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Looking for BRPF1 Presence in Differentiating Osteoclast Cells

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Abstract:
Through this semester-long experiment, professor Birnbaum and I attempted to identify the presence of BRPF1 (Bromodomain And PHD Finger Containing 1) in harvested RAW osteoclast cells. This was done through a series of steps, beginning with growing RAW osteoclasts, differentiating them, harvesting them and finally running a Western Blot test to look for our target protein. Unfortunately, no major discoveries were made with this project, but there is still reason to be optimistic about the presence of other members of the BRPF family (BRPF3, BRPF4). With some knowledge on our procedure, future researchers should be able to note specific points that could be tweaked for improvement to yield better results.

Introduction:
This research was done as a part of my senior thesis in conjunction with the Honors Program at Merrimack College. The results of this research paper were disseminated at the Research and Creative Achievement Conference on campus. Through this semester long research, professor Birnbaum and I built upon the research we had done of BRPF proteins in his Cellular Biology class the previous semester. BRPF1 is a protein that in humans is encoded by the BRPF1 gene located on 3p26-p25. It is a multivalent chromatin regulator that recognizes different epigenetic marks and activates three histone acetyltransferases (Moz, Morf and Hbo1). BRPF1 contains two PHD fingers, one bromodomain and one chromo/Tudor-related Pro-Trp-Trp-Pro (PWWP) domain. This gene is very conserved and has a critical role in different developmental processes of embryos as well as with tumors, which is what interested us the most. Brpf1 was reported to have a tumor suppressing role in malignant tumors. This conclusion was based on the observation that mutations in cancer cells appeared to diminish the function of Brpf1. However, oncogenic role of Brpf1 is also possible in cancer, as it can form a stable complex with Moz-Tif2, which could lead to the development of leukemia in humans. So, by looking for this gene's presence in RANKL treated osteoclast cells, we aimed to confirm its role in osteoclast differentiation, which could be important for future studies concerning BRPF1's role in osteoclast differentiation and tumor suppression/promotion. While studies have shown the RNA for BRPF1 being present in differentiating osteoclast cells, no one has ever confirmed the presence of BRPF1 protein itself.

Materials and Methods:
Osteoclast growth and differentiation using RANKL procedure: First, we diluted 45 µL of cell media that was combined with 5 µL of cells with 14 mL of 20% fetal bovine serum (FBS) and left the cells to incubate for 3 days on one culture plate. Next, we divided the cells in half and dispersed the 7 mL portions between two larger culture plates with fresh media to ensure we had enough osteoclasts to work with. Then, after a week of growth and re-feeding (replacing 7 mL of old media with new media every two days), the

cells were refed one last time, but only with 5 mL of media. The plate was then scraped and stirred to suspend the cells in fluid, and a portion of this mixture was removed and counted under a microscope using a hemocytometer and tryptophan blue stain to predict the total number of cells/mL of fluid. The approximate number came out to 2,400,000 cells/mL in our counting. We then calculated .125 µL + 10 mL to achieve a diluted sample of 30,000 cells/mL. Upon diluting and observing the diluted cells, they seemed too thin, so we combined 250 µL of cells from stock with 10 mL of media to achieve a diluted sample of 60,000 cells/mL. This combination of cells and media was then put in one well of a multi-well plate and incubated. After confirming sufficient growth two days later, the cells were re-fed with fresh media and combined with 2 µL of receptor activator of nuclear factor kappa-B ligand (RANKL) per well. RANKL is a key osteoclast differentiation factor, so we expected to see differentiation after three more days of incubation. After three days, a portion of the cells were again stained with tryptophan blue and observed under a microscope using a hemocytometer. The cells seemed far too dilute upon this observation (no more than 5 cells per 4x4 grid could be seen). It was apparent that the cells needed to be re-plated and re-fed with fresh media, given one more week to grow, and be diluted to a lesser degree. After observing these new cells once more under a microscope, they were re-fed and re-plated by putting 2.5 mL of the cell and stock solution into three new wells on the multi-well plate. This gave us four total wells, which we labeled as day 0 thru day 3 for harvesting. Day 0 was able to be immediately harvested, as this was the start of our 4-day interval.

Cell harvesting procedure: To harvest the cells from their plates, we first set up a plastic tube and put 1 protease inhibitor tablet in it. To this we added 10 mL Radioimmunoprecipitation assay (RIPA) Buffer and vortexed it until the tablet had completely dissolved in solution. Next, the well with the cells to be harvested had its media removed and it was washed with 2 mL of Phosphate-buffered saline (PBS). The PBS was then immediately removed and 250 µL of RIPA Buffer was added to the well. This plate was then vigorously scraped to ensure all cells were suspended in solution before the solution was removed and placed in an Eppendrof tube. This tube, containing harvested RAW osteoclast cells that had been treated with RANKL was our day 0 sample. The tube was placed in a -80 degree centigrade chiller for storage to prevent denaturation until we were ready to work with them again. Over the next 3 days, the harvesting process was repeated on the subsequent three plates to give us day 1, day 2 and day 3 samples. The next step was to run a protein assay, which required a Pierce™ BCA Protein Assay Kit.

