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14. ABSTRACT The purpose of this grant was to examine the mechanism by which *Sequestosome 1* (*SQSTM1*) mutations initiate the sporadic form of Paget's Disease of Bone (PDB). We found that somatic *SQSTM1* mutations in the osteoblasts of pagetic bone play a critical role in PDB formation. We isolated osteoblastic cells from pagetic lesions and analyzed their expression profiles using microarrays. From this, we developed a model in which mutant *SQSTM1* protein interacts with the MyD88 gene product to activate NF-κB via the Toll-like Receptor signaling pathway to activate chemokine signaling. We confirmed our results in an osteoblastic cell line that had a *SQSTM1* mutation that was isolated from a PDB patient and again when we introduced a *SQSTM1* mutation into a osteoblastic cell line wildtype for *SQSTM1*. This suggests that while regulation of osteoclastogenesis is a key process in PDB pathogenesis, osteoblastic cells containing *SQSTM1* mutations play critical role in the initiation and progression of the disease. This is paradigm-shifting as it suggests a multi-cellular model of PDB where both osteoblastic and osteoclastic cells play a role in the initiation and progression of the disease. This research opens new avenues for treatment rather than the current bisphosphonate therapies.

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Introduction

The purpose of this grant was to examine the mechanism by which Paget's Disease of bone (PDB) originated and the genetic events that took place in the affected bone of patients with the sporadic form of the disease. This was in contrast to earlier studies of PDB that had focused on the rarer, familial form of the disease. We wanted to understand how *Sequestosome 1* (*SQSTM1*) mutations, which have been linked to the familial disease could act to drive the formation of the pagetic bone in the sporadic form of the disease. We found that somatic *SQSTM1* mutations occurred in the osteoblasts in the pagetic bone and that these osteoblasts play a significant role in PDB formation. Using Laser Capture Microdissection to isolate homogeneous populations of mononuclear cells from the pagetic lesions, we found that the mutant-containing captured cells did not express osteoclast (OC)-specific genes but did express osteoblast (OB)-specific genes suggesting that the $SQSTM1^{mut}$–containing cells were of OB rather than OC origin. Moreover, when we performed pathway analysis on the expression data from these pagetic samples, we developed a model in which the mutant *SQSTM1* protein interacts with the MyD88 gene product to activate NF-κB via alteration of Toll-like Receptor signaling pathway and regulate chemokine and cytokine signaling. We found similar results when we compared cells from an OB cell line from a pagetic patient that had a *SQSTM1* mutation to cells from a normal OB cell line, and again when we introduced a *SQSTM1* mutation into a normal OB cell line. This suggests that while the regulation of osteoclastogenesis is a key process in PDB pathogenesis, OB cells play an important role in the initiation and progression of the disease as well. This discovery is paradigm-shifting as our evidence suggests a multi-cellular model of PDB in which both the OB and OC cells have a role in the initiation of the disease. PDB represents an example of a disease in which coupled bone remodeling occurs. If true, then this research could open new avenues for treatment rather than relying on the current non-specific bisphosphonates therapy.

Body: *(From Statement of Work)*

Specific Aim 1: ***To demonstrate the presence and nature of the subset of critical cells within the pagetic bone***

I. Laser Capture Microdissection (LCM) and RNA isolation

We took samples of pagetic bone identified by a pathologist (Figure 1) and prepared

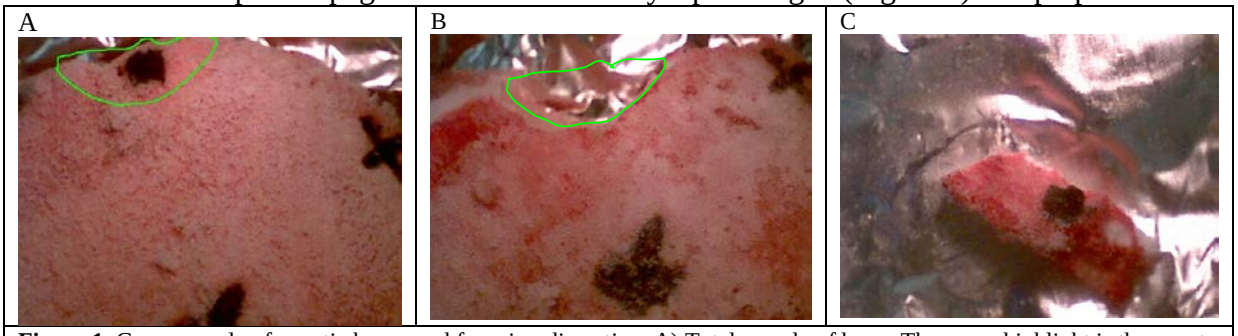

Figure 1. Gross sample of pagetic bone used for microdissection. A) Total sample of bone. The green highlight is the area to be excised for microdissection. B) The bone after the sample has been excised. C) The excised piece of bone.

them for LCM. Briefly, the procedure was as follows: 5μm thick serial sections were cut from each embedded bone sample and placed on clean glass slides. As in all procedures in which PCR amplification was used, careful attention was paid during sectioning and mounting to prevent carryover contamination of one specimen to another or transfer of material from one region of a section to another that could lead to spurious results. The microtome and cryostat used to cut sections were kept clean and excess embedding compound and tissue fragments were wiped

from the area with xylene between each block. A fresh cutting blade was used for each sample. Each section from which captured cells were to be taken was mounted on an uncharged/uncoated glass slide and H&E-stained following a modified procedure recommended by the manufacturer. The slides were not coverslipped prior to capture. The LCM was done using a PixCell IIe Laser Capture Microdissector. An example of the LCM capture is shown in Figure 2.

