The Role of Kindlin3 in Osteoclast Function

A Senior Research Thesis

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Abstract

Osteoclasts are a bone cell type responsible for the breakdown of bone. They display cell surface integrins that help activate signaling cascades that control the differentiation and function of osteoclasts. The kindlins are a family of proteins that are involved in activating integrin function and enhancing cell attachment to substrates. Kindlin3 is expressed solely in hematopoietic cells, including osteoclasts and their precursors. Mutations in kindlin3 are associated with a human syndrome, Leukocyte Adhesion Deficiency III (LAD-III) whose symptoms include osteopetrosis, which is bone brittleness due to non-functioning osteoclasts. The kindlin3 C-terminal tail is composed of three FERM (integrin-binding) domains, F1, F2, and F3 and one PH (membrane lipid-binding) domain that interrupts the F2 domain. The goal of this work is to determine the roles of kindlin3 and its domains in affecting osteoclast differentiation and function. Our studies demonstrated that kindlin3 is upregulated during differentiation indicating its role in activating differentiation. These data further showed that overexpression of the F2 and PH domains together in osteoclast precursors inhibited their differentiation. However, the overexpression of the PH domain alone did not. This work was continued to determine the role of the various tail domains in osteoclastic bone resorption activity. Overexpression of the F1, F2, F3 and PH domains individually affected the ability of osteoclasts to resorb bone. The three FERM domains caused a decrease in the clearing size and overall resorption while the PH domain also leads to a decreased clearing size but an increase in the overall resorption. These results are helping to dissect the functional domains of kindlin3 in an effort to understand how mutations can cause the osteopetrosis of LAD-III.

Introduction

The human body is composed of two hundred and six bones that work together to provide shape, support, and protection for the body's internal structures. The body consists of five different types of bones; long, short, flat, irregular, and sesamoid. Bone is a specialized connective tissue and consists mostly of collagen that is hardened by calcium phosphate (Datta et al, 2007). It is a living tissue that is constantly undergoing remodeling which allows the bone to heal itself and retain its slight flexibility to respond to stress.

Bone turnover relies on three cell types, osteocytes, osteoblasts, and osteoclasts. Osteoblasts and osteoclasts are required to work in a specialized balance to ensure a stable mass during bone remodeling(data et al, 2007). Osteocytes begin the process by responding to stress and chemical signals that results in the digestion of the endosteum collagen matrix. This recruits osteoclast precursors that fuse together causing activation. Activated osteoclasts are then able to mediate bone resorption. Finally, osteoblasts are recruited to lay down the new bone to be calcified. This tight interplay relies on the regulated differentiation and function of all bone cell types to ensure proper skeletal development, indicating a need to attempt to understand this unique biology.

Osteoblasts are mononucleated, specialized fibroblasts that are responsible for laying down new bone (Datta et al, 2007). Osteoblasts differentiate from mesenchymal stem cells via preosteoblasts (Tamama et al, 2006). Many signaling pathways regulate the differentiation of osteoblasts, including activation of a wnt signaling pathway and BMP (bone morphogenetic protein) signaling. The Runx2 transcription factor is essential for osteoblast differentiation along with the Osterix transcription factor (Kundu et al, 2002). Runx2 activates differentiation of mesenchymal stem cells into preosteoblasts and Osterix works next by activating differentiation

from preosteoblasts into osteoblasts. After differentiation, some osteoblasts continue to differentiate into the next bone cell type, osteocytes (Datta et al, 2007).

Osteocytes are terminally differentiated cells that are responsible for maintenance of bone structure using long dendritic processes for communication throughout the matrix (Zhao et al, 2002). Osteocytes are thought to respond to mechanical stress, like mechanosensory cells, to initiate bone remodeling and maintenance. Osteocytes are able to turn mechanical signals into biochemical signals that, through various signaling pathways, are able to regulate bone turnover. Osteocytes differentiate from osteoblasts that have become encased in the bone matrix via an unknown mechanism. However, it has been shown that type-1 collagen is responsible for proper osteocyte phenotype, including dendrite number (Holmbeck et al, 2005). It has been suggested that changes in dendrite number affects osteocyte function and viability. This in turn can affect various properties of bone.

Osteoclasts, the final cell types, are terminally differentiated, multinucleated cells that are responsible for resorbing bone. Both macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κB ligand (RANKL) are important in osteoclastogenesis (Wiktor-Jedrzejczak et al, 1990). M-CSF is a growth factor responsible for the development of macrophage colonies (Nakamura et al, 2011). Its importance in the differentiation of osteoclast progenitors was shown in osteopetrotic knockout mice that displayed a decrease in the number of osteoclasts. Reinjection of M-CSF rescues the mutant phenotype (Felix et al, 1990). RANKL is part of the TNF family that directly controls differentiation by activating RANK that can cooperate with other receptors to activate differentiation into osteoclasts (Anderson et al, 1997). Fully differentiated osteoclasts display specific cell surface markers indicating maturation, such as tartrate-resistant acidic phosphatase (TRAP). TRAP protein can be stained using biochemical

substances that lead to a colored precipitate that can easily be seen by light microscopy and can be used to detect differentiated osteoclasts. A summary of the processes and factors involved in osteoclast differentiation is shown in Figure 1.

After differentiation, osteoclasts must become polarized before beginning resorptive activity. The actin cytoskeletal network is important in osteoclast function. The osteoclast actin ring, also called the sealing zone, is an attachment structure required for bone resorption. The actin-rich sealing zone is required for tight attachment of osteoclasts to the bone matrix (Nakamura et al, 2011). This arrangement is required for bone resorption and the formation of the ruffled border where digestive enzymes such as V-ATPases and chloride channels reside to create the acidic environment required to resorb bone. The actin ring interacts with a variety of integrins that extend outside of the osteoclast to the bone surface and allow signaling initiation and recognition.

