

Understanding osteoclast development:
A first step in the identification of therapies for osteoporosis

Anthony Evans
Department of Biological Sciences, Fordham University
Bronx NY, 10458

Abstract:

Osteoporosis is a metabolic bone disease that impacts a large number of individuals over 50 years of age. It occurs when osteoclasts, cells that break down bone, are more active than the cells that build up bone. This leads to porous and easily broken bones. Current treatments of osteoporosis include the use of bisphosphonates, which over the long term, can also lead to an increase in fractures. To better develop treatments for osteoporosis, a better understanding of the induction of genes involved in osteoclasts development and function is needed. Cells were treated with vitamin D3 and Tetradecanoyl phorbol acetate to induce differentiation. RNA was harvested at several time points between 0 and 24 hours. Expression of osteoclast genes RANK, MMP9, and TRAP were monitored using quantitative RT-PCR. RANK expression was induced within the first 2 hours after treatment. MMP9 expression was induced next, with an increase of expression starting between 2 and 4 hours after treatment. TRAP mRNA expression did not begin until around 8 hours after treatment. All three genes expression increased up through 12 hours. This indicates that, for future studies, 12 hours after treatment is a sufficient point at which to monitor the effectiveness of compounds on osteoclast gene expression.

Introduction:

The human skeleton is the primary source of structural support for the human body. For bones to maintain their structural integrity, they must be continuously remodeled, which is dependent on bone resorption. Osteoclasts are multinucleated cells, derived from myeloid precursor cells, which attach to bone, and secrete enzymes that will dissolve bone minerals and the extracellular matrix; effectively breaking down the bone (Vaananen, 2000). Once bone has been resorbed, osteoblasts are recruited to replace the old bone by assisting in the mineralization of bone matrix. In a normally functioning system, a very delicate balance between the activity of osteoclasts and osteoblasts maintain the needed remodeling of bone (Crockett, 2011).

Osteoporosis is a metabolic bone disease that affects 50% of women and 25% of men over the age of 50 (Teitelbaum,2007). Osteoporosis occurs when osteoclast function becomes abnormally higher than the function of osteoblast. When this occurs, a greater amount of bone is broken down, than is built back up causing the bone to become brittle and porous. This leads to higher rates of bone fracture, and in severe cases even disability (Lewiecki, 2009).

One of the most common treatments for osteoporosis is treatment with a molecule bisphosphonates. Bisphosphonates are molecules that when introduced to the bone, will incorporate themselves into bone matrix. When osteoclasts begin to breakdown bone, these bisphosphonates are released and induce apoptosis in the osteoclasts. This treatment is effective, but in some cases of long term treatment can actually cause other problems with the bone (Brown 2014).

Several genes are important in the differentiation and function of osteoclasts. RANK (receptor activator of nuclear factor-KappaB) is a gene that encodes a receptor that, when bound to its ligand initiates the signal for differentiation of myeloid cells into osteoclasts (Xing, 2005).

MMP9(matrixmetallopeptidase 9) is a gene that encodes for a metalloprotease. The MMP9 protein is secreted from fully mature osteoclasts, and then assists other osteoclast secreted proteins in the breakdown of bone matrix (Bruni-Cardoso, 2010). Tartarate-resistant acid phosphatase(TRAP) has been widely used as a marker for osteoclast function (Minkin, 1982). TRAP is a protein secreted by osteoclasts, which biological function and natural substrate is not yet known (Hayman 2000).

Current work is being done in the development of new treatments for osteoporosis. These studies focus on compromising osteoclast function by altering gene activity. To determine how effective these new treatments are in altering activity of osteoclast specific genes, a better understanding of the induction of genes in osteoclasts is needed. In previous literature, it has been shown that treatment of myeloid progenitor cells, undifferentiated cells that grow in bone marrow, with a combination of 1,25-Dihydroxyvitamin D3(D3) and 12-O-tetradecanoylphorbol-13-acetate(TPA) induce the in vitro differentiation of the progenitors into osteoclasts (Kido, 2003). This study focused on using quantitative reverse transcriptase polymerase chain reaction(qRT-PCR) to gain a better understanding of the induction of RANK, MMP9, and TRAP in the first 24 hours after myeloid cells were treated with vitamin D3 and TPA.

