Adseverin is a Key Regulator and Marker of Osteoclastogenesis

by

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Faculty of Dentistry
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Abstract

The intricate processes of osteoclastogenesis are highly dependent on the dynamic regulation of the actin cytoskeleton. Adseverin, a member of the gelsolin superfamily of actin binding proteins, regulates actin remodeling by severing and capping actin filaments in a calcium dependent manner. The objective of this project was to characterize the role(s) of adseverin during osteoclastogenesis, by assessing adseverin expression throughout osteoclastogenesis and through differentiation assays using a knockdown strategy. **Methods:** qRT-PCR and immunoblot analyses were used to examine adseverin expression during osteoclastogenesis. A stable adseverin knockdown macrophage cell line was generated using a retroviral shRNA construct. **Results:** Adseverin expression increased significantly in response to RANKL during the early phases of osteoclastogenesis, and adseverin was highly expressed in mature osteoclasts. Adseverin knockdown macrophages experienced a major osteoclastogenesis defect, most likely caused by a defect in pre-osteoclast fusion. **Conclusion:** Adseverin is a RANKL induced early and pro-fusion marker of osteoclastogenesis.
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Abbreviations

Ads – Adseverin
AP -1 Activator Protein 1
BM – Bone Marrow
BMM – Murine Bone Marrow Monocytes
BMP -2 - Bone Morphogenic Protein-2
BMU – Basic Multicellular Units
DAP12 - DNAX-Activating Protein 12
ECM- Extracellular Matrix
FACS - Fluorescence-Activated Cell Sorting
FBGC - Foreign Body Giant Cells
FBS – Fetal Bovine Serum
FcRγ -Fc Receptor Common γ chain
FOV – Field of View
GPCR - G-Protein Coupled Receptor
IκB - Inhibitors of κB
IgSF – Super Family of Immunoglobulins
IL – Interleukin
ITAM - Immunoreceptor Tyrosine-based Activator Motif
LPA - Lysophosphatidic Acid
MAPK – Mitogen Activated Protein Kinase
M-CSF – Macrophage Colony Stimulating Factor
MARCKS - Myristoylated Alanine-Rich C Kinase Substrate
MGP - Matrix GLA Protein
MITF - Microphthalmia-Associated Transcription Factor
NFAT - Nuclear Factor of Activated T cells
OCG – Osteoclastogenesis
OCN - Osteocalcin
OPG – Osteoprotegerin
PBS – Phosphate Buffered Saline
PI3K- Phosphatidylinositol 3-Kinase
PI – phosphatidylinositol
PIP - Phosphatidylinositol-4-monophosphate
PIP2 - Phosphatidylinositol-4,5-bis-phosphate
PIR-A - Paired Immunoglobulin-like Receptor A
PKC - Protein Kinase C
PMSF - Phenylmethylsulfonyl Fluoride
PS – Phosphatidylserine
qRT-PCR – Quantitative Real-Time Polymerase Chain Reaction
RANKL – Receptor Activator of NF-κB Ligand
RAW- RAW 264.7 Macrophages
RGD - Arg-Gly-Asp peptide sequence
SIRP β1 - Signal-Regulatory Protein β1
TBST – Tris Buffered Saline with 0.05% v/v Tween 20
TGF-β – Transforming Growth Factor beta
TIRF – Total Internal Reflection Fluorescent microscopy
TNF-α - Tumor Necrosis alpha
TRACP - Tartrate-Resistant Acid Phosphatase
TRAF - Tumor Necrosis Factor Receptor-Associated Factor
TRAM-2 - Triggering Receptor Expressed in Myleoid cells-2
WASP - Wiskott Aldrich Syndrome Protein
WIP – WASP Interacting Protein
WT – Wild Type
Chapter I
Literature Review

I. Mammalian Skeletal Bone Turnover

A. Characterization of Mammalian Bone Matrix

The bone extracellular matrix (ECM) has historically been described as a static and protective scaffold composed of organic fibrils imbedded in an amorphous matrix with the sole purpose of providing a backdrop for cellular adhesion and mineral nucleation (1). However, not only is the bone ECM highly dynamic and subject to periodical remodeling, but the molecular composition of the ECM can have significant implications on cellular behavior (1,2). For instance, resident ECM molecules can activate second-messenger signal pathways, alter gene expression and influence adhesion, migration, proliferation, differentiation and apoptosis of bone residing cells. Through this intimate interaction with bone residing cells, the bone ECM can actually mediate its own composition in a feedback process known as “dynamic reciprocity” (3). Furthermore, the ECM serves as a protective warehouse for secreted growth factors by prolonging the half life and minimizing diffusion of sequestered growth factors and chemokines (1). The remainder of this section will provide a brief description of the composition of the bone ECM in terms of its inorganic mineral phase as well as it organic phase.

i. The Mineral Phase of Bone Extracellular Matrix

The cardinal characteristic of mineralized tissue is calcium phosphate. In bone, osteoblasts control the deposition of calcium and phosphate into the bone matrix through the secretion of hydroxyapatite ([Ca\textsubscript{10}[PO\textsubscript{4}]\textsubscript{6}][OH\textsubscript{2}]), thus making bone a significant storehouse for calcium (1). Secretion of hydroxyapatite and bone matrix mineralization is highly sensitive to and regulated by extracellular phosphate concentrations (4). On the other hand, bone mineralization is inhibited by the presence of crystal inhibitors such as pyrophosphate (5) and matrix GLA protein (MGP) as well as osteocalcin (OCN) (4). These mineralization inhibitors are ubiquitously expressed in the bone matrix and inhibit mineralization by preventing the incorporation of phosphate ions into
to newly formed hydroxyapatite crystals. Therefore, in order for mineralization to occur, the factors that promote mineralization must outweigh the factors that inhibit crystal nucleation (1).

**ii. The Protein Phase of Bone Extracellular Matrix**

In addition to the mineralized matrix, the bone ECM is also rich in organic matter. Collagen constitutes more than 90% of the organic component of the bone ECM and is essential for proper hydroxyapatite formation (6). Type I collagen is the predominant collagen found in the bone matrix, although other collagens (e.g., collagen type III, V, XI and XIII) are also present within the bone ECM. In addition, the bone ECM contains over 160 non-fibrillar proteins, most of which are not directly involved in bone formation but rather serve important regulatory roles by influencing the dynamic reciprocity of the ECM (1,7). The non-collagenous ECM protein can be characterized by their structure into four groups: γ-carboxyglutamic acid-containing proteins, glycoproteins, sialoproteins and proteoglycans (for a detailed review see ref. (1)).