Osteoclasts display αvβ3 integrins that are recognized by proteins containing an RGD sequence on the bone surface. Integrins are the main receptor for binding the extracellular matrix (Moser et al, 2009). Integrins are heterodimeric membrane proteins that signal inside and outside of the cell by structural changes that activate and inactivate the integrin. All integrins contain both an α and β subunit that span the entire cellular membrane. The extracellular domain binds specific amino acid sequences of extracellular proteins and the intracellular domain binds actin filaments via talin. $\alpha_v\beta_3$ integrins are important adhesion proteins in osteoclasts and are located where sealing zones attached to the bone (Nakamura et al, 2011). This is associated with intracellular signaling, including an increase in Ca^{2+} and phosphorylation to cause osteoclast activation. The $\alpha_v\beta_3$ is important in osteoclast resorption. This was shown in β_3 knockout mice that showed a late on-set osteopetrosis and the osteoclasts in the mice failed to spread out and

form actin rings(McHugh, 2002). This $\alpha_v\beta_3$ integrin domain has been shown to interact with IL-1 to cause osteoclast adhesion and, through a signaling pathway, cause activation of osteoclasts. An overview of the role of $\alpha_v\beta_3$ signaling in osteoclasts is shown in Figure 2. Integrins are also responsible for leukocyte adhesion (Malinin et al, 2010). The kindlin family of proteins has been shown to be necessary for the inside-out activation of integrins and is also thought to regulate actin-based cellular attachment to other cells or substrates (Harburger et al, 2009).

There are three known isoforms of kindlin; kindlin1, kindlin2, and kindlin3 (Malinin et al, 2011). Kindlins are expressed in a tissue-specific way with kindlin1 expressed in epithelial cells, kindlin2 in solid tissue and kindlin3 in hematopoietic cells (Ussar et al, 2006). This gives all of the kindlins a different tissue specific function. Kindlin1 is responsible for creating a layer of adherent cells, kindlin2 for controlling the three dimensional matrix scaffold in organs, and kindlin3 for cell adhesion. Kindlins are able to bind to the cytoplasmic domains of the β_1, β_2 and β_3 integrins (Schmidt et al, 2011). The binding triggers a conformation change that quickly shifts the integrin from an inactive to an active state. This is known as inside-out signaling, transmission of information from intracellular regulatory proteins to the outside of the osteoclast. The function of kindlin requires cooperation with talin-1 to allow inside-out signaling activation of integrins (Tadokoro et al, 2003).

The kindlin family has a highly conserved structural arrangement consisting of three FERM domains (F1, F2, and F3), which are interaction domains with the F3 domain being responsible for integrin binding (Shi et al, 2007). All of the kindlin F2 domains are interrupted by a PH domain which acts like a phospholipid binding domain to bind ligands (see Figure 3). Kindlin1 is found throughout the cytoplasm, kindlin2 in cell-cell contact areas and kindlin3 throughout the cell and surrounding the nucleus (Ussar et al, 2006). Mutations in all three kindlin

isoforms lead to human genetic disorders. Kindlin1 deficiency results in the first characterized kindlin disease, Kindler Syndrome (Sharma et al, 2003). Symptoms include skin blistering, periodontitis and poikilodermia and results in part due to keratinocyte defects (Nikloay et al, 2010). Human deficiencies in kindlin2 have not been reported, most likely because they are embryonic lethal as they are in mice and zebrafish knockout models (Dowling et al, 2008). Kindlin3 deficient mouse models die within the first few weeks after birth from severe bleeding (Kruger et al, 2008). However, human patients with kindlin3 deficiencies are able to survive with phenotypic mutations (Svensson et al, 2009). Patients with these mutations have a disease termed Leukocyte Adhesion Deficiency III (LADIII). LADIII is characterized by severe bleeding, frequent infections, and osteopetrosis (increased bone mass) (Malinin et al, 2009). Patients with LADIII have shown normal levels of integrins but the ligands were unable to bind and therefore activate signaling and adhesion. These data demonstrate kindlins are necessary for the activation of integrins and kindlin3 is specific to hematopoietic cells, including osteoclasts.

Our lab's preliminary data demonstrated that kindlin3 was located throughout the cell and surrounding the nuclei as well as podosomes of osteoclasts, the area responsible for creation of sealing zones (Figure 4). Malinin et al. (2010) similarly showed that kindlin3 is located in podosomes. Kindlin3 activates classes of integrins, controlling the ability of osteoclasts to spread out and organize their cytoskeleton (Schmidt et al, 2011). Loss of kindlin3 leads to the failure to form podosomes and sealing zones, inhibiting the ability of the osteoclasts to resorb bone. Kindlin3 knockout mice show the importance of kindlin3 in osteoclast function. In the knockout mice, the number of osteoclasts, determined by TRAP staining, was shown to greatly increase, however, the surface area of bone covered by osteoclasts was significantly lower (Schmidt et al, 2011). This indicates the osteoclasts are functionally impaired, which was confirmed with

resorption studies showing a decrease in the resorption area. Kindlin3 deficient osteoclasts also demonstrated a decrease in adhesion in comparison to wild type osteoclasts. Also noted was a decrease in the level of integrins. This demonstrated the importance of kindlin3 in osteoclast adhesion through integrin signaling. Kindlin3 deficient mice were able to form the initial actin core, however, they were unable to from podosomes or sealing zones which are important in bone resorption (Schmidt et al, 2011). These data demonstrate the importance of kindlin3 in osteoclast bone resorption.

Our lab's preliminary data demonstrated that the kindlin3 protein is upregulated during differentiation. This is not contrary to previously published data showing no change in kindlin3 levels across the seven days of differentiation because Schmidt et al. only looked at RNA levels, not protein levels (2011). Additionally our lab overexpressed full-length kindlin3 in pre-osteoclasts by twofold (Figure 5A). Following treatment with RANKL, the degree of differentiation in cells overexpressing kindlin3 was found to be less than that of control cells (Figure 5B/C). These data indicated the importance of kindlin3 in osteoclast differentiation, leading to the further studies described here.

This current research was aimed at investigating the role of kindlin3 in osteoclast differentiation and determining whether specific protein domains have any other effect on osteoclast function. The goal was to overexpress kindlin3 domains to swamp out any protein binding sites normally accessible to full-length kindlin3, thereby working in a dominant negative fashion. Expression plasmids were made containing each of the kindlin3 domains. Overexpression in differentiating osteoclasts showed that only the F2 subunit influenced differentiation. In contrast, overexpression of each kindlin3 domain resulted in decreased resorption capacity, indicating the importance of every domain in mature osteoclast function.

