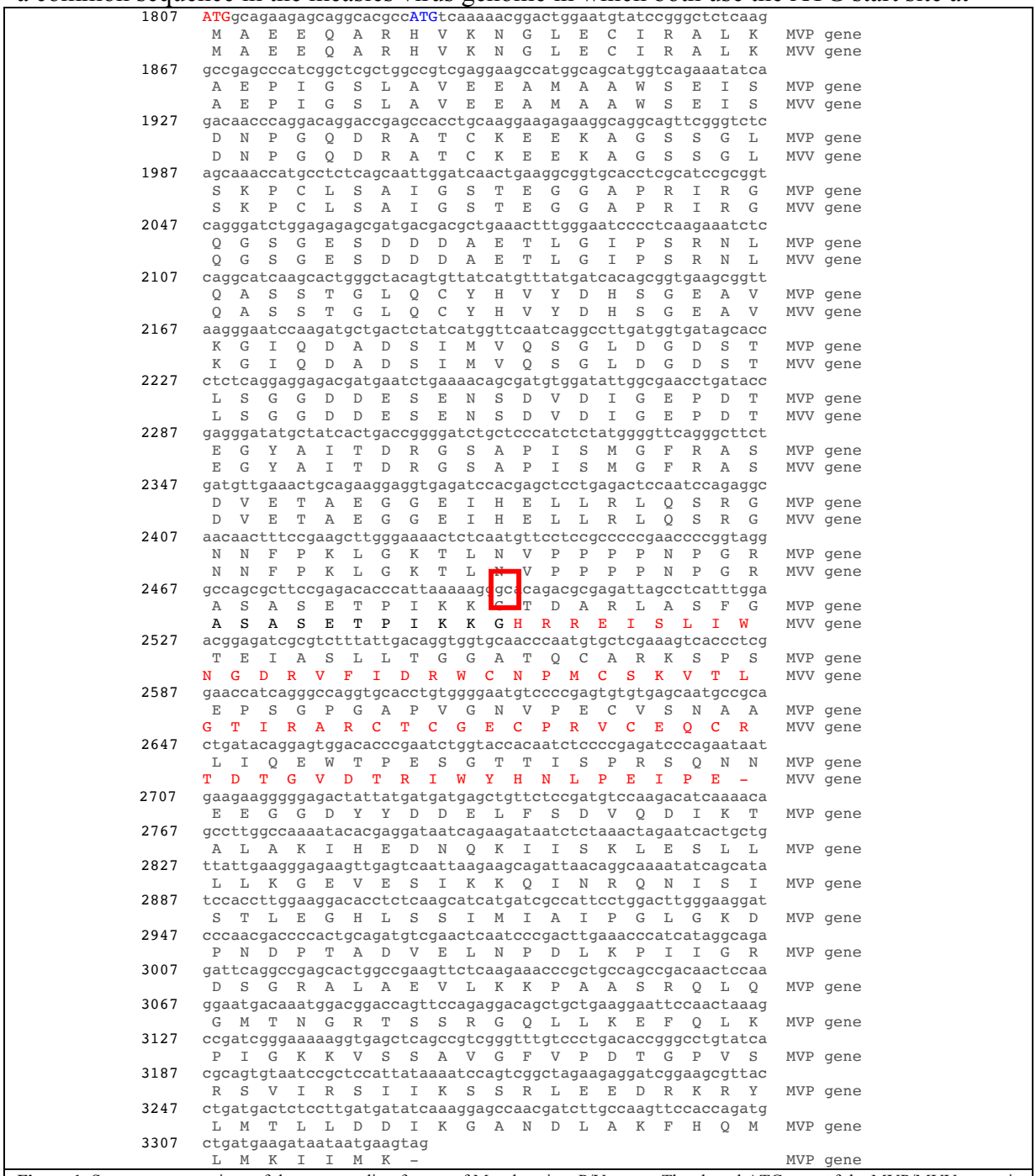

Figure 1. Sequence comparison of the open reading frames of Measles virus P/V genes. The shared ATG start of the MVP/MVV genes is shown in red. The shared amino acid sequence of MVP/MVV is shown in black. The unique amino acid sequence of MVV, caused by a non-templated G insertion into the sequence at nucleotide 2499 (site of insertion is boxed in red, inserted base not shown), is shown in red. The start ATG for MVC and the unique amino acid sequence are shown in blue. Sequence from GenBank NC_001498.1.

nucleotide 1807 and from 1807 to 2498 show a common translated amino acid sequence. The two sequences diverge due to a non-templated insertion of a G nucleotide at nucleotide 2499 in

the MVV sequence resulting in a frameshift and altered reading resulting in a unique amino acid sequence for MVP and MVV from that point on. Our attempts at cloning this sequence of the MVV gene from the measles' genome using a PCR-based strategy in which we assemble the MVV gene from the measles virus genomic RNA using overlapping primer sets were ultimately unsuccessful.