**B. Bone Remodeling Cycle**

The mineralized and organic connective tissue matrix of the skeleton is organized in a manner to provide maximal strength with the minimal mass (8). During growth and development, the skeleton is consistently sculpted to achieve its final shape and size. In maturity the skeletal regeneration continues via periodic removal and deposition of bone, which allows the skeleton to maintain its strength and integrity (8,9). The task of skeletal remodeling falls primarily in the domain of the two major bone residing cells; osteoclasts which function to degrade the inorganic and organic phases of bone (10) and osteoblasts, which are responsible for producing and secreting the matrix proteins in addition to regulating matrix mineralization (11). Therefore skeletal remodeling requires spatial and temporal orchestration of osteoclast and osteoblast function. *In vivo* this harmonious process is achieved through the formation of transient structures called basic multi-cellular units (or BMUs), comprised of a central vascular capillary and nerve supply fronted by a cluster of bone resorbing osteoclasts and rounded out in the rear by a team of matrix secreting osteoblasts. As the BMUs travel across the bone surface, the frontier osteoclasts excavate and degrade old bone all the while trailing osteoblasts, migrate into the freshly excavated areas and secrete new matrix (11). Therefore, as demonstrated by the spatial organization of BMUs, bone resorption and formation are not separate, independently
regulated processes, but rather highly intertwined and “coupled” (11,12). Recent advances in bone biology have identified the molecular mechanisms that “couple” bone formation and resorption at the macroscopic level. Specifically, osteoblasts synthesize and secrete the microenvironment that supports osteoclast growth, maturation and function. Furthermore, osteoblasts express and secrete a series of cytokines that control osteoclast formation and activity (e.g., M-CSF, IL-1, IL-6 and RANKL) (13). Conversely, osteoblast differentiation and activation are regulated by osteoclast activity. For instance, bone resorption by osteoclasts releases latent TGF-β, which serves as a potent stimulant of osteoblastogenesis (14). In addition, osteoclasts secrete two potent inducers of osteoblast differentiation in the form of bone morphogenic protein-2 (BMP-2) (15) and cathepsin K (16), as well as Mim-1, a chemoattractant of osteoblast precursors (17). All together, these factors potentiate osteoblast differentiation and function, leading to increased bone formation (18). This clear continuity and connection between osteoblasts and osteoclasts is what orchestrates the sequential cycle of bone degradation and formation leading to homeostatic bone remodeling (10).

C. Bone Remodeling Disorders and Their Impact on Human Health

Under normal circumstances bone destruction and formation are in steady state equilibrium. However, imbalances in bone remodeling can result in perturbations of skeletal structure, integrity and function as well as morbidity and shortened lifespan (8). The following section will describe some common problems that arise from imbalances in bone remodeling and their impacts on human health.

i. Osteoporosis

Osteoporosis is a skeletal disease caused by a systemic loss of bone density and mass, which leads to the deterioration of the skeletal microstructure and formation of fragile bones (19). Due to the loss of bone mass, osteoporosis is associated with increased risk of fractures, with hip, vertebral body and forearms fractures being the most common (20). Osteoporotic fractures pose a major risk to the elderly population, as fractures can immobilize patients for prolonged periods and increase the risk of mortality by up to 20% in the first year following the fracture. However, the mortality risks associated with osteoporotic fractures vary greatly depending on the type of
the fracture and few deaths are directly linked to the bone fractures. Instead, most osteoporosis related deaths are a result of chronic illnesses that caused the fall/fracture in the first place or acute events such as infections and postoperative complications (19). In recent years, the percentage of the worldwide population over 65 years of age has grown dramatically and is projected to steadily increase over the next 20 years. With age being one of the major risk factors of osteoporosis, osteoporotic related disorders are becoming an ever growing public health concern (19). As of 2003, over 70 million people worldwide suffered from osteoporosis (8) and it is now estimated that more than 50% of Caucasian women and 20% of Caucasian men over 50 years of age will experience an osteoporotic fracture in the remainder of their lifetime (21). Therefore, as the incidence of osteoporotic fracture rise so will it’s economic burden. Recent estimates on the combined annual costs of all osteoporotic fractures have been estimated to be over 50 billion dollars in the US and Europe (22) and over 130 billion dollars worldwide (23). Based on these numbers it is easy to see why so much time and money has been devoted to understanding the mechanisms that lead to osteoporosis. At the cellular level osteoporotic bone loss occurs because of an imbalance between the normally synchronous activity of bone resorbing osteoclasts and bone depositing osteoblasts (19). This imbalance can be caused by a number of factors including age, sex steroid deficiencies, excess glucocorticoids, reduced mechanical loading, multiple myelomatosis and hyperparathyroidism (9). For instance, excess glucocorticoids suppress osteoblastogenesis and promote osteoblast apoptosis (24), thus leading to decreased bone formation, decreased trabecular wall thickness and bone necrosis (11). With age, bone formation during each remodeling cycle decreases (25) and the rate of bone loss starts to overtake the rate of bone formation, leading to systemic bone loss (9). This age induced imbalance is in part caused by a shift in differentiation of multipotent mysenchymal cells towards adipocytes instead of osteoblasts, leading to fewer marrow osteoblast progenitors and mature osteoblasts as well as diminished bone deposition (26). Another cause of osteoporosis is estrogen deficiency (in postmenopausal women), which creates an imbalance in bone remodeling by promoting the expression of cytokines that induce osteoclast differentiation and activation (e.g., RANKL, TNF-α, IL-6, M-CSF and prostaglandin E) while suppressing the expression of osteoclastogenesis inhibiting agents (e.g., OPG) (10,27). Therefore, estrogen induced osteoporosis is predominantly caused by enhanced bone resorption rather than diminishing bone formation (28).
ii. **Osteopetrosis**

Osteopetrosis is a family of inheritable conditions characterized by marbled-like bone with increased mass and density as well as radiographic loss of distinction between the cortex and marrow cavity (13). In severe osteopetrotic cases the marrow cavity is filled with endochondral bone decreasing the size of the marrow cavity. The diminished marrow is unable to support adequate hematopoiesis, resulting in extramedullary hematopoiesis and hepatosplenomegaly (13). Children with severe osteopetrosis can also suffer from cranial-nerve dysfunction, visual defects, thrombocytopenia and anemia which can be fatal within the first decade of life (29). In less severe cases of osteopetrosis, patients experience normal life expectancy, but have brittle bones that are more susceptible to fractures (30).