Methods

A. Cell culture: The RAW264.7 mouse macrophage cell line was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. This cell line has the potential to differentiate into osteoclasts for further study. To differentiate these cells, 50 ng/ml recombinant GST-RANKL was added and cells were cultured for 7 days, giving fresh medium every 2-3 days (Krits et al, 2002). By day seven the RAW264.7 cells have fully differentiated into osteoclasts.

B. Quantification of mRNA and protein levels. Mouse osteoclasts were differentiated over a standard seven day period as described above, and total RNA and protein were extracted on each day. RNA was extracted using RNA-B, while protein was extracted using M-PER buffer. Kindlin 3 mRNA levels were tested by quantitative RT-PCR using an internal standard (McMichael et al, 2007). To determine levels of kindlin proteins, western blots containing lysates from a seven-day differentiation period were stripped and probed using specific antibodies and detection reagents from Pierce Biotechnology.

C. Isolation and subcloning of kindlin3: Total RNA from mouse osteoclasts was isolated. RT-PCR was run to generate and amplify cDNAs of the kindlin3 FERM and PH domains. These cDNAs were subsequently subcloned into mammalian expression vector pEF6/V5-His using restriction enzymes and ligase to insert the cDNA and close the gaps. Then plasmid stocks were prepared. These were stably transfected into the RAW264.7 (pre-osteoclast) cell line using Lipofectamine and Plus reagents(McMichael et al, 2007). Cells were grown in blasticidin to select clones expressing the desired plasmids.

D. Quantifying the total level of differentiation. The differentiated osteoclasts containing plasmids with each of the three FERM domains and the PH domain were stained to determine

the degree of differentiation. TRAP, a marker of differentiation on the surface of osteoclasts, leads to a biochemical reaction turning cells that have differentiated (TRAP+) a purple color. Photomicrographs were taken of the stained osteoclasts and the degree of differentiation was quantified numerically and displayed graphically.

E. Resorption Assay: Stable cell lines were created using RAW264.7 cell lines with the three kindlin3 FERM domains and the PH domain inserted via cloning into the pEF6/V5-His vector. These cell lines were differentiated into osteoclasts for four days and then allowed to resorb synthetic bone substrate for three days. After this, the cells were removed from the substrate and pits were photographed using a light microscope. The resorption area or the resulting pits were quantified for both the size of the individual pit and the totally resorption area on the synthetic bone using a program, SigmaScan Pro (McMichael et al, 2007).

Data

Protein levels of kindlin 3 increase but RNA levels remain constant— Schmidt et al. (2011) showed that kindlin3 (K3) mRNA expression on days 1, 3, and 5 of differentiation remained constant. Because our lab had previously determined that overexpression of K3 influences osteoclast differentiation, we decided to further investigate K3 levels during the differentiation process. We examined both mRNA and protein levels days 0-7 of differentiation. We found by western analysis that kindlin 3 protein levels initially dropped on day 1, but then increased more than 3-fold by day 7 (Figure 6, upper panel; in the middle panel, beta-tubulin is used as a loading control). We also performed competitive RT-PCR to show that Schmidt et al. was correct in that the RNA levels did not change over the same time points (Figure 6, lower panel). This finding demonstrates that K3 expression during differentiation is regulated at a translational or post-translational level.

Cloned K3 tail domains into expression vector— To begin our studies to better define the domains of K3 important in osteoclast differentiation, we cloned five different K3 cDNA segments: F1, F2, F3, PH, and ALL (containing all the domains). All five regions were successfully cloned into a pEF6/V5-His vector (Figure 7). All tail domains were confirmed by sequencing. We utilized the pEF/V5-His vector so that we would get a low level of overexpression due to the human elongation factor promoter in the construct. Previous studies in the laboratory had determined that osteoclasts poorly tolerate high levels of overexpression stimulated by the more commonly used CMV or SV40 promoters. The five plasmid constructs were then transfected into RAW264.7 cells to make a stable cell line expressing each domain.

The K3 F2 region regulates differentiation in osteoclasts — TRAP stain analysis, (a marker of osteoclast differentiation), showed that overexpression of the region containing the F2 and PH domains led to a decrease in osteoclast differentiation, while overexpression of the PH domain alone did not (Figure 8). As seen in Figures 3 and 7, the F2 domain is split into two by the PH domain so the F2 necessarily also contains the PH domain. Our results suggested that either the F2a or the F2b subdomain may be affecting osteoclast differentiation as the PH domain alone did not do so. The F2b subdomain was successfully cloned and expressed in cells and stable cell lines were created. The PCR of the F2a domain was successful but cloning into the pEF6/V5-His expression vector has not yet been completed. Therefore the relative contributions of each of the F2 subdomains to osteoclast differentiation could not be assessed as comparisons between the two subdomains is not yet possible.

All four domains affect osteoclast resorption --- In addition to the studies examining K3 domains in osteoclast differentiation, we also investigated their potential role in mature osteoclast function. The F1, F2, F3 and PH domains were expressed in cells that were differentiated into osteoclasts. After differentiation, the osteoclasts were placed on synthetic bone and allowed to resorb the substrates. Photomicrographs were taken of the synthetic bone after five days and the white spots, known as resorption clearings, were measured. Results indicated that all four domains resulted in a decrease in clearing size in comparison to the control (Figure 9). While all four led to decreased individual clearing sizes, the quantified data showed that only the F1, F2, and F3 domains all led to an overall decrease in resorption. The PH domain, however, led to an increase in overall resorption due to an increase in the number of clearings. This demonstrates

that although the F2 subunit is responsible for osteoclast differentiation, all four domains plan a role in osteoclast resorption.

Discussion

Osteoclasts are the cell type responsible for resorption of bone. Osteoclast precursors first differentiate into mononucleated pre-osteoclasts with the introduction of M-CSF and RANKL. M-CSF is released by osteoblasts and binds receptors on osteoclasts to induce differentiation. RANKL directly controls differentiation by binding to RANK receptors on osteoclasts surfaces. These mononucleated pre-osteoclasts then fuse together forming multinucleated osteoclasts through mechanisms that are not well established. Once the osteoclasts fuse, they are activated by RANKL and other cytokines such as IL-1 to allow resorption.