Protein assay procedure: First, a preparation of Diluted Albumin (BSA) Standards had to be made. This was done by diluting the contents of one BSA ampule into several clean vials (using the same diluent as the sample). Each 1 mL ampule of 2 mg/mL BSA was sufficient to prepare a set of diluted standards for a working range of 25-2,000 µg/mL concentration. First, vials labeled A-I loaded with the BSA were given specified amounts of diluent,

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shown in figure 1.1 below. Next, vials A-C were given different amounts of stock solution, vials D-H were given dilutions from other vials and vial was left without any stock/BSA dilution. Upon doing this, a ladder could now be made with BSA concentrations ranging from 25- 2,000 µg/mL.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Fig. 1.1

Next, a preparation of BCA working reagent (WR) was made using the formula (# standards + # unknowns) x (#replicants) x (volume of WR per sample) = total volume WR required. The WR reagents were prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. 2.0 mL of this working reagent was added to.1 mL of both the sample to be assayed in a and with the appropriate BSA standard in a test tube, Next, the solution in the test tubes was able to be transferred to cuvettes and run through the spectrophotometer (along with a blank for control) to yield an absorbance rate at 562 nm wavelength. Results of this test can be seen in fig 1.2 in the results

section. Day 3 harvested osteoclasts were initially observed to have extremely low protein levels, so a new set of osteoclasts had to be treated with RANKL and incubated for 3 days before another day 3 sample could be pulled and run through the spectrophotometer. Once we had appropriate absorbance readings for protein presence in all four of our samples (day 0- day 3), we were able to move onto preparation for the Western Blot.

Western Blot Procedure: To execute the final step of our procedure, the Western Blot, we first needed to run an electrophoresis gel. During the electrophoresis, our negatively charged proteins moved toward the positively charged anode, which is what propagates proteins in any gel to move down through the gel. Our samples were prepared and loaded onto a gel, along with a standard protein ladder that gave us a benchmark of where proteins would end up on the gel based on their size. This ladder ranged from 250 kDa to 25 kDa. In order to further analyze the proteins after they had completed a sufficient run time (we knew it weighed 137 kDa, so we looked at the standard that weighed 150 kDa and waited for that to run approximately half way down the gel), they were transferred onto a membrane in a procedure called blotting. This was done by putting the gel on top of a membrane for transfer, between two buffer-soaked filter sheets (in order to prevent unwanted membrane-protein interaction in the following steps) and finally on the anode floor of the blotting machine where it was covered by a cathode plate and allowed to run. To visualize our protein of interest after the transfer was complete, the membrane was first

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washed with TBST without milk, then it was probed using a primary protein-specific antibody with TBST and milk with washes in between. Once this had been applied, we applied a labeled secondary antibody that would reveal our target protein if it was present on the membrane.

Results: Seen in fig. 1.2 are the results of our protein assay, which gave us a good measurement of exactly how much protein we had in our day 0- day 3 samples. Using an excel spreadsheet, we were able to make a graph from the BSA concentration ladder. Off of the protein concentration vs standard protein average absorbances at 562 nm, we could determine where on the graph our protein would fall, given its absorbance at 562 nm. This was done by using every second number (in red) in column A, standard protein absorbance @ 562 nm, except for the last number in the column, where the first number in red was used instead (1.944, here we used 1.489 instead because it stayed in bounds on our trend line). Every first number in column A represents a cuvette that contained 20 µL of our cell solution, and every second number represents a cuvette that contained 100 µL of our cell solution. The resulting concentrations of our proteins went as follows: day 0 had a concentration of 495 µg/mL, day 1 had a concentration of 660 µg/mL, day 2 had a concentration of 950 µg/mL and day 3 had a concentration of 1450 µg/mL. Initial day 3 harvested osteoclasts (not shown) were redone because they gave us a 20 µL reading of .008 absorbance, and a 100 µL reading of .027 absorbance, which would correlate to a concentration less than 100 µg/mL based on our standard graph.

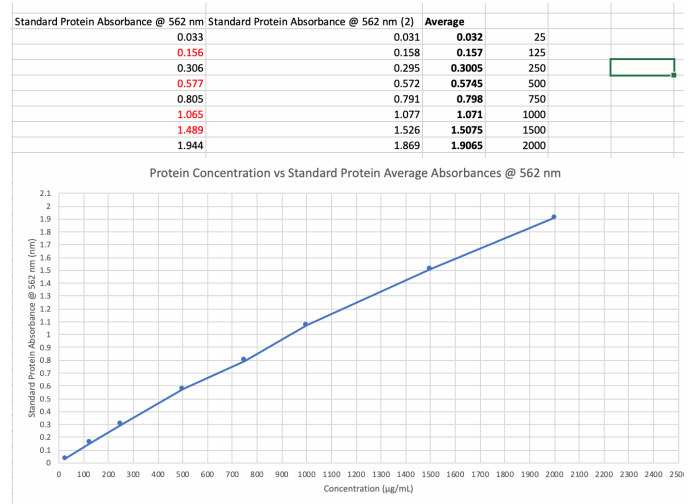| Standard Protein Absorbance @ 562 nm	Standard Protein Absorbance @ 562 nm (2)	Average	
0.033	0.031	0.032	25
0.156	0.158	0.157	125
0.306	0.295	0.3005	250
0.577	0.572	0.5745	500
0.805	0.791	0.798	750
1.065	1.077	1.071	1000
1.489	1.526	1.5075	1500
1.944	1.869	1.9065	2000