Osteopetrosis can be caused by a number of defects that impair osteoclastogenic bone resorption. Specifically, any mutation that arrests osteoclastogenesis or inhibits osteoclast function and activation can cause osteopetrosis (for review see (13)). For instance, loss of function mutations in genes that are involved in precursor commitment to the osteoclast lineage and/or in the early phases of osteoclast differentiation such as: *mcsf* (31), c-fms receptor (32), PU.1 (33) and Mitf (34), all cause osteopetrosis. In addition, transgenic mice over-expressing the osteoclastogenesis inhibitor osteoprotegerin (OPG), lack osteoclasts and are severely osteopetrotic (35). Similarly, loss of function mutations in genes involved along the osteoclast specific RANKL-RANK signaling axis, such as: c-fos (36), NF-κB (37) and TRAF6 (38) cause osteopetrosis. Furthermore, osteopetrosis is caused by mutations in genes involved in mature osteoclast function including c-src, cathepsin K, TRAP, the vitronectin integrin receptor avβ3, α3 subunit of the ATP-dependent proton pump Atp6i, carbonic anhydrase II, and the chloride channel CLC-7 (39-44). It should be noted that the osteopetrotic phenotype observed in the aforementioned transgenic mice models was specifically caused by the inability of osteoclasts to polarize, spread and degrade the bone matrix and not by a loss of function in osteoclast precursor populations (13).

iii. **Inflammatory Bone Disorders**

Inflammatory osteolysis, common in disorders such as rheumatic and psoriatic arthritis, is characterized by the destruction of articular cartilage by excessive bone resorption resulting in
joint collapse (9,45). Under inflammatory conditions, the synovial membrane is rich in macrophages. These macrophages are stimulated to undergo osteoclastogenesis in response to osteoclastogenic cytokines (e.g., RANKL, IL-6, IL-11, IL-13, IL-17) (46) secreted by synovial fibroblasts and activated T-cells (45). Furthermore, many inflammatory cytokines present in the synovial fluid including TNF-α, IL-1, IL-6 and MCP-1 can enhance osteoclast differentiation and resorptive activity (47). Although no single cytokine is responsible for osteoclast formation and activation leading to bone erosion and rheumatoid arthritis (9), animal models have shown that a blockade in RANKL mediated osteoclastogenesis leads to significantly dampened bone erosion even in the face of severe inflammation (48). Another common form of inflammatory bone diseases is periodontal disease. Periodontal disease is caused by an accumulation of plaque producing bacteria that destroys the cellular and structural components of the periodontium. This creates an inflammatory immune response that stimulates osteoclast recruitment and activation leading to alveolar bone destruction in a molecular process similar to that operating under rheumatoid arthritis (9).

iv. Paget’s Bone Disease

Paget’s disease is a bone remodeling disease characterized by excessive bone formation and resorption due to the heightened presence of large, hyperactive osteoclasts. As a result the remodeled bone has a disorganized, woven appearance and is highly vascular, bulky, weak and prone to bowing and fractures. Paget’s disease occurs in nearly 3% of U.K women over the age of 40, with slightly lower prevalence in North American women. The disease transmission appears to be caused by an autosomal dominant mutation as approximately 40% of patients have a first-degree relative with the disease (9,45). However, viral inclusions are also observed within the nuclei of osteoclasts isolated from Paget’s disease patients, which suggests that the disorder might result from infection with viruses of the paramyxovirus class such as measles, respiratory syncitial and canine distemper viruses (49).

v. Therapies

Understanding the molecular mechanisms of bone metabolism is crucial for development of novel drugs for treating bone-related disease (50). Even though homeostasis in bone remodeling
requires the collaborative action of osteoblasts and osteoclasts, the common thread to all of the aforementioned bone disorders is abnormal or defective osteoclast bone resorption. In fact, efforts aimed at creating therapeutic agents designed to treat bone disorders primarily concentrate on the development of drugs that inhibit bone resorption through decreasing either the formation or activity of osteoclasts. To date, all successful anti-osteoporosis agents identified such as estrogens and bisphosphonates target osteoclasts (reviewed in (9,45)).
II. Osteoclasts

Mature osteoclasts are large (50-100 µm diameter), terminally differentiated multinucleated cells with well developed Golgi apparatus and abundant mitochondria, lysosomes, vacuoles and ribosomes (51). Osteoclasts also possess unique plasma membrane projections known as the ruffled border that is surrounded by an actin rich area called the clear zone or sealing zone, which together form the resorptive machinery of the osteoclast. The sealing zone also mediates the attachment of the osteoclast to the bone matrix while clearly defining the quarantined microenvironment immediately underneath the osteoclasts that will be degraded (11,45,52). Once firmly attached to the bone matrix, osteoclasts degrade the mineral and organic phases of the bone matrix via acidification and proteolytic degradation, respectively (53). The degraded matrix components are then endocytosed into the osteoclast, transcytosed across the cell and exocytosed through the basolateral membrane facing the bone marrow (54).

Osteoclastic bone resorption can be subdivided into multiple steps including: proliferation of osteoclasts progenitors, differentiation of the progenitors into mononuclear pre-osteoclasts, fusion of pre-osteoclasts into multinucleated osteoclasts and mature osteoclast bone degradation (53). This section will provide a detailed summary of the major steps involved in each of these processes.

A. From Monocytes to Osteoclasts: Molecular Mediators of Osteoclastogenesis

Osteoclasts are tissue specific multinuclear giant cells derived from hematopoietic stems cells (55,56) of the macrophage/monocyte lineage (57). The intricate pathways that regulate the osteoclast formation or osteoclastogenesis have yet to be fully elucidated. However, a number of major signaling molecules present in the bone marrow environment have emerged as critical osteoclastogenesis signals. This section will provide a detailed background on a number of the most important signaling molecules.

i. PU.1 and Microphthalmia-Associated Transcription Factor

The earliest known molecular mediator of osteoclastogenesis is the ETS domain transcription factor PU.1. PU.1−/− mice suffer from osteoclast-deficiency induced osteopetrosis, yet PU.1 null
cultures can generate cells with monocytic characteristics (58). Slightly downstream of PU.1, the microphthalmia-associated transcription factor (MITF) family of proteins (including TFE3, TFEB, TFEC and MITF) is required for the differentiation and survival of osteoclast progenitors (59). Similar to PU.1 null mice, MITF null (mi/mi) mice have abundant macrophages but fail to form functional multinucleated osteoclasts and are therefore osteopetrotic (59). In addition, mononuclear mi/mi osteoclasts experience accelerated death due to diminished expression of the anti-apoptotic molecule Bcl2 (60). Therefore PU.1 and MITF are critical for the early differentiation of bone marrow progenitors into osteoclast precursors. Both PU.1 and MITF are also involved in the transcriptional control of a number of osteoclast-specific genes such as TRAP, carbonic anhydrase II, cathepsin K and OSCAR (61-64).