Previous studies were focused on the role of kindlin3 in osteoclast function. Our lab has found that kindlin3 protein is upregulated during osteoclast differentiation, suggesting that kindlin3 plays an important role in the osteoclast differentiation process. Published data indicated that the levels of kindlin3 mRNA do not change during differentiation. However, these results do not contradict our findings as Schmidt et al. (2011) only looked mRNA levels, not proteins levels as we did. Since the levels of kindlin3 protein changes during differentiation but not the level of kindlin3 RNA, it is not the rate of transcription that is changing during differentiation. Therefore, the change could either be due to an increase in translation in the cytoplasm creating more protein as differentiation proceeds or it could be due to post-translational processes. Further studies are needed to determine the method of kindlin3 protein increase during differentiation.

We have been able to narrow down the location on the kindlin3 protein that was influencing differentiation to the F2 region. The overexpression of the F2 domain was the only domain that led to a decrease in differentiation of osteoclasts during early differentiation due to

the decreased number of TRAP+ cells. This information demonstrated that the F2 domain alone was responsible for influencing osteoclast differentiation. Although the F2 domain leads to a decrease in differentiation, this trend is only seen in the early days of differentiation. Eventually, differentiation of osteoclasts in the presence of the overexpressed F2 domain leads to a complete degree of differentiation by the end of the differentiation period. These data indicate that the F2 subunit is important for proper differentiation of osteoclasts. Although the $\alpha_v\beta_3$ integrin complex is the most important integrin in osteoclast signaling, other integrins also function with osteoclasts. It is possible the F2 subunit is binding partially or completely to one of those integrins which is also playing an important role in osteoclast differentiation leading to the observed data. Also, since the F2 domain is a FERM domain and the function of FERM domains is primarily in binding other molecules, it is possible the F2 subunit is responsible for recruiting some molecule that is necessary for proper differentiation.

Although the F2 domain has been shown to solely influence osteoclast differentiation and affect resorption, it is still the F3 domain that has been shown to binds to integrins to allow cell signaling. The effect of the F3 domain on osteoclast differentiation was studied in differentiation and resorption. When overexpressed in osteoclasts, the F3 domain was not shown to have an effect on osteoclast differentiation in comparison to the empty vector control. This indicates that binding to the β integrin alone is not enough to active differentiation. The effects of the F3 domain on osteoclast resorption were looked at as well. When the F3 domain was overexpressed in RAW cells, differentiated into osteoclasts, and allowed to resorb on synthetic bone for three days, we saw changes in resorption. In comparison to the control, the F3 domain led to a decrease in clearing size and total resorption. This demonstrates that the F3 domain plays a role in osteoclast resorption. Due to the decrease in resorption by overexpressing just the F3 domain

in a dominant negative fashion, it can be hypothesized that the F3 domain positively regulates resorption.

The effects of the overexpression of the F1 domain were also studied. The overexpression of F1 did not lead to a change in the percent TRAP+ cells when studying differentiation indicating it does not play a role influencing differentiation. When resorption was studied, it was shown that the clearing size and the total resorption decreased in comparison to the control. This also indicates that the F1, like the F3, domain influences resorption. Since all three FERM domains were shown to decrease clearing size and total resorption, it can be hypothesized that all three domains are necessary to activate proper resorption.

When the PH domain was inserted into a plasmid and introduced into osteoclasts to create a stable cell line overexpression the PH domain, TRAP staining demonstrated no change in the degree of differentiation in comparison to the control, even though it divides the F2 subunit which does play a role in resorption. Although the PH domain can bind the cell membrane and recruit molecules, it does not appear that is binds any molecules that activate differentiation. The effects on resorption were also examined using synthetic bone. After three days of resorption, the clearing size decreased in comparison to the control, but the total resorption increased. This can be explained by the increase in the number of pits as seen in Figure 8A. This indicates the possibility that the PH domain negatively influences resorption. The role of the PH domain in wild type kindlin3 in osteoclasts would be to inhibit resorption to work in concert with the three FERM domains that act to activate resorption in order to keep osteoclast function at the required rate for healthy bones.

In conclusion, this study has demonstrated some of the effects of kindlin3 on osteoclast function. It was shown that the overexpression of kindlin3 lead to a decrease in differentiation

which was narrowed down to the F2 subunit. Future studies will hopefully indicate which of the two F2 subdomains, the F2a or the F2b, region is responsible for influencing osteoclast differentiation and possible binding partners that work in conjunction for proper osteoclast function. It has also been demonstrated that all three FERM domains and the PH domain contribute to osteoclast resorption, potentially by interacting with each other to give healthy bone turnover.

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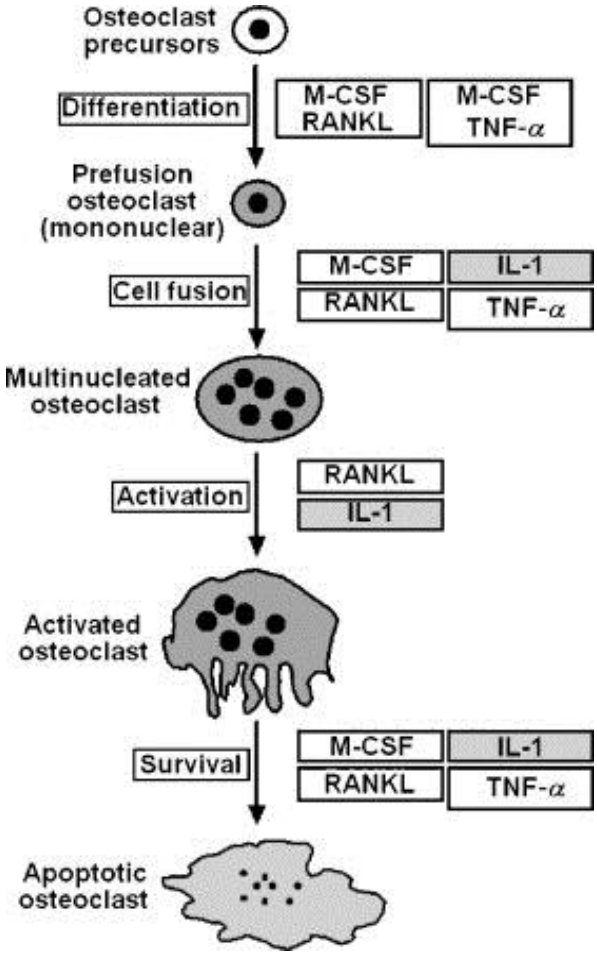


Figure 1 A depiction of the process of osteoclast differentiation and activation (Nakamura et al, 2006).