Methods:

Cell Culture:

HL-60 cells were generously donated by Dr. Sylvia Anderson. Eleven wells(on two six well plates) were plated with 5×10^5 /ml HL-60 cells suspended in 2ml of RPMI media that had 10% fetal bovine serum added to it. At time zero, 9 of the wells were treated with 10 nM Vitamin D3 and 50 nM TPA. The final two wells with cells were left untreated, to act as a control. The cells were then incubated at 37° C until it was time for RNA to be extraction.

RNA Extraction:

At time zero, the control plate was taken out, the cells were taken out of the plate, placed in a 1.5 ml Eppendorf tube, and centrifuged at 2 rpm for 6 minutes to form a pellet. The media was then taken off the top of the pellet. The cells were then re-suspended in 350 μ l of RLT lysis buffer plus B-mercaptoethanol and frozen at -80° C until RNA purification. This was repeated at time points of: 0,0.5, 1,2,4,6,8,12,24,24 hours for the treated cells.

RNA Purification:

All lysed samples were placed through a qia-shredder column (Qiagen) and spun at 16xg for 2 minutes to remove genomic DNA. The RNA was then purified using the Qiagen RNeasy plus mini kit in the Qiagen Qiacube automated system, according to the manufacturer's protocol. The RNA samples were analyzed by UV-spectrometry for concentration and purity. All samples were then standardized to 5($\text{ng}/\mu\text{l}$) stock solution. The RNA samples were then stored at -80° C.

Quantitative RT-PCR:

Quantitative reverse transcriptase polymerase chain reaction(qRT-PCR) was performed for all samples. 20 μ l reaction volumes, on a 96-well micro-amp plate, were made with sybr green, 4 μ l of RNA template and a.5 μ M primer concentration(primers shown in fig. 1). The RANK primers used were: forward-gatcggctacagtcgaggaag, reverse-tgctgagttgaggagtg. MMP9 primers were: forward-ttctacggccactactgtgc, reverse- gaatcgccagtacttccatc. The primers for the amplification of TRAP were: forward- tcttggtcaagaacagctg, reverse- ctcatcttcaggtactgcaga. The primers for RANK both sit in exon 10 because RANK has a high number of variants, and exon 10 is highly conserved. To ensure that genomic DNA was not amplified, a qia-shredder column was used in RNA purification, as explained above.

The qRT-PCR was performed using the applied biosystems real-time machine, following the sybr green program template. All qRT-PCR's were done in triplicate. The results were obtained and the compared at a threshold level(Ct) 20. The samples were then run on 1% agarose gels to ensure that only one product of the correct size was present(not shown).

Figure 1. Diagram of the RANK, MMP9, and TRAP transcripts, with primer locations dictated by the red arrow.

Results:

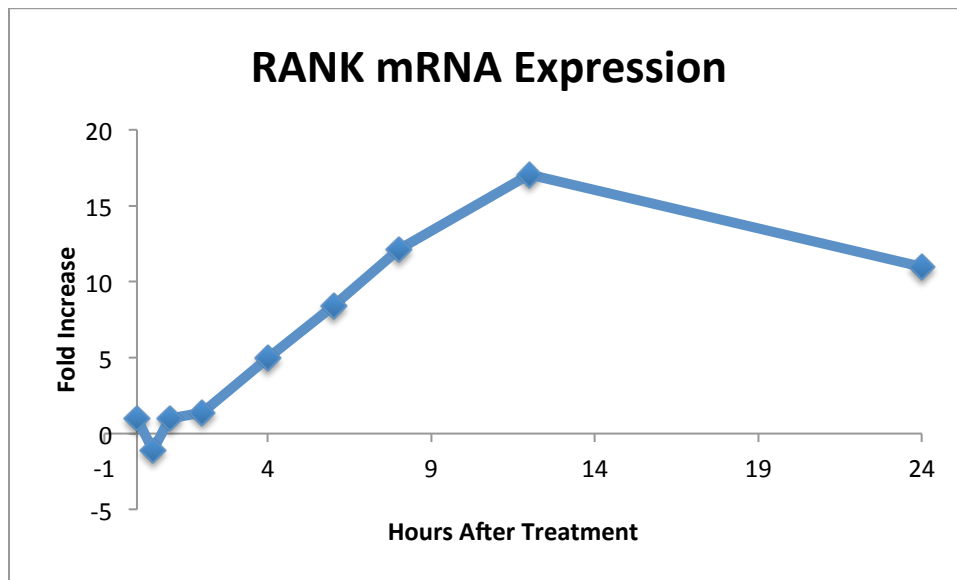


Figure 2. RANK mRNA transcript expression increased until 12 hours after treatment, where expression of the transcript leveled off.