ii. Macrophage Colony Stimulating Factor

A significant breakthrough in the understanding of osteoclastogenesis was made when cocultures of stromal and bone marrow cells yielded osteoclasts (57). This finding suggested that cell-cell signaling between osteoclast progenitors and stromal mesenchymal cells was required for osteoclastogenesis (64). We now know that stromal cells, particularly marrow osteoblasts, express two critical hematopoietic cytokines: Macrophage Colony Stimulating Factor (M-CSF or CFS-1) and Receptor Activator of NF-κB Ligand (RANKL), both of which are essential and sufficient for osteoclastogenesis (8). The pivotal role of M-CSF during osteoclastogenesis was first described in op/op mice, which lack functional M-CSF, have no mature osteoclasts and are osteopetrotic. We now recognize that M-CSF regulates osteoclastogenesis downstream of PU.1 and MITF by promoting the survival and proliferation of osteoclast precursors, which increases the size of the osteoclast committed precursor pool (65). M-CSF exerts its effects through a receptor tyrosine kinase, c-Fms, found on the surface of both osteoclast precursor and mature osteoclasts. The binding of M-CSF to c-Fms activates downstream signaling cascades including the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) as well as the mitogen-activated protein kinase (MAPK) pathways which are critical for osteoclast precursor survival and proliferation (45). M-CSF signaling also activates the MITF transcription factor, which binds to the Bcl-2 promoter and induces the expression of the anti-apoptotic protein Bcl-2 (60). In mature osteoclasts, M-CSF promotes cell spreading (66), migration and cytoskeletal rearrangements (53) through signaling cascades involving c-Src and PI3K (reviewed in (59)).
iii. **Receptor Activator of NF-kB Ligand**

M-CSF is clearly an essential survival and proliferation factor for osteoclast precursors. However, arrested osteoclastogenesis exhibited in csf-1 mutant mice can be overcome via the over-expression of the anti-apoptotic protein Bcl-2 (67). Therefore, another factor must be involved in directly controlling the differentiation process during osteoclastogenesis. Using a series of genetically modified animal models, it is clear now that RANKL and downstream signaling from its receptor RANK, are indispensable for osteoclastogenesis (68). Evidence supporting RANKL’s pivotal role during osteoclastogenesis comes from three sources. Firstly, RANK and RANKL deficient mice with normal macrophage numbers failed to generate osteoclasts and were severely osteopetrotic. Conversely, RANKL over-expressing animals had hyper-resorptive osteoclasts and suffered from severe osteoporosis. Furthermore, the soluble RANKL decoy receptor, OPG, inhibited osteoclast formation and caused severe osteopetrosis in animal models (69,70). Therefore, RANKL and RANK signaling are both absolutely necessary during osteoclastogenesis.

RANKL (also known as ODF, TRANCE and TNFSF11), is a member of the Tumor Necrosis alpha (TNF-α) family of proteins and is produced by marrow stromal cells and osteoblasts in response to a variety of stimuli including: calcitriol, parathyroid hormone, TNF-α, IL-1, IL-11, LPS and low gravity (reviewed in (12)). RANKL exerts its osteoclastogenic influence by interacting with its receptor RANK, found on the surfaces of macrophages and mature osteoclasts. The RANK receptor lacks intrinsic enzymatic activity in its cytosolic domain, and therefore transduces signals primarily by recruiting the adaptor molecule Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6). RANK also activates Activator Protein 1 (AP-1) and calcium signaling via c-Fos and DNAX-activating protein 12 (DAP-12), respectively. Ultimately, TRAF6, c-Fos and calcium signaling pathways culminate in the induction of the master regulator of osteoclastogenesis nuclear factor of activated T cells (NFAT) c1 transcription factor (reviewed in (64)). The following section will describe the signaling cascade downstream of the RANK receptor, beginning with the recruitment of TRAF 6.
iv. **TNF Receptor-Associated Factor 6**

There are seven members in the TRAF family of adaptor proteins (TRAF 1 through 7) all of whom mediate signals induced by the TNF family of cytokines, including RANKL. The cytoplasmic domain of RANK can bind to TRAFs 1, 2, 3, 5 and 6. However, knockout studies have identified TRAF6 as the only essential adaptor molecule linking RANK to osteoclastogenesis (71). There is controversy regarding the exact role of TRAF6 in osteoclastogenesis as two independently Traf6−/− strains had two distinct osteopetrotic phenotypes. In one strain, osteopetrosis was caused by a complete lack of osteoclasts (72), whereas the second strain had abundant yet dysfunctional osteoclasts (38). *In vitro* studies support the former phenotype, as the addition of permeable peptides complementary to the RANK’s TRAF6 binding site inhibited osteoclastogenesis, which was supportive of a role of TRAF6 during osteoclast differentiation (73). The mechanism of TRAF activation is not yet fully understood. What is clear is that binding of TRAF6 to RANK induces the trimerization of TRAF6, which leads to downstream activation of the transcription factor NF−κB, as well as the activation of PI3K/Akt, and all three MAPK pathways (reviewed in (45,59,64,74)). Interestingly, other inflammatory cytokines, such as Interleukin and TNF-α, also use TRAF6 as a signaling adaptor. However, these factors don’t directly induce osteoclastogenesis. Therefore, it is necessary to outline the unique TRAF 6 signaling cascades downstream of the RANK receptor during osteoclastogenesis.

v. **Nuclear Factor kappa B (NFκB)**

NF−κB is one of the earliest RANKL activated molecules during osteoclastogenesis, and is required for precursor survival (75) as well as osteoclast differentiation (76). Deletion of NF-κB leads to osteopetrosis caused by the absence of multinucleated bone-resorbing osteoclasts, even in the presence of macrophages (77). Further, NF−κB inhibition had more profound consequences on osteoclastogenesis if administered during the early phases of osteoclastogenesis rather than the late phases of osteoclastogenesis. Together these observations suggest that NF-κB is an important early molecular mediator of osteoclastogenesis, slightly downstream of the aforementioned PU.1 transcription factor (59). The NF-κB family consists of 5 dimeric transcription factors that recognize κB DNA motifs including: Rel (cRel), RelA (p65), RelB, NF-
κB1 (p50) and NF-κB2 (p52) (74). Osteoclast differentiation seems to be under the direct control of both p50 and p52, as mice deficient in both p50 and p52 suffer from osteopetrosis due to a defect in osteoclastogenesis (77). NF-κB proteins normally reside in the cytoplasm of unstimulated cells where they exists as heterodimeric complexes with inhibitors of the κBs (IκBs). Upon stimulation with NF-κB activating cytokines (e.g., RANKL, TNF-α and IL-1) IκB kinases (α and β) are activated which phosphorylate serine residues of IκBs thus targeting them for ubiquitin-dependent proteasome degradation. IκB degradation exposes the NF-κB nuclear localization signal, permitting their nuclear transport within minutes (78). The interaction between TRAF6 and RANK is sufficient to activate NF-κB. However, dominant negative TRAF molecules cannot fully suppress NF-κB activation suggesting that TRAF-independent pathways are also involved in activating NF-κB (79). There is also evidence that RANKL induced calcium signaling (see below) is involved in the nuclear translocation of NF-κB, thus promoting osteoclast survival and differentiation (78).