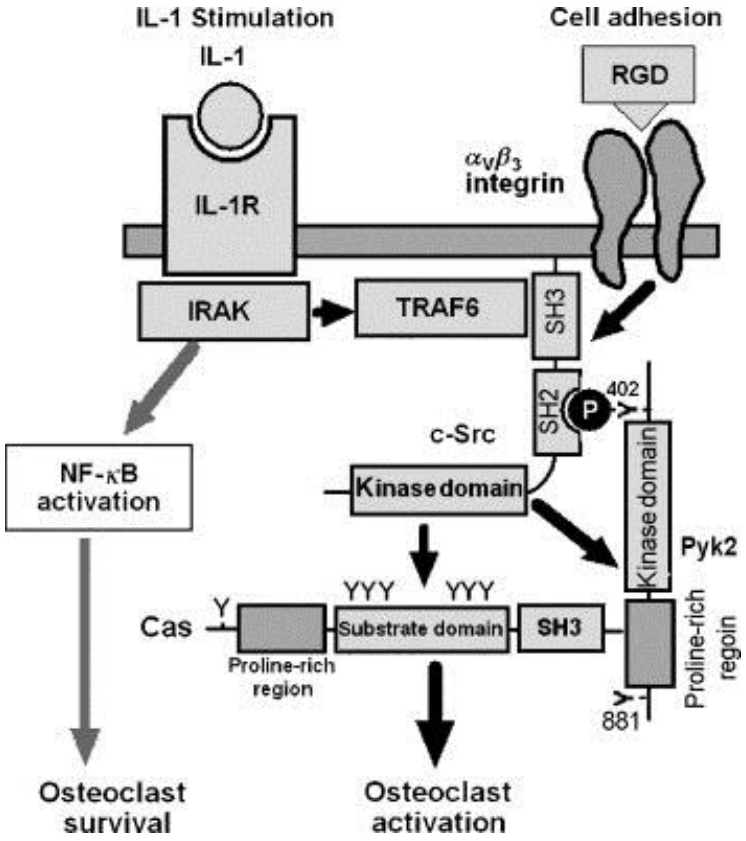


Figure 2 A signaling pathway in osteoclasts that relies on IL-1 and the RGD region of αvβ3 integrin (Nakamura et al, 2006).

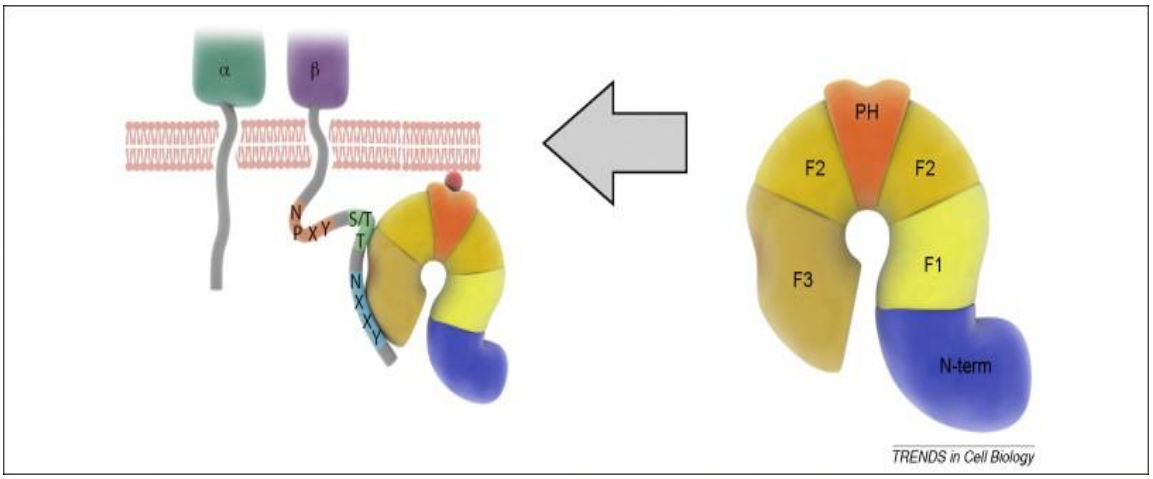


Figure 3 A depiction of kindlin3, folded in its native form, binding through the F3 domain to the β integrin tail (Meves et al, 2009)

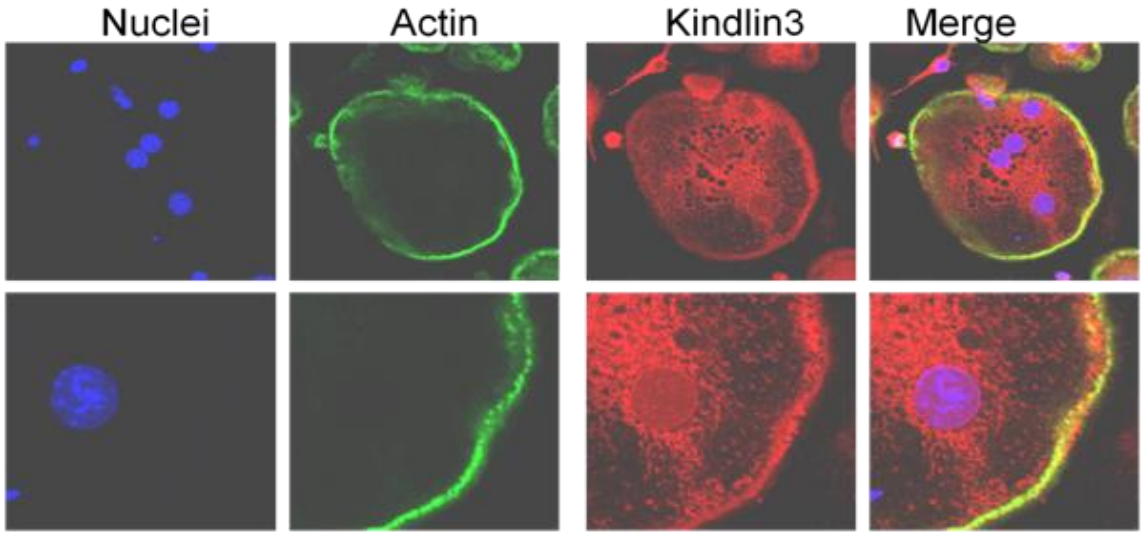


Figure 4 Cell staining showed that kindlin3 is found throughout the cell and surrounding the nucleus.