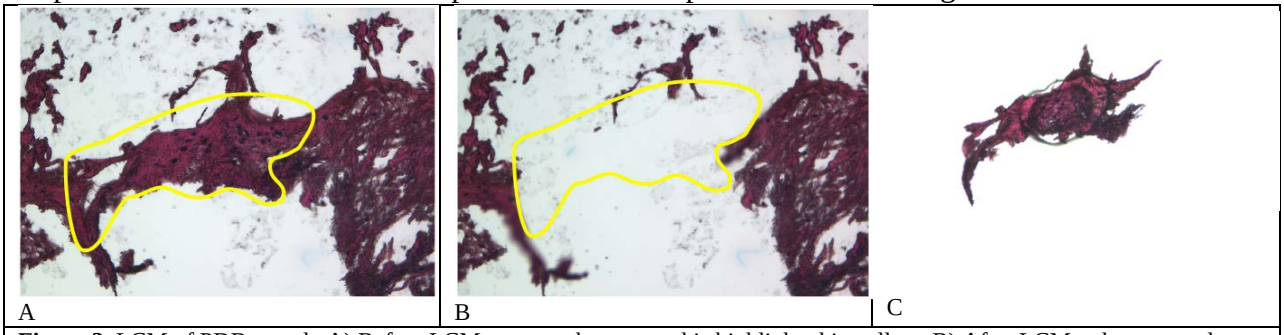

Figure 2. LCM of PDB sample A) Before LCM – area to be captured is highlighted in yellow. B) After LCM – the captured area is highlighted in yellow. C) The LCM captured material.

During the LCM procedure, the tissue sample was viewed on a video monitor. A joystick was used to move the microscope stage to locate the cells of interest and to position the film carrier over the cells. A low power infrared laser was then activated and the desired cells were adhered to the film carrier. Approximately 1000-5000 mononuclear cells were captured from a total of 1-5 serial sections. Following LCM, the film carrier carrying the captured cells was lifted off the tissue surface to remove the captured cells. The film carrier was then placed directly onto a standard microcentrifuge tube and RNA isolated from the LCM-captured material. RNA isolation was done using the Roche High Pure RNA Paraffin kit as recommended by the manufacturer. Quantitation of the RNA was done using a NanoDrop spectrophotometer.

II. Allele-specific in situ PCR amplification to identify cells carrying a mutant allele of *SQSTM1*

We had planned to use a modification of the SNaPshot® Multiplex kit (Applied Biosystems, Inc.) that adapts the SNaPshot protocol to an in situ PCR-based protocol. The idea was that the combination of SNaPshot and in situ PCR would fluorescently mark individual cells within the affected bone that carry a mutant allele of *SQSTM1*, which would allow us to assess the frequency and distribution of $SQSTM1^{mutant}$-containing cells within the pagetic lesion. However, after extensive testing, we were unable to reproducibly adapt the SNaPshot protocol to the FFPE specimens. The background noise was too great to reliably detect the mutant-containing cells. We therefore focused our efforts on signaling molecules in the pagetic bone that would recruit normal bone cells to the growing lesion.

Specific Aim 2. ***To determine whether a subset of cells containing the SQSTM1 mutation can affect the overall nature of bone cell cultures containing cells with and without the mutations.***

We wanted to determine whether the presence of the *SQSTM1* mutation affected the overall nature of the pagetic bone. To do that, we took RNA isolated from the LCM-captured cells of the pagetic bone with and without *SQSTM1* mutations and analyzed it by microarray. The isolated RNA was amplified using the NuGen WT-Ovation FFPE kit, which is a whole transcriptome amplification system designed to allow global gene expression analysis on small and/or degraded total RNA isolated from FFPE samples. 50ng of RNA from each sample was used as an input into the amplification protocol. Each sample was amplified in quadruplicate. The resulting amplified cDNAs were tested for quality by qRT-PCR using 18S RNA and β-actin primers.

(Figure 3).