Upon nuclear translocation, NF-κB acts in concert with other transcription factors to regulate osteoclastic specific gene expression (78). But what is the major NF-κB targeted gene during the early phases of osteoclastogenesis? As will be discussed in more detail below, NF-κB mediated induction of NFATc1 expressions appears to be the hallmark event during determination of the osteoclastic fate (80). Evidence in support of this claim comes from the observation that NF-κB is recruited to the NFATc1 promoter immediately after RANKL stimulation. In addition, the NFATc1 promoter contains κB DNA binding motifs. Furthermore, NF-κB inhibitors along with genetic mutations in p50/p52 NF-κB subunits can suppress RANKL-stimulated NFATc1 induction (74).

vi. c-Fos, Activator Protein-1 Complex and Mitogen Activated Protein Kinases

RANKL recruitment of TRAF6 also activates c-Fos and MAPK signaling pathways that ultimately lead to the activation of the transcription factor complex AP-1 (80). AP-1 is a transcription factor complex composed of Fos (c-Fos, FosB, Fra-1, Fra-2), Jun (c-Jun, JunB, JunD) and ATF (ATFa, ATF2, ATF3, ATF4, B-ATF) proteins (reviewed in (80)). c-Fos appears to be critical in AP-1 activation as well as RANKL mediated signaling, such that c-Fos null mice develop severe osteopetrosis due to a complete block of osteoclast differentiation (81). In
addition, c-Fos is recruited to the NFATc1 promoter within 24 hours of RANKL simulation, which promotes the autoamplification of NFATc1 (82). c-Jun is also critical for the activation of AP-1 and osteoclastogenesis, as transgenic mice expressing dominant negative c-Jun fail to develop osteoclasts and are osteopetrotic. Unlike, c-Fos however, members of the Jun family play redundant roles such that conditional knockout in any one of the three members do not lead to a complete blockage of osteoclastogenesis (83).

In vitro studies have also suggested that MAPKs are involved in the activation of the AP-1 complex (84). For instance, both p38α and β isoforms are involved in osteoclast formation and expression of osteoclast specific genes including cathepsin K (85). In addition, ERK1/2 kinases are activated by RANKL. However, their inhibition seems to potentiate osteoclastogenesis suggesting that they may negatively regulate osteoclastogenesis (86).

vii. Nuclear factor of activated T cells (NFAT) c1

NF-κB and AP-1 are not only activated by RANKL but by other inflammatory cytokines that are not known initiators of osteoclastogenesis, such as IL-1 (74). What sets RANKL signal transduction apart from other inflammatory cytokines is that RANKL signaling ultimately leads the activation of the transcription factor NFATc1. In fact, NFATc1 activation downstream of TRAF6, NF–κB and c-Fos/AP-1 pathways is now believed to be the most specific and strongly induced gene in RANKL mediated osteoclast differentiation (64).

The essential role of NFATc1 in osteoclastogenesis has also been demonstrated through the genetic knockout NFATc1 and exogenous over expression of NFATc1 which inhibited osteoclastogenesis and induced osteoclastogenesis in the absence of RANKL, respectively (80). NFATc1 is just one member of the NFAT transcription family (NFATc1-c5), originally identified in T-cells. The activation and nuclear translocation of NFAT transcription factors in these systems is under the control of the calcium/calmodulin regulated phosphatase calcinuerin (87). Similar mechanisms may also be involved in activating NFATc1 during osteoclastogenesis, as inhibition of calcinurin (with FK506) suppressed the induction of NFATc1 during RANKL mediated osteoclastogenesis. Furthermore, it appears that NFATc1 is recruited to its own promoter and is possibly involved in an auto-amplification cycle (74). Once activated, NFATc1 forms an osteoclast-specific transcriptional complex containing AP-1 (Fos/Jun), PU.1 and MITF
leading to the efficient induction of a number of osteoclast specific genes, including TRAP, cathepsin K, MMP-9, calcitonin receptor and β3 integrin. However, the components of the NFATc1 complex are not always the same. For instance, PU.1/MITF are not involved in calcitonin receptor expression but are involved in cathepsin K and OSCAR promoter induction (74,88).

viii. RANKL Activated Calcium Signalling

Calcium signaling is an important mediator in NFAT activation in a number of biological systems, including RANKL mediated osteoclastogenesis (89). The mechanism through which RANKL activates calcium signaling leading to the induction of NFATc1 is not immediately clear, as the RANK receptor and other TNF receptors are not directly involved in calcium signaling. Recent studies have identified a number of receptors including: OSCAR, Triggering Receptor Expressed in Myleoid cells-2 (TRAM-2), Signal-Regulatory Protein β1 (SIRP β1) and Paired Immunoglobulin-like Receptor A (PIR-A) that upon activation with unidentified ligands recruit two adaptor proteins, DNAX-Activating Protein 12 (DAP12) and Fc Receptor Common γ chain (FcRγ). The DAP12 and FcRγ adaptor proteins contain an Immunoreceptor Tyrosine-based Activator motif (ITAM), which is critical for calcium activation in a number of immune cells (reviewed in (64,74)). ITAM mediated calcium signaling via FcRγ and DAP12 appear to be critical during osteoclastogenesis as FcRγ−/− DAP12−/− double knockout mice suffer from severely impaired osteoclast differentiation (90). Furthermore, the induction of NFATc1 was suppressed in FcRγ and DAP12 deficient mice and the over expression of NFATc1 rescued osteoclastogenesis in these double knockout mice, which suggests that NFATc1 is a critical signal downstream of ITAM mediated calcium signaling (90). However, ITAM mediated calcium signaling is insufficient to induce osteoclastogenesis in the absence of RANKL, suggesting that these signals act in cooperation with RANKL (64). It is not clear how RANKL induces osteoclastogenesis in cooperation with ITAM, but RANKL does up regulate the phosphorylation of ITAM (90). Phosphorylated ITAM (DAP12 or FcRγ) recruits Syk kinases, which lead to the activation of the Phospholipase C γ (PLCγ). PLCγ induces the release of calcium from intracellular stores causing transient elevation of intracellular calcium and activation of calcium dependent singling pathways, such as the nuclear translocation of NF-κB and the activation of NFATc1 (78,89).
B. The Early Phases of Osteoclastogenesis

The rate of bone degradation is the product of osteoclast number and function. Osteoclast number is a reflection of the proliferative and differentiation capacity of osteoclast precursors plus the rate at which mature osteoclasts undergo apoptosis, while osteoclast function is a measure of the bone resorptive capacity of mature osteoclasts (91). As discussed in detail, a great number of molecular mediators are involved in the differentiation of monocytes into mature, multinucleated osteoclasts. It is also important to examine the events of osteoclastogenesis at the cellular level. This section is devoted to describing the functional steps involved during the early phases of osteoclastogenesis including: chemotaxis and migration, induction of fusion competency followed by cell-cell adhesion and fusion (92).