Fig 1.2

As displayed in fig. 1.3, there is a questionable indication of the BRPF1 protein on this Western Blot. We see our protein standard ladder in the lane on the far right, but there is no definitive indication of BRPF1 presence anywhere else on this membrane. From left to right, we expected to see lines somewhere between the second and third standard lines on the ladder. The second standard line translated to a molecular weight of about 150 kDa, and the third line translated to a molecular weight of about 100 kDa. The molecular weight of BRPF1 is approximately 137 kDa, meaning it theoretically should have fallen somewhere between these two lines. Furthermore, we hypothesized to notice a pattern from left to right of the BRPF1 containing bands getting darker. This would have signified that as time went on (from day 0 to day 3) there was differentiation occurring in the osteoclasts that promoted further expression of the BRPF1. Instead of seeing these results, though, we instead saw two lines (that got fainter from day 1 to day 2). Circled in figure 1.3 are these two possible bands that we found on this membrane, but its reliability is

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debatable. The faintly observable band is not near its supposed molecular weight of 137 kDA, but rather up near the top line of the ladder, which signifies a molecular weight somewhere around 250 kDA. While this is a highly unlikely position for the protein to be positioned at, considering that the standard ladder run on the right side of the membrane ran reliably and without any problems, it is a possibility that this is BRPF1. We can say this because we used a protein specific antibody, meaning the antibody was tailored to only stick to BRPF1 and nothing else. Further testing would be required placed our mel
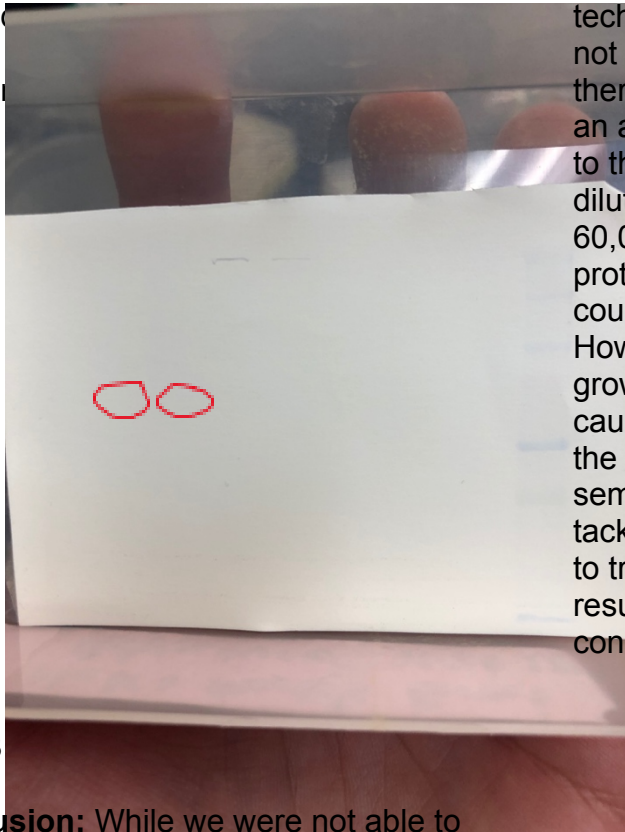
Fig 1.3

Conclusion: While we were not able to get the exact results we hypothesized to see, this does not mean that there is no BRPF1 to be found in osteoclasts. The presence of its RNA still supports the proteins presence and the two bands that we did see on the gel means we cannot confidently rule out the incidence of BRPF1 in our differentiated osteoclasts. There could be some

procedural changes to make that might confirm or deny our initial hypothesis to a greater degree. Some reasons for our potential inability to find the protein could be dilution beyond a threshold of observability. Having to regrow and harvest our day 3 samples was also an inconvenience, as it gave us two separately grown sets of osteoclasts (day 0- day 2 and day 3). For consistency in experiments like this, it is always preferred to have all the cells come from one batch to minimize potential error. The largest source of error was likely related to these technical details. Simply put; there might not have been enough cells, meaning there wasn't enough protein to give us an accurate reading. This could go back to the dilution of our cells (to an initial dilution of 30,000 cells/mL and then to 60,000 cells/mL). To garner higher protein and cell density, more cells could have been initially added. However, one must be careful to not grow too many cells because this might cause overcrowding, which means that the osteoclasts won't differentiate. Next semester, Professor Birnbaum will tackle this research with another student to try and get more comprehensive results and draw a more definite conclusion on our experiment.

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References:

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