The expression of the RANK transcript started to increase between 1 and 2 hours after cells were treated with vitamin D3 and TPA. The transcript continued to increase up until 12 hours after treatment. At 12 hours, transcript expression reached its maximum, and expression then plateaued.

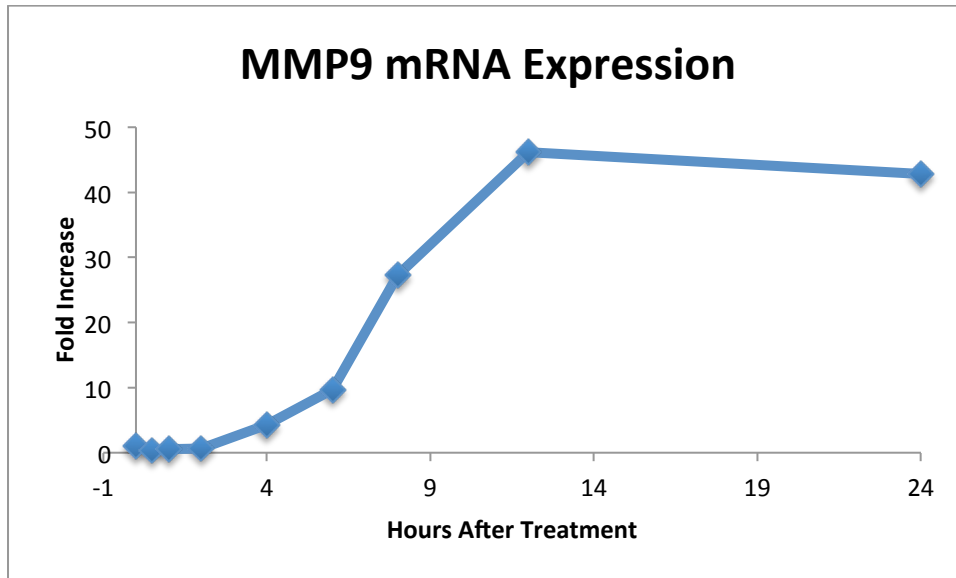


Figure 3. MMP9 mRNA transcript expression increased until 12 hours after treatment, where expression of the transcript plateaued.

Expression of the MMP9 transcript started to increase between 2 and 4 hours after the cells were treated. The expression continued to increase until 12 hours after treatment where the expression leveled off.