i. Migration

In bone, the formation of multinucleated osteoclasts is dependent on the fusion of hematopoietic/monocytic osteoclast precursors, while a similar mechanism involving fusion of macrophages at sites of chronic inflammation can lead to the formation of giant multinucleated macrophages. In order for fusion to occur, macrophages must first migrate towards each other (92). Macrophages are well known for their migratory capabilities, and it is their chemotactic ability that endows us with nearly all of our nonspecific immune response towards pathogenic entities (93). Much like in fibroblasts, migration in macrophage can be broken down into a number of steps including: i) the formation of small (20µm) surface protrusions called filopodia as well as directed protrusion of membrane-enclosed cytoplasm, or lamellipodia, at the leading edge of the cells, ii) attachment of the leading edge to the substratum, iii) contraction of cytoplasm and iv) release from the contact site at the tail of the cell (94). All of these events are heavily dependent on the dynamic regulation of the actin cytoskeleton. For instance, filopodia are supported by a core polymerized actin bundles while, the actin network within the lamellipodia are associated with numerous structural and regulatory proteins that create the molecular locomotive engine required for migration (95). Furthermore, the actin cytoskeleton is a key mediator of macrophage polarization and chemotaxis (94). Chemotaxis in macrophages starts with the interaction of a chemotactic factor with a specific heptahelical receptor embedded in the plasma membrane that induces signals via heterotrimeric G proteins. Downstream of these
G protein complexes, a number of target enzymes including PLC and PI3K are activated that lead to intracellular signaling cascades that culminate in cytoskeletal rearrangements and a chemoattraction response (96). In addition, macrophages/monocytes also respond to chemoattractants that signal through receptor tyrosine kinases, including M-CSF receptor (97). In fact, studies have shown that the addition of M-CSF to quiescent macrophages induces rapid rearrangements of the actin cytoskeleton, loss of adhesive structures and formation of lamellipodia leading to directed cell motility within minutes (98).

### ii. Fusion Competency

Cellular fusion is a common property of multicellular organisms and is required for fertilization as well as the formation of muscle, placenta, foreign body giant cells (FBGC) and osteoclasts (64). Cell-cell fusion during fertilization as well as myotube and placental formation is a natural component of development. However, macrophage fusion leading to the formation of FBGC and osteoclasts are physiologically rare events, because macrophages normally reside in tissues as mononuclear cells and only undergo fusion under specific tissue conditions. Therefore, in order for macrophages to fuse, they must first gain fusion competency to avoid uncontrolled fusion (92). Many of the studies carried out on macrophage fusion have focused on IL-4 stimulated FBGC formation. However, many of the characteristics involved in IL-4 induced FBGC formation are interchangeable with RANKL mediated osteoclastogenesis (92). IL-4 stimulation induces the formation of giant multinucleated macrophage due to the up regulation of fusion mediators including DAP12, CCL2, DC-STAMP, E-Cadherin, Rac1, DOCK180, phosphatidylserine (PS) and SIRPα/CD47. All of these fusion mediators, except for DOCK180 and PS, have been shown to be up-regulated in RANKL mediated macrophage fusion as well (reviewed in (92)). In addition to exogenous stimuli such as IL-4 or RANKL, macrophage proximity can also induce the up regulation of fusion mediators. For instance, mice deficient in the chemokine CCL2, fail to undergo chemotaxis and also fail to form giant multinucleated macrophages in response to IL-4. The CCL2 deficient mice also have lower expression levels of the important fusion receptor DC-STAMP, suggesting that proximity of macrophage is a prerequisite for macrophage fusion competency (99).
iii. Fusion and Fusion Receptors

Mononuclear phagocytes are unique in that they are ubiquitously expressed in tissues and under specific circumstances can be programmed to undergo fusion. Macrophage fusion requires an incredible physiological investment from each cell and therefore must serve a biologically beneficial purpose. This raises the questions, why do macrophages fuse to form multinucleated cells? And why don’t single, mononuclear macrophages simply work together as they do when they are phagocytic cells? The answer comes from the added value mononuclear phagocytes obtain upon fusion. Multinucleation increases the surface area and size of each cell, which allows them to degrade larger targets more efficiently and more importantly, extracellularly (100). Therefore, multinucleation allows one binucleate osteoclast to do what two mononuclear macrophages cannot (59). Despite the biological relevance of macrophage fusion, relatively little is known regarding the exact mechanisms that drive membrane fusion in macrophages. Much of what is known of membrane fusion comes from studies that have examined intracellular membrane fusion or viral fusion with the host cell membrane (92). Using viral fusion and intracellular vesicle trafficking as models we know that fusion is often mediated via a specific set of transmembrane proteins or fusion receptors (100). This section will provide a brief background on a number of surface molecules considered to be macrophage fusion mediators.

1. DC-STAMP

DC-STAMP was originally identified as an IL-4 inducible 53 kDa transmembrane protein with no considerable homology to other known macrophage fusion mediators (101). DC-STAMP is an essential mediator of macrophage fusion during RANKL induced osteoclastogenesis, because no multinuclear osteoclasts were identified in bone sections of DC-STAMP null mice, nor could multinucleated osteoclasts be generated in vitro using DC-STAMP null osteoclast precursors (102). Furthermore, mice lacking DC-STAMP develop a mild form of osteopetrosis (103). Interestingly, DC-STAMP deficient macrophages were capable of differentiating into mononuclear, tartrate-resistance acid phosphatase (TARCP) positive bone resorbing osteoclasts in response to RANKL, suggesting that DC-STAMP is not involved in osteoclast differentiation but rather cellular fusion. In addition, a co-fusion assay by Yagi and colleagues (102) demonstrated that DC-STAMP expression is only required on one of the two fusing partners. In this experiment, GFP expressing, DC-STAMP null bone marrow macrophages were cultured
with genetically wild-type macrophages. After differentiation, multinucleated, GFP expressing osteoclasts were observed suggesting that fusion occurred between a wild type “founding” cell and a DC-Stamp null, GFP expressing “follower”. This new binucleate cell then becomes a “master” fuser which incorporated other mononuclear precursors (104). Although it is not entirely clear how DC-Stamp mediates macrophage fusion, structural analysis of DC-Stamp suggests that it might function as a G-protein coupled receptors (GPCR). The structure of DC-Stamp is reminiscent of the HIV co-receptor and CXCR4 and Ste2, Ste3 and Ste4 yeast GPCRs that are required the initiation of fusion (105). Unfortunately, the ligand that activates DC-Stamp signaling has yet to be identified, but progress has been made. Recent studies have suggested that this putative ligand is likely a surface membrane protein expressed by macrophages (104), but the possibility of a soluble ligand cannot be excluded; further studies are required.