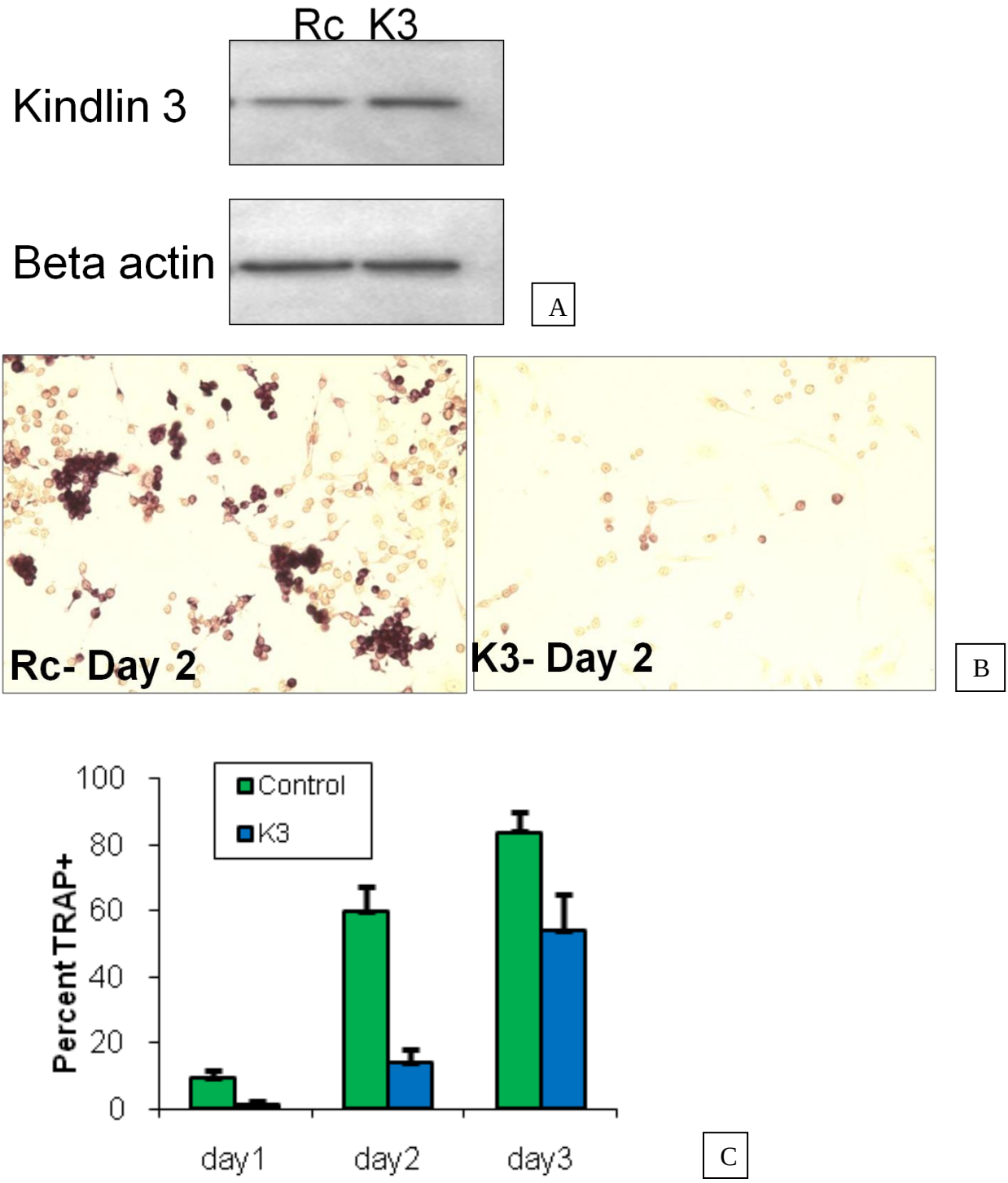


Figure 5 A. Western analysis showed the kindlin3 is overexpressed two fold in osteoclasts. B. TRAP staining showed that overexpression of kindlin3 lead to decreased differentiation. C. The number of TRAP+ cell was quantified to show a consistent decrease in differentiation over the first three days in comparison to the control.

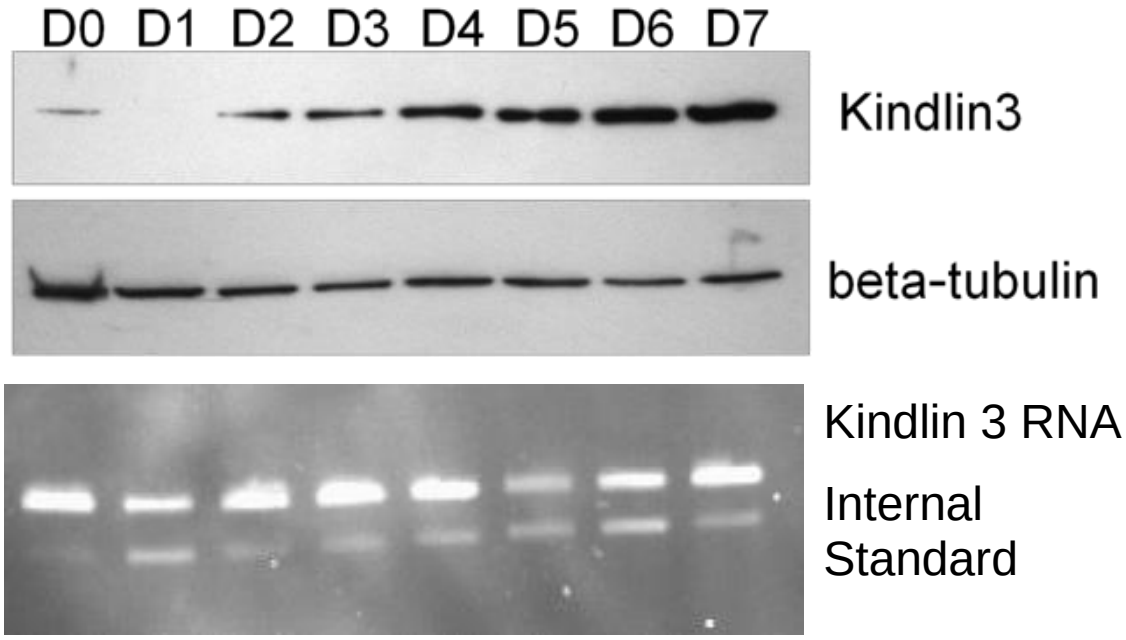


Figure 6. Western analysis showed the kindlin3 is unregulated during seven days of differentiation. Competitive RT-PCR shows the RNA level remain constant over the same seven days differentiation period.