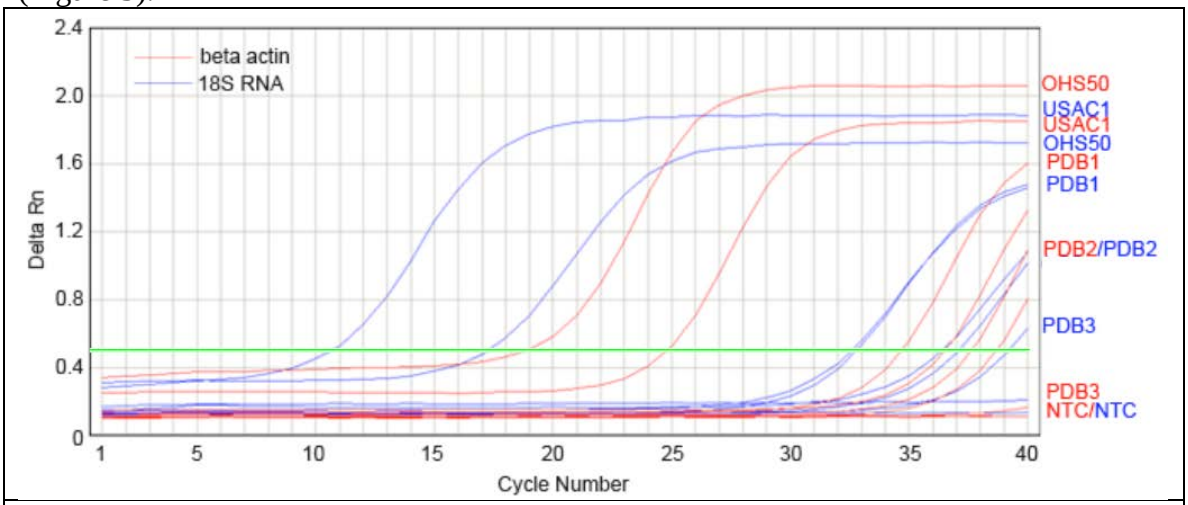

Figure 3. qPCR analysis of LCM-captured and amplified cRNA samples from PDB samples. RNA isolated from two cell lines, OHS50 and USAC1, acted as positive controls. NTC – no template controls. Three samples of PDB (PDB1, 2 and 3) were analyzed. The green line indicates the threshold for positive amplification (Ct).

The amplified cDNA was then labeled according the NuGen Illumina Solution Protocol Application Note #2 and final concentration of the labeled cDNA was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality of the cDNA was tested using a Model 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) (Figure 4).

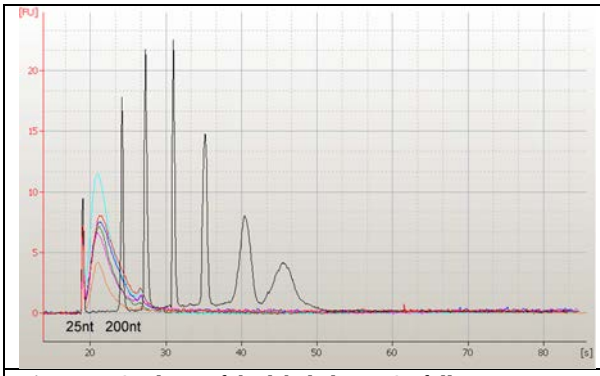

Figure 4. Analysis of the labeled cDNAs following fragmentation and labeling of the amplified cRNA obtained from the LCM-captured PDB samples. The black peaks are the sizing ladder with sizes shown below the graph. As can be seen, all of the samples produced labeled cDNA material of the appropriate size for use in our microarray analysis.

Hybridization to the Illumina Human Ref-8v2 Genome-Wide Expression BeadChip Array was done in the UCHC Translational Genomics Core. The arrays were hybridized, washed and scanned according to the Illumina protocol except that hybridization was done at 48°C because of the altered hybridization kinetics of cDNA/DNA pairs rather than cRNA/DNA pairs. Correlation coefficient of the replicates was typically ≥ 0.98.

The raw hybridization data was normalized using quantile normalization using the software package Bioconductor by Affymetrix (Affymetrix Inc,; Santa Clara, CA). The normalized data was analyzed using the BeadStudio software (Illumina) packages for statistical analysis and hierarchical clustering analysis. Quality control filtering was done using the detection p-values. Differentially expressed genes were identified using a two-sample t-test using False Discovery Rate for multiple test correction and the resulting data was analyzed by hierarchical cluster analysis similarities calculated using the Pearson correlation similarity metric and expressed as a dendrogram. We found that the samples that had somatic *SQSTM1* mutations clustered discretely from those that had no detectable *SQSTM1* mutations (Figure 5).