As our major objectives for this last year of the grant, we proposed two alternative strategies for this aim: The first was to adopt a two-step procedure where a truncated MVP gene sequence (approximately nucleotides 1800 - 3000) was amplified by PCR and cloned into a vector and then the non-templated MVV G nucleotide at 2499 would be inserted into the cloned MVP gene sequence by a second PCR reaction or restriction reaction. Our second alternative was to isolate the MVV RNA from cells infected with the intact Measles virus and then use that RNA as template to clone the MVV gene. Unfortunately, both of those strategies also ultimately failed. We were unable to clone the MVV gene from the measles genome.

With regard to our second objective, in which we proposed to examine the RNA from pagetic cells, we were able to capture cells by LCM, however, we were unable to distinguish amplification of the MVV gene from the MVP gene as the primer sets that we were able to develop for PCR amplification did not include the region containing the non-templated G nucleotide.

Therefore, as an attempt to salvage the ultimate goal of the project, which was to understand the effect of the measles' genome on the initiation of the pagetic phenotype, we altered the focus of the work towards a new approach, which was an *in vitro* analysis to compare cells in which both the *SQSTM1* mutation and the measles' genome were already present with those of a normal human cell into which the *SQSTM1* mutation was introduced without the accompanying measles' genome and to then examine the genomic signatures of the two cell lines by RNA-seq analysis to see what genes were differentially expressed in the two cells. We could then also expose these two cell lines to environmental triggers (LPS and Vitamin D3) and see what genes were differentially expressed in the cells carrying both the *SQSTM1* mutation and the measles virus genome compared to those that carried the *SQSTM1* mutation alone.

Cell	$SQSTM1^{P392L}$	Measles virus genome	LPS/Vitamin D
hFOB1.19	–	–	–
hFOB1.19	–	–	+
hFOB1.19	+	–	–
hFOB1.19	+	–	+
PSV10	+	+	–
PSV10	+	+	+

Table 1. Experimental conditions for samples collected for RNA-seq analysis.

The experimental approach is outlined in Table 1. For our normal human cell carrying the *SQSTM1* mutation, we stably introduced the $SQSTM1^{P392L}$ predisposing mutation into the human fetal osteoblast cell line hFOB1.19[13] under the CMV promoter. We have found that the hFOB cell line approximates the genomic expression pattern of normal primary human osteoblasts without the difficulty of using primary human cell cultures. For our pagetic osteoblast, we used the PSV10 osteoblast cell line isolated from a patient with hereditary Paget's disease[14] that is positive for measles virus genome. We treated these two cell lines (and the hFOB cells without the SQSTM1 mutation as a control) with bacterial lipopolysaccharide (LPS) and Vitamin D to simulate the environmental trigger to initiates Paget's disease of bone. We then isolated RNA from the treated and untreated cells and subjected them to whole genome RNA-seq[15, 16].

The RNA-seq was performed by the Beth Israel-Deaconess Genomics, Proteomics, Bioinformatics, and Systems Biology Center and DF/HCC Cancer Proteomics Core (Towia Liberman, Director). The RNAs were analyzed for quality control by Agilent Bio-analyzer and

then the sequencing libraries were prepared using Qiagen GeneRead Library Prep kits (GeneRead DNA Library I Core Kit, GeneRead DNA I Amp Kit and GeneRead Adapter I Set 1-plex). The samples were barcoded to allow multiplexing. The libraries were then sequenced using an Illumina HiSeq2500 Next Generation sequencing system and 20 million single-pair reads were performed for each sample. The sequencing data was then returned to us as FASTQ files. This happened on 15 September 2015. Using Bowtie2, a software package commonly used for sequence alignment and sequence analysis in bioinformatics[17] combined with TopHat, Cufflinks and CummeRbund software packages, we were able to generate a genetic signature from this comparison of genes that were turned on or turned off in the presence of the measles' genome or in the presence of the *SQSTM1* mutation. This genomic signature is shown in Table 2.

Upregulated linked to Measles	Downregulated linked to Measles	Upregulated linked to mutant SQSTM1	Downregulated linked to mutant SQSTM1
Gene Name	Gene Name	Gene Name	Gene Name
BCAT1	BCL2A1	ARPP21	FBN2
CNR1	CDK18	CCL3	
CSTA	CKB	CCL5	
CXCL5	ERG	CD22	
DCLK1	FBXO44	GRAP2	
DGKD	GNG11	IFNL1	
DLG1	IFNL2	PCSK6	
EDIL3	IFNL3	SERPINB10	
EFCAB4B	IGFBP6	SERPINB2	
EFEMP1	IL11	SPINK1	
FAM129B	IRAK2	TMEM100	
FAM171A1	KLF4		
FHL1	MAPK10		
FOXF2	PAX8		
MYO10	PTGS2		
NLGN4Y	SALL1		
PCDHB2	SAT1		
PCDHB3	VGF		
PCDHB5	ZBP1		
PREX1			
SATB1			
SETD7			
SH3BP4			
SIPA1L2			
SMG1			
SUSD5			

Table 2. Preliminary genomic signature of effect of measles virus genome and mutant SQSTM1 on gene expression.