2. SIRPα/CD47

SIRPα also known as Macrophage Fusion Receptor (MFR), was the first ever identified molecular mediator of macrophage fusion. The critical role of SIRPα during macrophage fusion was realized when four monoclonal antibodies that had the ability to block macrophage fusion in culture, all recognized a 150 kDa protein, that was specifically and transiently expressed in macrophage at the onset of fusion (106). Interestingly, only SIRPα surface expression is induced at the onset of fusion, as abundant mRNA levels were detected in freshly isolated macrophages prior to the introduction of fusogenic conditions. This finding suggests that either SIRPα surface expression is regulated by post-translation modifications or that SIRPα is stored intracellularly until the onset of fusion (106). SIRPα, which also exists as a lower-weight isoform called MFR-s, belongs to the superfamily of immunoglobulins (IgSF) (100). The extracellular domains of SIRPα and MFR-s both bind to another IgSF protein essential for macrophage fusion, called CD47. Much like SIRPα, blocking antibodies against CD47 also blocked macrophage fusion. However, unlike SIRPα, CD47 is continuously expressed (107). One theory explaining the interaction between SIRPα and CD47 is that CD47 initially binds to SIRPα to secure the attachment of adjacent macrophages. Subsequently, CD47 switches to MFR-s to bring apposing plasma membranes to within 5nm of one another, which greatly increases the likelihood of spontaneous fusion (100). It is also believed that much like fusion
events in mating yeast or myoblast formation (108,109) CD47 can trigger cell-cell fusion by promoting calcium entry in to cell possibly through the formation of pores (110).

3. CD44

CD44 is another cell surface receptor that is strongly and transiently induced at the onset of macrophage fusion, suggesting it plays a role during fusion (111). Unfortunately, no cell surface ligand for CD44 has been identified. However, it has recently been discovered that the extracellular domain of CD44 is cleaved by MT1-MMP during fusion, which allows for more intimate interactions between opposing macrophage membranes thus facilitating fusion (100). In addition, cleavage of CD44 extracellular domain induces that cleavage and nuclear translocation of the intracellular domain which is thought to promote the activation of the osteoclastogenic transcription factor, NF-κB (100).

4. Phosphatidylserine

Membrane lipids have long been considered an inert barrier between the cell and the matrix. However, in recent years a growing body of evidence has suggested that the structural properties of the lipid bilayer such as the thickness of it hydrophobic core as well as the phospholipid composition of the bilayer can regulate intercellular interactions (112). This claim is perhaps best exemplified by the multidimensional role of the membrane lipid phosphatidylserine (PS). PS is best known for its ability to induce programmed cell death. Under normal circumstances, PS is actively sequestered to the inner leaflet of the plasma membrane bilayer. However, upon exposure to the outer leaflet, PS can be a potent apoptotic signal, shown to efficiently induce phagocytosis (113). In addition, when platelets are activated by thrombin and collagen, PS is rapidly exposed to the platelet outer leaflet. The exposed PS region becomes the assembly site of the tenase and prothrombinase complexes of the proteolytic cascade leading to the formation of fibrin matrix of the clot (114). Furthermore, studies have reported that PS exposure and recognition is a prerequisite for efficient cell-cell fusion during the formation of FBGC, myoblast fusion and trophoblast formation (112).

Even though PS exposure seems to be involved in fusion, it is not clear how it is regulated. Due to it’s potent and diverse signaling capabilities, PS is almost exclusively restricted to the inner
leaflet of the plasma membrane bilayer (112). In fact cells invest a great deal of energy to sequester PS to the inner leaflet of the bilayer. PS analogues introduced to the outer leaflet of the plasma membrane are rapidly transported to the inner leaflet via an ATP-dependent transporter called translocase. While, translocase acts to establish and maintain the PS asymmetry, another membrane protein acts to disrupt the membrane lipid asymmetry in response to elevated cytosolic calcium levels. Calcium elevations not only inactive translocase, but also activate a non-specific bi-directional lipid flip-site called scramblase. The activity of scramblase, rapidly randomizes the transbilyer lipid distribution to produce a lipid-symmetric membrane, which results in the exposure of PS on the cell surface (reviewed in (115)).

PS exposure, recognition or capture has not been implicated in osteoclast formation. But, PS exposure and recognition is required for macrophage fusion leading to the formation of FBGC (116). PS exposure can lead to phagocytosis thus making it difficult to distinguish between the process of the cell-cell fusion and phagocytosis. One hypothesis as to how cells circumvent this problem is that recognition of PS by a scavenger receptor or fusion receptors (e.g., CD36 or SIRPα respectively) can direct macrophage to undergo fusion or celllocytosis instead of phagocytosis (92).

C. Mature Osteoclast Function

i. Matrix Adhesion

Formation of an intimate physical contact between osteoclasts and the bone matrix marks the end of osteoclastogenesis and signals the start of bone degradation cycle by osteoclasts (59). Cellular attachment to the extracellular matrix is often mediated by integrins that anchor cells to the ECM and allow for the transmission of singles from the ECM into the cell (117). Osteoclast precursors and macrophages normally express the integrin Avβ5. However, during osteoclastogenesis Avβ5 is quickly replaced by Avβ3 (42), suggesting that Avβ3 is crucial for osteoclast attachment to the bone matrix. Avβ3 does in fact recognize several bone matrix proteins containing the RGD amino acid sequences including osteopontin, bone sialoprotein and vitronectin (118). In addition to Avβ3, osteoclasts also express other integrins, albeit at lower levels, such as the collagen/laminin receptor A2β1 and the vitronectin/fibronectin receptor Avβ1. All three of these
integrins are responsible for mediating the attachment of osteoclasts to the bone matrix (53). However, of all the integrins that mediate osteoclast adhesion to the ECM, Avβ3 seems to be the most critical (119). The following section will describe the role of Avβ3 integrin in osteoclast adhesion, polarization and signal transduction.

ii. Osteoclast Polarization

Osteoclasts derived from β3 null mice can only be described as highly dysfunctional as they have abnormal ruffled membranes, lack actin rings characteristic of active osteoclasts, fail to properly spread and migrate on bone and fail to efficiently resorb bone. As a result, β3 null mice suffer from progressive osteopetrosis, have low blood calcium levels and have increases skeletal mass (42,53). It is believed that the osteopetrotic defect seen in β3 null mice is not caused by an inability of osteoclasts to firmly attach to bone, but rather by a failure to transmit bone matrix derived signals that promote intracellular events responsible for initiating osteoclast polarization. Activation of Avβ3 leads to the recruitment of the tyrosine kinase, Syk which is phosphorylated and activated by another tyrosine kinase c-Src. Activated Syk phosphorylates Vav3, which in turn activates Rac. Activated Rac leads to rapid cytoskeletal rearrangements that lead to the osteoclast polarization characterized by the allocation of nuclei to the anti-resorptive surface, formation of the ruffled membrane and actin sealing zone as well as cell spreading, all of which are pivotal to bone resorption (45,51,53).