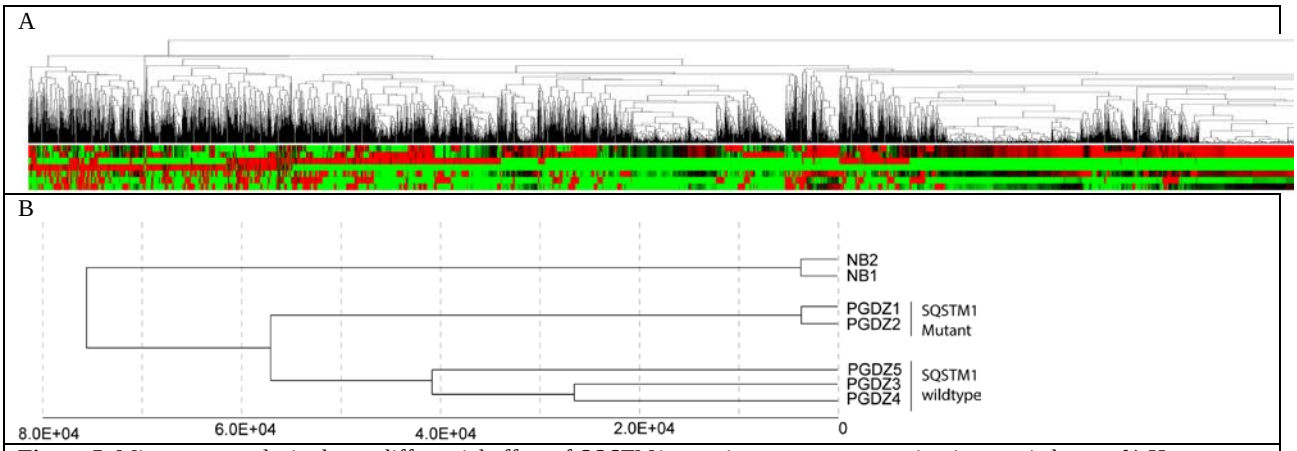

Figure 5. Microarray analysis shows differential effect of *SQSTM1* mutation on gene expression in pagetic bone. **A)** Heat map of expression data of the 332 most significant genes from the Illumina Human Ref-8v2 hybridization analysis. From top to bottom, this dendrogram shows 2 normal bone samples and 5 pagetic bone samples. **B)** Dendrogram showing that the pagetic samples containing the *SQSTM1* mutations cluster distinctly from the pagetic samples with wildtype *SQSTM1*. NB – normal bone

Differential expression of chemokines in PDB samples with SQSTM1 mutations relative to those with wildtype SQSTM1: We found that *RANKL* was upregulated in all PDB bone samples regardless of *SQSTM1* mutation status (Figure 6). This is consistent with previous reports that *SQSTM1*^{P392L} – carrying cells express higher levels of RANKL than normal cells in response to stimulation by 1,25-$(OH)_2D_3$ (1). We also found that OPG, an inhibitor of RANK signaling, was downregulated in the *SQSTM1*^{P392L} mutant samples while RANK expression was slightly upregulated in the *SQSTM1*^{P392L} mutant samples (Figure 6). We found several other chemokines that were upregulated in the *SQSTM1*^{P392L} bone samples including known OC signaling molecules *CCL2* (MCP1), *CCL3* (MIP1A), *CCL5* (RANTES), *CCL20* (MIP3A) (2, 3). We also found *CXCL6*, a gene implicated in inflammation and wound healing (4) was upregulated (Figure 6).

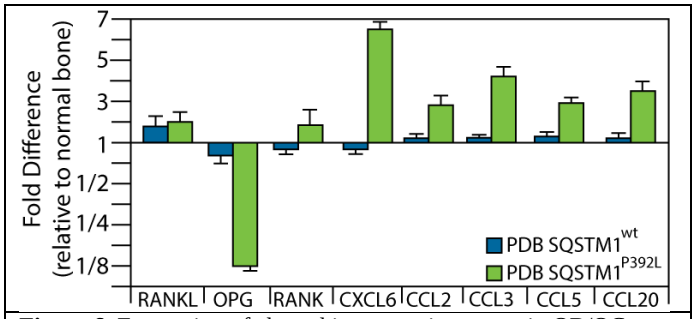

Figure 6. Expression of chemokine genes important in OB/OC signaling is elevated in PDB samples with *SQSTM1* mutations. Expression normalized to normal bone.

Comparison of expression patterns of an OB cell line carrying a SQSTM1 mutation with an OB cell line carrying wildtype SQSTM1: Menaa et al. (5) isolated and immortalized a marrow stromal cell line from a pagetic lesion (PSV10). They showed that RANKL mRNA was increased in the PSV10 cells compared to a normal stromal cell line or normal marrow from uninvolved bones from PDB (5). We obtained the PSV10 cell line from Dr. G. David Roodman (Indiana University School of Medicine) and tested the cells for a *SQSTM1* mutation. As shown in Figure 7, the PSV10 cell line has a heterozygous *SQSTM1* P387L mutation.