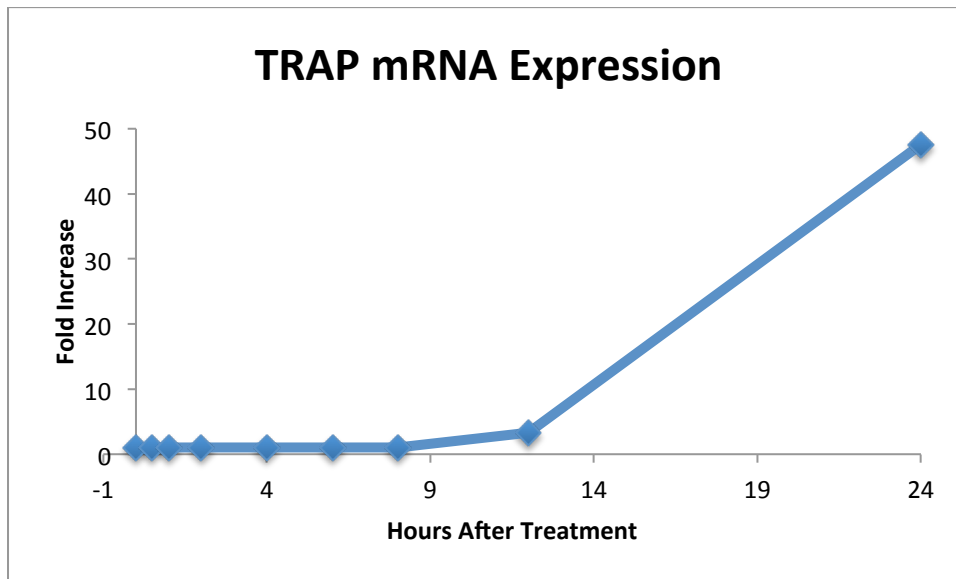


Figure 4. Expression of the TRAP transcript began to increase between 8 and 12 post treatment.

TRAP transcript expression started to increase between 8 and 12 hours after the cells were treated. The expression of the transcript continued to increase through the 24 hour time point.

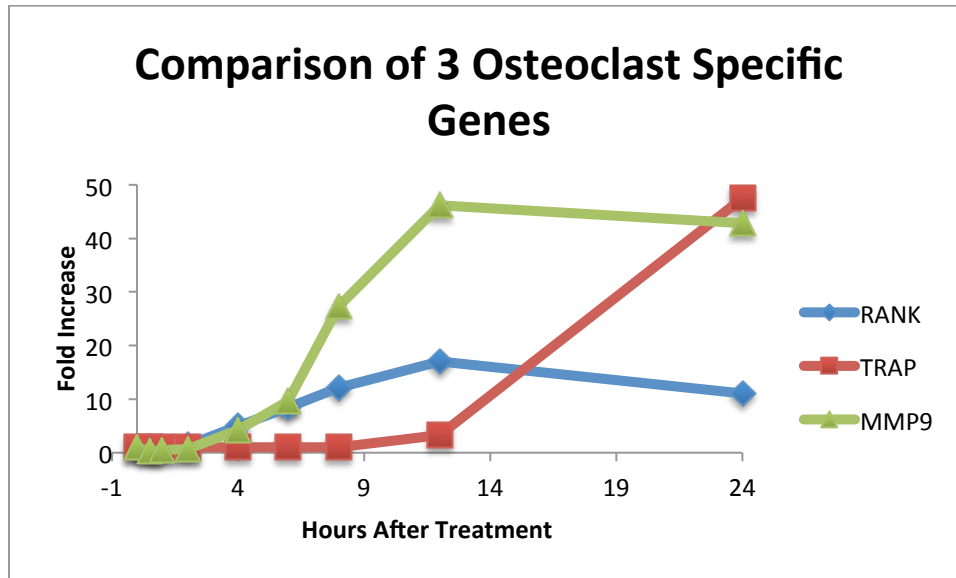


Figure 5. The RANK transcript started to increase first, followed by MMP9 and TRAP.

The expression of the 3 osteoclast specific genes was compared. The expression of the RANK transcript began first, between 1 and 2 hours after treatment. MMP9 and TRAP then began to increase at 2 and 8 hours respectively. All three transcripts increase up to the 12 hour point.

Discussion:

The expression of the RANK transcript began between 1 and 2 hours after the HL-60 cells were treated with a combination of vitamin D3 and TPA. This could indicate that the cells are beginning to differentiate two hours after treatment. This expression increase continues until 12 hours after treatment, which could indicate that cells are becoming fully differentiated around 12 hours after treatment, and therefore no longer need RANK signaling to occur. Further studies on the presence of the RANK receptor need to be completed before this can be a definitive conclusion. MMP9 and TRAP expression began to increase after RANK expression. This was the expected result, because RANK is the signal for myeloid cells to differentiate (Xing, 2005), where as the proteins encoded by MMP9 and TRAP are known to be involved in the function of fully formed osteoclasts (Bruni-Cardoso, 2010). It has been shown that RANK, MMP9, and TRAP mRNA expression did increase up to 12 hours post treatment. This indicates, for future

studies in the development of treatments for osteoporosis, that looking at transcript expression levels 12 hours after incubation with vitamin D3 and TPA should be sufficient in determining the effectiveness of a treatment in compromising the function of osteoclast gene expression.

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