We are presently validating the genomic signature qPCR and analyzing it for insights into altered genetic pathways. One example of the types of genetic interaction information that can be gleaned from this work is shown in Figure 2. The genes that are upregulated in response to the measles virus genome were loaded into the GeneMania web interface (http://www.genemania.org) and analyzed for genetic interactions. The green lines represent known genetic interactions between these genes.

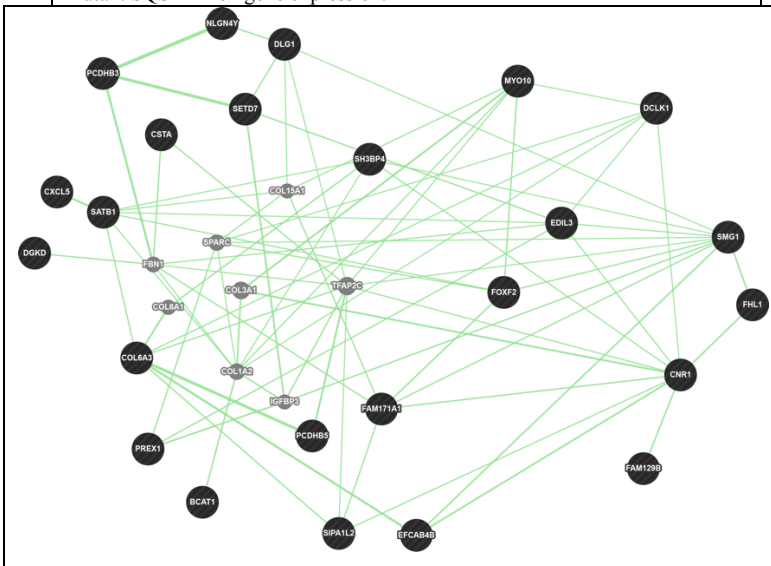

Figure 2. Pathway analysis of the genes whose upregulation is linked to the measles' virus genome. Dark circles are the genes in the genomic signature, the gray circles represent links in the genetic pathway. From GeneMania (http://www.genemania.org). © University of Toronto 2016

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

Nothing to report

How were the results disseminated to communities of interest?

Upon completion of our validation of the genomic signature, we intend to submit a

manuscript on the results of out comparison. At that time, we will submit the RNA-seq data to the NCBI GEO database so that it will be available to the research community.

What do you plan to do next?

This is the final report – therefore there is nothing to report. We plan to complete the validation of the genomic signature and publish that signature as well as the complete dataset.

4. Impact

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

At this stage, we are still completing the validation of the genomic signatures to allow us to write manuscripts about the data generated. Therefore nothing to report. Once the signature is validated and published, it should have a significant impact on the field of Paget's disease.

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

5. Changes/Problems

Changes in approach and reasons for change

As noted above, we were unable to clone the MVV gene or detect its expression in the pagetic samples. We therefore changed the experimental plan to examine the effects of the measles' genome in the context of a mutant *SQSTM1* gene allele in an *in vitro* approach in which cells from a familial Paget's disease patient that already had the measles genome and a mutant *SQSTM1* allele present in the cell were compared to cells that had only a mutant *SQSTM1* allele. The change in approach is described in the Accomplished Goals section above. The goal was to develop a genomic signature that would allow us to determine what genetic pathways are altered by the expression of the measles virus genome in these cells.

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

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

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

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals.
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

6. Products

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

We have obtained a preliminary genomic signature for cells containing the measles virus genome and a mutant *SQSTM1* allele as well as a genomic signature of cells with a mutant *SQSTM1* allele. Once this is validated, it will be submitted for publication and the genomic sequencing data submitted to the NCBI GEO database.

7. Participants and Other collaborating organizations

What individuals have worked on the project?

Name	Marc F. Hansen
Project Role	PI
Person Months worked	1.2 (no change from last funding period)
Contribution to Project	Overall supervision of project, data analysis, manuscript preparation
Additional funding support beyond this grant	University of Connecticut Health Center
Name	Michael Mogass
Project Role	Postdoctoral Fellow
Person Months worked	3.0 (no change from last funding period)
Contribution to Project	Dr. Mogass performed cell culture and transfections and RNA preparation for sequencing also assisted with data analysis and manuscript preparation
Additional funding support beyond this grant	University of Connecticut Health Center
Name	Cindy Alander

Project Role	Research Assistant
Person Months worked	2.4 (no change from last funding period)
Contribution to Project	Ms Alander performed routine cell culture and RNA preparation as well as stock maintenance
Additional funding support beyond this grant	University of Connecticut Health Center

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?

No other organizations were involved as partners. The RNA-seq analysis was done as work for hire by the Beth Israel-Deaconess Genomics, Proteomics, Bioinformatics, and Systems Biology Center and DF/HCC Cancer Proteomics Core (Towia Liberman, Director).

8. Special Reporting Requirements

Not applicable

10. Appendices

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