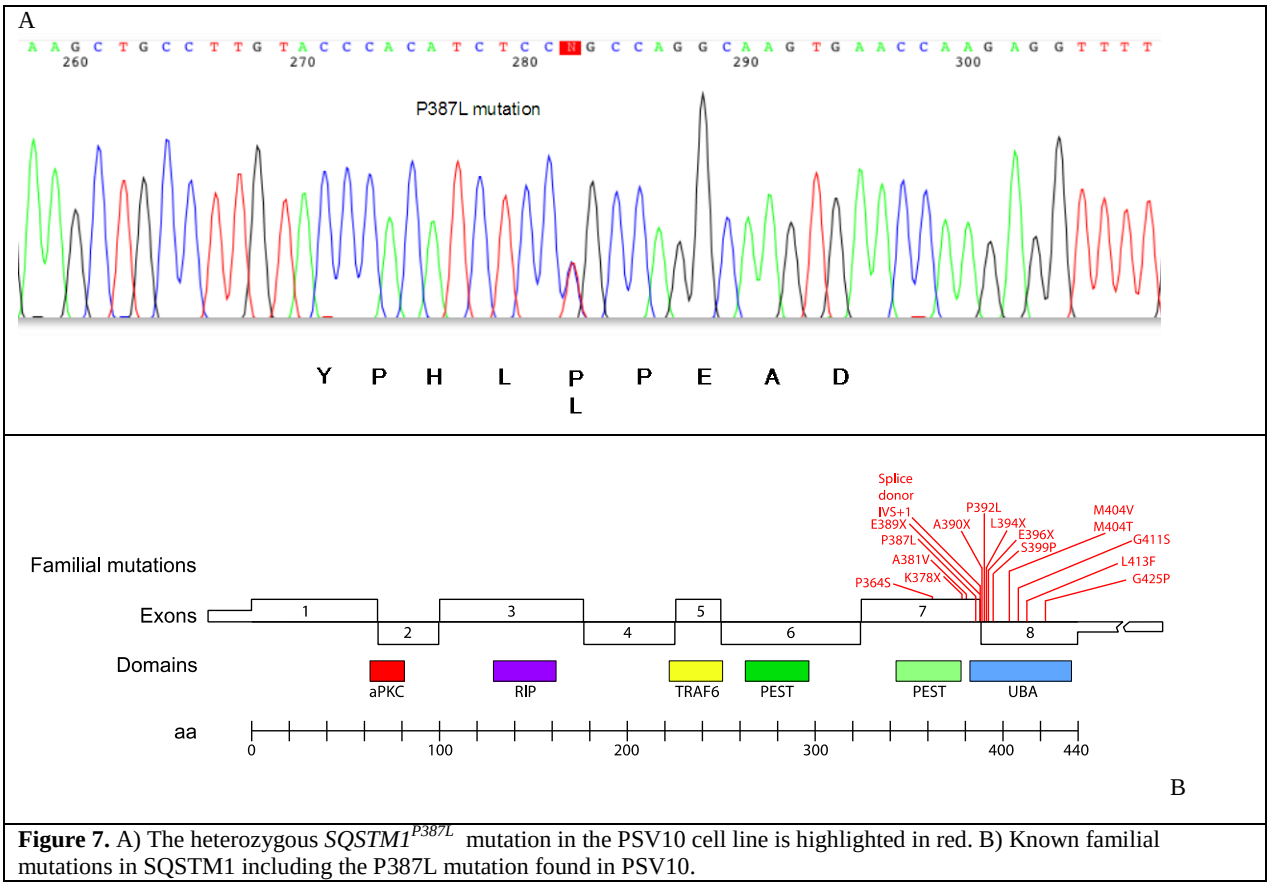

Figure 7. A) The heterozygous *SQSTM1*^{P387L} mutation in the PSV10 cell line is highlighted in red. B) Known familial mutations in SQSTM1 including the P387L mutation found in PSV10.

This mutation has been previously reported in PDB families (6-9) and appears to behave similarly to other PDB *SQSTM1* mutations (10). We confirmed by qRT-PCR that the PSV10 cell line was of OB origin and expressed OB lineage-specific genes *BGLAP* and *COL1A1* (*data not shown*). We then examined the expression of the chemokine genes that were found to be elevated in the primary PDB samples in the PSV10 cell line and compared them with those found in an OB cell line with wildtype *SQSTM1*, hFOB1.19 cells (hFOB, ATCC) which is an immortalized pre-osteoblast cell line that carries a temperature sensitive mutation (tsA58) of SV40 T-antigen that is permissive for proliferation at 33.5°C. When the culture temperature is raised to the restrictive temperature of 39.5°C, the cells differentiate along the OB lineage and mineralize in approximately 14 days (11). We tested and confirmed that hFOB cells have a wildtype *SQSTM1* gene (*data not shown*). We then grew the PSV10 and hFOB cells with and without 10^{-8}M 1,25-$(OH)_2D_3$ and assayed the cells for expression of a variety of chemokines by quantitative RealTime PCR (qRT PCR) as well as by PCR arrays (SA Biogen SuperArrays). The results are shown in Figure 8. Following stimulation by 1,25-$(OH)_2D_3$, we found that PSV10 showed upregulation of many of the same chemokines that had also been shown to be upregulated in our analysis of the *SQSTM1*^{P392L} PDB bone samples. These included *RANKL*, *CXCL6*, *CCL2*, *CCL3*, *CCL5*, and *CCL20*. We also found that the PSV10 cells, like the *SQSTM1*^{P392L} PDB bone samples, downregulated *OPG* in response to 1,25-$(OH)_2D_3$.

Introduction of the SQSTM1 mutation into hFOB cells and upregulation of chemokine genes: We next tested whether the increased expression of these chemokines was the result of the *SQSTM1*^{P392L} mutation, by introducing a *SQSTM1*^{P392L} mutation into the hFOB cells. We introduced the P392L mutation into the full-length human *SQSTM1* cDNA clone (GenBank ID:

BC001874.1, IMAGE:3535436) by PCR mutagenesis and then cloned both the mutant and wildtype *SQSTM1* cDNA into a lentiviral vector under the control of the constitutive EF1α promoter (pPS-EF1-LCS-T2A; Systems Biosciences). The incorporation of a "self-cleaving" T2A followed by an EGFP fluorescent marker allowed us to monitor transfection efficiency by immunofluorescence or by FACS analysis in the UCHC Flow Cytometry Core Facility. We transiently transfected the mutant and wildtype *SQSTM1* clones into hFOB cells and treated the cells with 10^{-8}M 1,25-$(OH)_2D_3$ and assayed for expression of the chemokines that we had found to be upregulated in the PSV10 cell line by qRT PCR. As shown in Figure 9, we were able to show in the *SQSTM1*^{P392L} –transfected hFOB cells the same increased expression of the chemokines that was seen in the *SQSTM1* mutant pagetic bone and PSV10 cell lines.