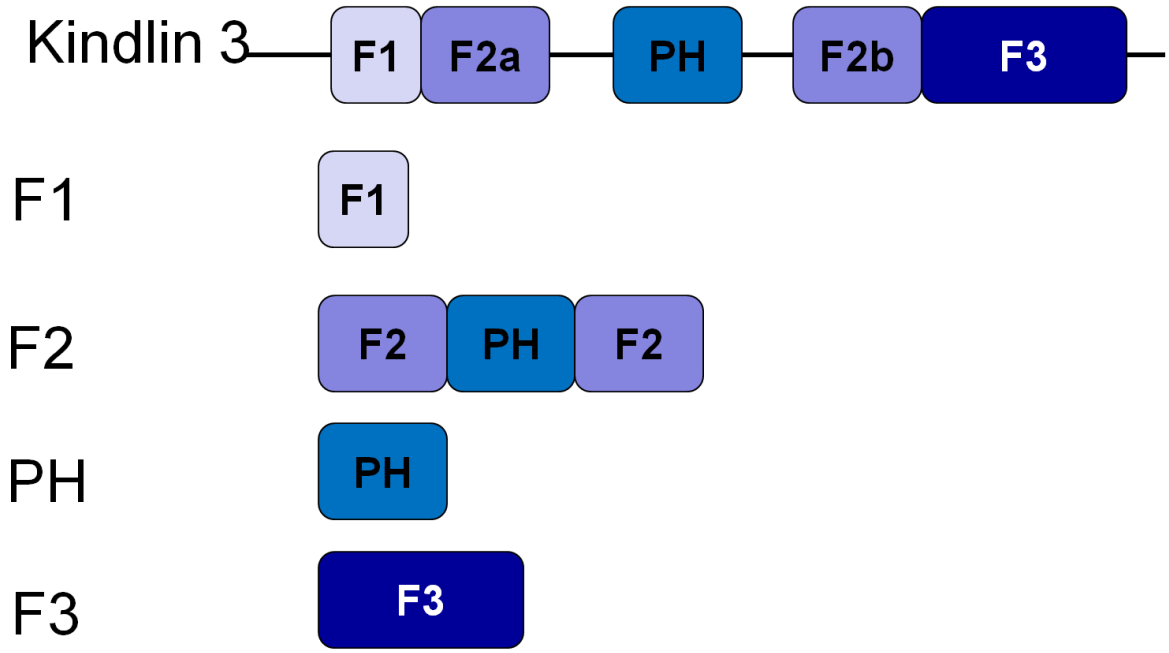
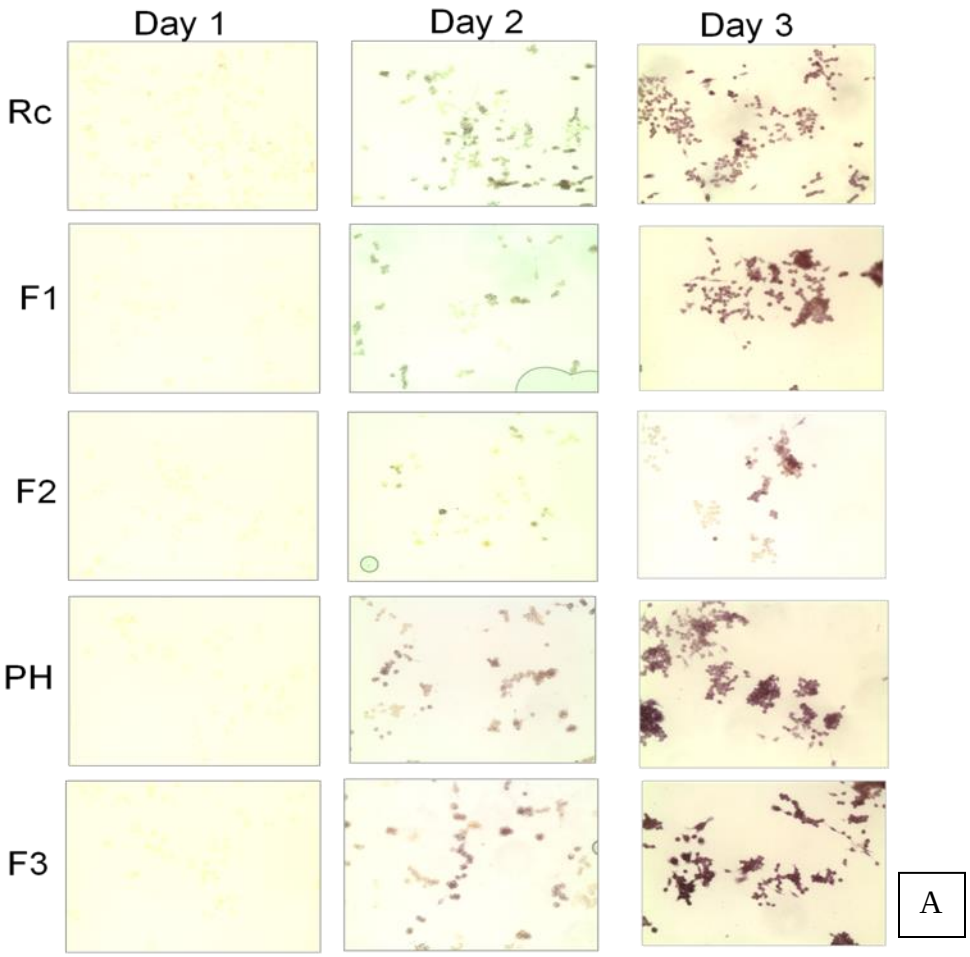