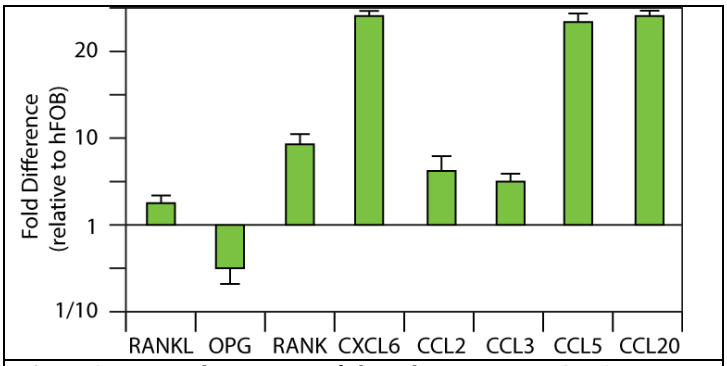

Figure 8. Increased expression of chemokine genes in PSV10 following stimulation with 10^{-8}M 1,25-$(OH)_2D_3$ for 1 hour prior to harvesting. Expression was normalized to untreated cells and then presented as the fold difference between PSV10 expression and hFOB expression.

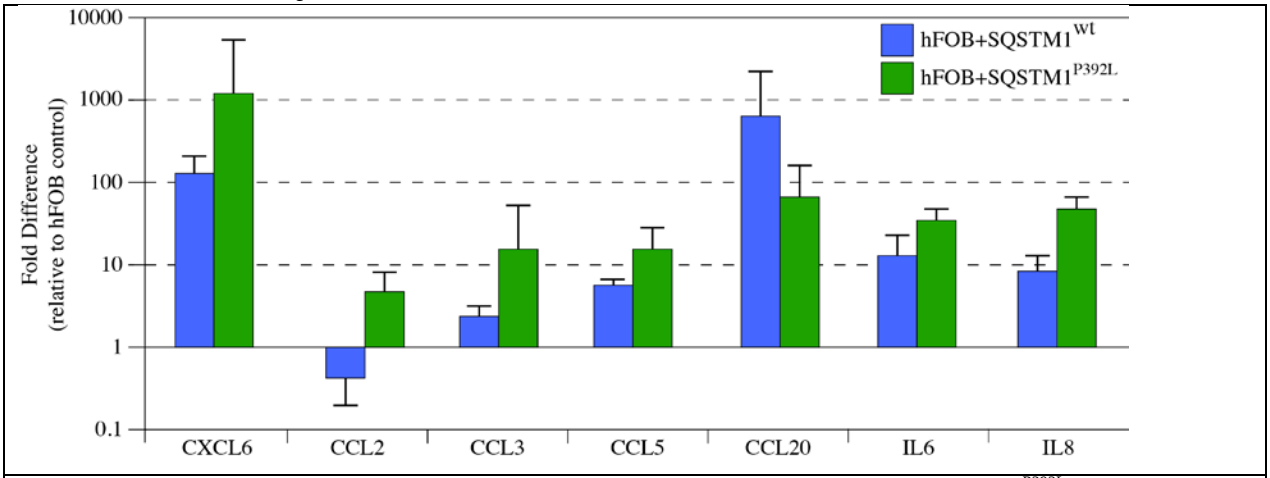

Figure 9. Expression of chemokine genes in hFOB transfected with virus expressing either the *SQSTM1*^{P392L} mutation or the *SQSTM1*^{wildtype}. Expression was measured following stimulation with 10^{-8}M 1,25-$(OH)_2D_3$ for 1 hour prior to harvesting.normalized to untransfected hFOB cells and presented as the fold difference relative to the control.

The SQSTM1 mutation results in activated MyD88-dependent Toll-like receptor signaling: We examined different signaling pathways to see which pathways were differentially affected based on the *SQSTM1* status. We found that downstream targets and members of the MyD88-dependent Toll-like Receptor (TLR) signaling pathway were altered (Figure 10). Significantly, the MyD88-dependent TLR signaling pathway regulates CCL2, CCL3 and CCL5 as well as IL6 and IL8 expression.

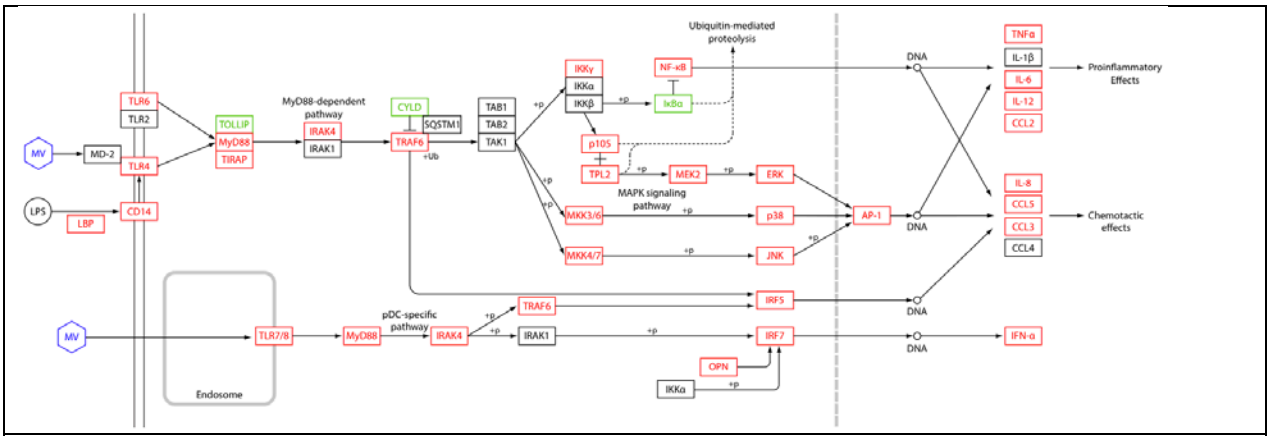

Figure 10. Effect of *SQSTM1* mutation on TLR signaling pathway. Genes shown in red are upregulated in *SQSTM1* mutant PDB samples while genes shown in green are downregulated in *SQSTM1* mutant PDB samples. Two exogenous stimulators of the TLR signaling pathway are shown: MV – measles virus and LPS – Lipopolysaccharide.

A proposed mechanism for how mutations in *SQSTM1* could act to affect TLR signaling is shown in Figure 11. SQSTM1 binding to MyD88 and TRAF6 and excluding CYLD would stabilize the complex and allow TRAF6 to become ubiquitinated, which would in turn activate NF-κB.

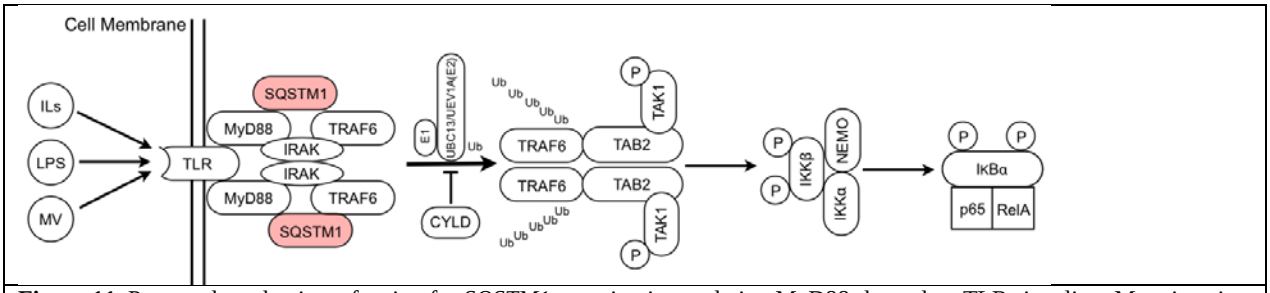

Figure 11. Proposed mechanism of action for *SQSTM1* mutation in regulating MyD88-dependent TLR signaling. Mutations in *SQSTM1* stabilize the MyD88/IRAK/TRAF6 complex, which upon stimulation by Interleukins (ILs), LPS or measles, leads to ubiquitination of TRAF6 and binding of the ubiquitinated TRAF6 to the TAB2/TAK1 complex, which phosphorylates the IKKβ/IKKα/NEMO complex, which in turn phosphorylates IκBα, which is degraded leading to activation and nuclear localization of the p65/RelA (NF-κB) and activation of NF-κB-dependent expression.

Activation of NF-κB can be detected by its translocation into the nucleus. As seen in Figure 12, PSV10 cells show an activated NF-κB response to LPS that is not seen in the hFOB control. This is an indication that our model is plausible and that the *SQSTM1* mutant can affect NF-κB activation through the MyD88-dependent TLR signaling pathway.

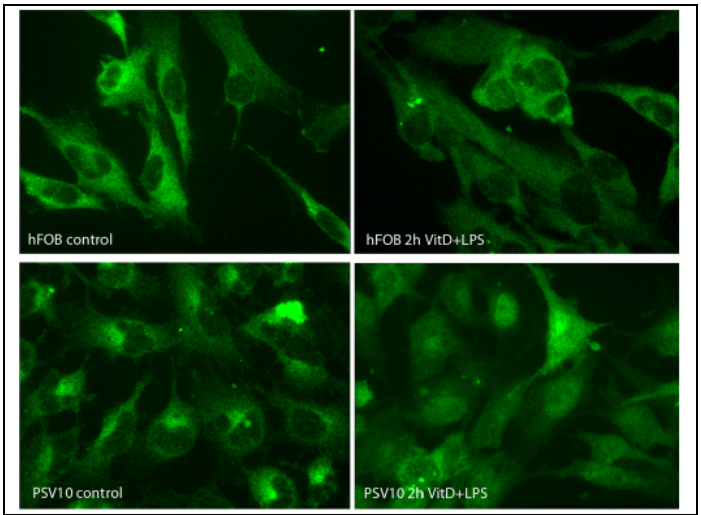

Figure 12. Differential activation of NF-κB depending on *SQSTM1* mutation status. NF-κB activation as measured by nuclear localization can be seen 2 hours after treatment with LPS in the PSV10 cells.