Figure 7 Four different plasmids were made containing the various tail domains



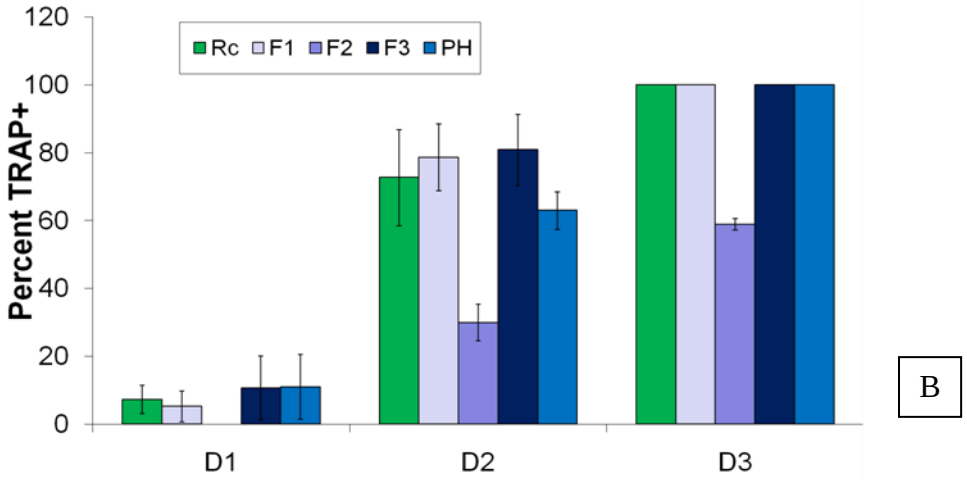
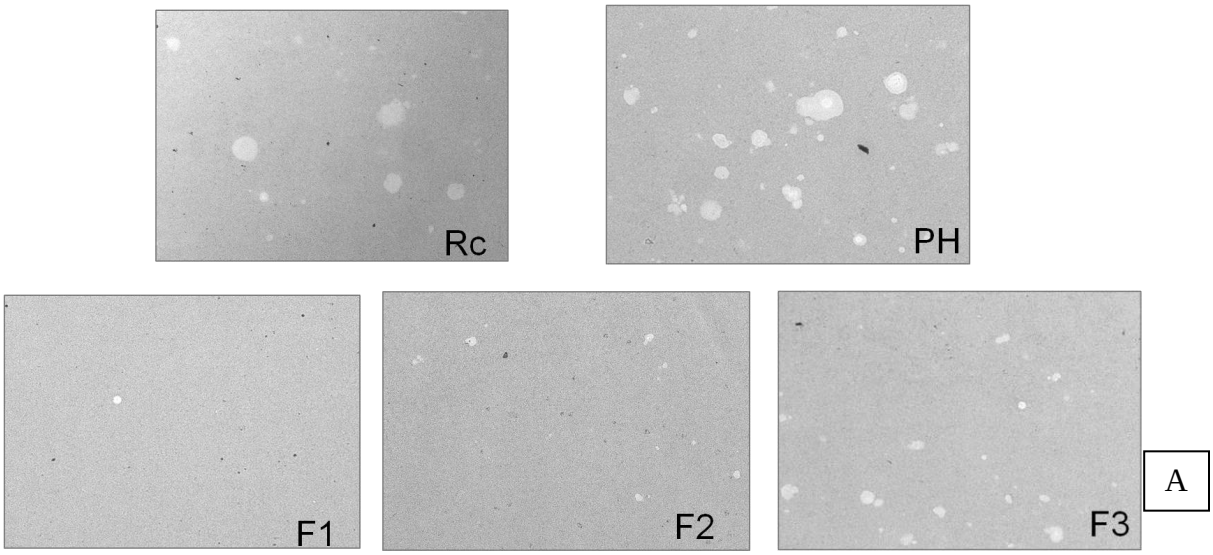


Figure 8 A. TRAP staining shows that the overexpression of the F2 subunit leads to decreased differentiation. B. Quantitative analysis shows the number of TRAP+ cells is consistently decreased for the F2 subunit over the first three days of differentiation.



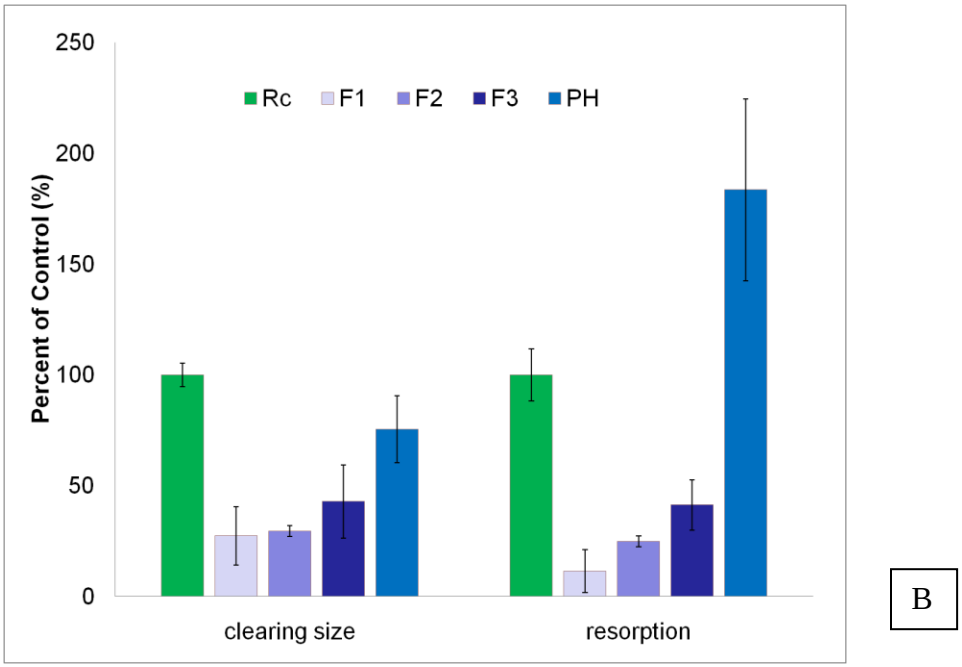


Figure 9 A. Stable cell line clones of kindlin3 subunits were differentiated and placed on synthetic bone to assess resorption. B. All five subunit changed osteoclast resorption. The FERM subunits all decrease resorption and clearing size. The PH domain decreases clearing size but increases resorption due to an increase in number of pits.