Key Research Accomplishments

- Discovery that in affected bone from sporadic PDB patients, somatic mutations in *SQSTM1* are found in the osteoblast lineage cells rather than in the osteoclast lineage cells.
- Successful isolation of RNA from LCM-captured samples from affected bone of sporadic PDB patients.
- Successful microarray analysis of RNA isolated from LCM-captured samples from affected bone of sporadic PDB patients.
- Discovery that chemokines important in osteoblast:osteoclast signaling are preferentially upregulated in $SQSTM1^{P392L}$ bone samples.
- Discovery that the PSV10 osteoblastic cell line derived from a PDB patient carries a heterozygous $SQSTM1^{P387L}$ mutation similar to that found in several familial PDB families.
- Discovery that the PSV10 cell line shows similar patterns of expression of chemokines important in osteoblast:osteoclast signaling as the LCM-captured $SQSTM1^{P392L}$ bone samples.
- Discovery that transfection of the $SQSTM1^{P392L}$ mutation into the hFOB osteoblastic cell line results in similar patterns of expression of chemokines important in osteoblast:osteoclast signaling as the LCM-captured $SQSTM1^{P392L}$ bone samples and PSV10 cell line.
- Discovery that the $SQSTM1^{P392L}$ mutation results in activated MyD88-dependent TLR signaling.
- Discovery that the $SQSTM1^{P392L}$ mutation in the LCM-captured $SQSTM1^{P392L}$ bone samples results in activated NF-κB through the MyD88-dependent TLR signaling.
- Discovery that the $SQSTM1^{P387L}$ mutation found in the PSV10 cell line also results in activated NF-κB through the MyD88-dependent TLR signaling suggesting that this is a key element of the mechanism by which mutations in *SQSTM1* are involved in PDB initiation.
- Development of a model in which somatic *SQSTM1* mutations in the osteoblasts upregulate chemokine expression in a MyD88-dependent, TLR signaling-dependent fashion, which then attracts pre-osteoclasts to the mutant osteoblasts. These pre-osteoclasts would then attract normal osteoblasts to the lesion. Thus the pagetic lesion would have a sub-population of osteoblasts containing the *SQSTM1* mutation and other osteoblasts that would not, but that were attracted to the lesion by chemokine signaling.

Reportable Outcomes

- We are currently preparing a manuscript of the results of this study.
- The microarray data that was obtained from the LCM-captured bone will be placed in the NCBI GEO database.
- We have characterized the PSV10 cell line for the SQSTM1 mutation status and for expression of the critical chemokines. This data will also be entered into the GEO database.
- We are preparing an R01 application to the NIH for continued funding of this work.
- We have successfully obtained further CDMRP PRMRP funding for a related aspect of this work; the role of measles virus in the delay of onset of PDB.

Conclusion

Our laboratory has shown that *SQSTM1* mutations also occur in the affected bone of PDB patients with no family history of PDB and that these mutations were not present in matched peripheral blood samples, suggesting that these mutations were somatic, not constitutional (12). Moreover, our analysis suggested that the mutations were present in only a subset of cells in the affected tissue. Furthermore, when we examined the laser-capture microdissected samples of pagetic bone with somatic *SQSTM1* mutations, we found that the mutant-containing samples did not express osteoclast-specific genes but did express osteoblast-specific genes suggesting that the $SQSTM1^{mut}$-containing cells were of osteoblastic rather than osteoclastic origin. We then were able to show that these cells upregulated chemokines important in osteoblast:osteoclast signaling in a MyD88-dependent TLR signaling-dependent fashion that resulted in activation of NF-κB. We were then able to replicate these findings, first in an osteoblast cell line derived from a SQSTM1 mutation positive PDB patient and then in a normal osteoblast cell line into which the *SQSTM1* mutation was transfected. We were able to develop a model that incorporates these findings to explain the role of *SQSTM1* in the onset of PDB.

"So What Section"

PDB affects approximately 1.5 million people in the U.S., making it the second most common metabolic bone disease after osteoporosis (13, 14). Yet despite the frequency of the disease, the etiology of the disease remains elusive and highly controversial. We have been able to develop a model that explains the origins of PDB as well as the experimental observation that not all cells in the pagetic lesion carry the initiating mutation. Our model is paradigm-shifting as the current model for PDB suggests that PDB is a disease of osteoclast origin. Our evidence suggests a multi-cellular model of PDB in which PDB acts like a hyperplastic disease characterized by polyclonal reactive tissue growth in which osteoblastic cells with *SQSTM1* mutations act as the nidus of the disease and recruitment of normal pre-osteoclastic and osteoblastic cells occurs through upregulation of chemotactic signals that attract these normal cells to the growing pagetic lesion. If true, then this research could open new avenues for treatments that target the mutant osteoblast cells rather than relying on the current non-specific therapy of bisphosphonates that targets all osteoclast cells in the body. Our discovery is also important because PDB is a disease of bone remodeling and represents an important window into the regulation of bone remodeling. Unlike osteoporosis, which is an uncoupled disease of bone remodeling, PDB represents a coupled disease of bone remodeling, which makes it more relevant to understanding how normal bone remodeling takes place.

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