iii. Podosomes

Osteoclasts adhere to bone by forming distinct focal adhesion structures called podosomes, which consist of a central column of filamentous actin (F-actin), referred to as the podosome core, surrounded by a loose network of radial actin cables, called the actin cloud. Both the actin core and the actin cloud participate in osteoclast adhesion. The actin core adhesion is mediated by the receptor CD44 whereas adhesion in the actin cloud is dependent on Avβ3. Interestingly, osteoclasts remain adherent in the absence of CD44 mediated actin core attachment or in the absence of Avβ3 mediated actin cloud attachment, suggesting that they are both involved in the adhesion, possibly in a complimentary manner (119). A fascinating feature of podosomes is that they serve a larger function than simple adhesion. Podosomes appear early during osteoclastogenesis, and evolve into complex superstructures such as, transient rings during
intermediate stages of osteoclastogenesis and peripheral belts or sealing zones in mature osteoclasts (119). The sealing zone is one half of the osteoclast’s resorptive machinery, and is what allows osteoclast to specifically isolate the resorptive microenvironment from the general extracellular space (10). In addition, the rapid turnover of podosomes seems to be essential for the ability of osteoclasts to migrate (120). When osteoclasts are adherent on calcium apatite, they alternate between a resorptive phase and a migratory phase. During the resorptive phase, osteoclasts are rich in podosomes and they degrade the substrate outlined by the sealing zone (119). Shortly after, osteoclasts enter a migratory phase where they begin to spread, the sealing zone disassembles and non-podosomal integrins move into the lamellipodia to mediate migration (93,119). Therefore, the turnover of podosomes is what allows for osteoclast to alternate between migration, adhesion and bone resorption(121).

iv. Bone Degradation

In order for osteoclasts to acidify the extracellular space they require protons as well chloride ions. Protons are initially generated by carbonic anhydrase II (CAII), which converts carbon dioxide and water into bicarbonate ions and protons within the osteoclasts (59). The bicarbonate ions are then exchanged for chloride ions by a passive Cl⁻/HCO₃⁻ exchanger on the anti-resorptive surface of the osteoclast. Next, through extracellular derived signals, possibly transduced by the integrin Avβ3, a ruffled membrane is formed on the bone residing surface of the osteoclast (122). The ruffled membrane is formed in a process fairly reminiscent of vesicular exocytosis (10), where lysosomal-like structures containing proteases and integral chloride ion channels charge-coupled to electrogenic proton pumps (H⁺-ATPase) are transported and incorporated into the plasma membrane (123). The proton pumps acidify the extracellular space to a pH of approximately 4.5 thereby mobilizing the mineral and exposing the organic phases of the bone ECM (59). Secreted lysosomal, collagenolytic enzymes then degrade the exposed organic component of ECM with low pH optimum such as cathepsin K (10,11). Finally, the degradation products are endocytosed at the ruffled boarder, transcytosed to the anti-resorptive surface and released (54).

Clearly, much is known regarding the mechanisms by which osteoclasts degrade bone. Comparatively, little is known regarding the signals that arrest bone degradation. One idea is that
when plasma membrane receptors sense high calcium concentrations in the resorptive space, they initiate the termination of bone resorption and promote the detachment of the osteoclast from the bone surface (10). This theory is supported by the observations that individual osteoclasts only have a half-life of 2-3 days and that once osteoclast mediated bone resorption is complete, they undergo apoptosis and are quickly removed by phagocytes (11).
III. Actin Cytoskeleton

The actin cytoskeleton is a highly fluid structure involved in many diverse and fundamental facets of eukaryotic cell biology. The actin cytoskeleton is particularly important in aspects of cellular physiology that are associated with membrane dynamics such as maintenance of cell shape and migration as well as endocytosis and exocytosis (92,124,125). For instance, during migration, coordinated polymerization of actin filaments against the plasma membrane induces the formation of membrane protrusions such as filopodia and lamellipodia. During endocytosis, actin polymerization is the driving force for membrane invaginations and splitting of endocytic vesicles from the plasma membrane (126), while during exocytosis, polymerized cortical actin filaments form a fusion barrier along the plasma membrane thus preventing the un-stimulated fusion of exocytic vesicles (125,127).

A. Actin Cytoskeleton in Osteoclasts

The intricate processes of osteoclastogenesis as well as mature osteoclast function are highly dependent upon the rearrangement and dynamic regulation of the microfilament cytoskeleton (8). For instance, migrating macrophages as well as mature osteoclasts generate protrusive membrane extensions at the leading edge of the cell supported by focal adhesions, which stabilize the contact with the substratum and provide the driving force required to pull the cell forward. The formation of adhesions structures at the leading edge is coordinated with the down regulation of adhesion sites at the trailing edge, which allows the cell to migrate in the desired direction (95,118). Furthermore, when differentiated osteoclasts contact bone they undergo major cytoskeletal reorganization eventuating in cellular polarization, formation of resorptive apparatus consisting of the sealing zone and ruffled boarder all of which are heavily dependent on the actin cytoskeleton and require dynamic regulation (42).

B. Control of Actin Filament Assembly

The microfilament cytoskeleton is a complex network of flexible actin filaments (F-actin) composed of polymerized globular actin (G-actin) subunits. Due to the intrinsic polarity of each actin subunit, actin filaments are polarized with distinct barbed (plus) and pointed (minus) ends.
The barbed ends of the actin filaments have a disproportionately higher affinity for actin monomers than the pointed ends. Therefore, the barbed end is considered to be the site of actin polymerization while the pointed end is considered to be the site of actin depolymerization. Although, this linear model of barbed end polymerization and pointed end depolymerization is suitable for describing actin dynamics in a cell free system, it is an oversimplification of the processes that are occurring in living cells (124,128). Living cells organize their actin filaments into complex three-dimensional networks, bundles and gels via interaction with a variety of actin binding proteins. These protein include: proteins that promote the nucleation of new actin filaments (e.g., Arp2/3 complex, formins, Cobl, Spire, leiomodin), proteins that promote actin filament severing and depolymerization (e.g., ADF/cofilins, gelsolin, adseverin), proteins that regulated actin filament polymerization by binding to and sequestering actin monomers (e.g., profilin, twinfilin, b-thymosins), proteins that that regulate actin filament polymerization by capping the barbed end of F-actin strands (e.g., capping protein, Eps8, Ena/VASP) and finally actin binding proteins that bundle and cross link pre-existing actin filaments (e.g., a-actinin, fascin) (reviewed in (124,129-131)). Together, these actin binding proteins mediate the length, flexibility and the viscosity of the actin network which can dramatically alter the properties of cytoskeleton, leading to changes in cell morphology and function (131).

C. Gelsolin Superfamily of Actin Binding Proteins

The gelsolin superfamily of actin binding proteins consists of seven highly conserved members found in both mammals and non-mammals alike (131). Together these seven proteins control actin organization by severing filaments, capping filament ends and nucleating actin filament assembly (132). In addition, these proteins have specific and often non-overlapping roles in a number of cellular processes such as motility, apoptosis, phagocytosis and regulation of gene expression. The members of the gelsolin family which include, gelsolin, adseverin (also known as scinderin), villin, capG, advillin, supervillin and flightless I, are classified together because they all contain homologous repeats of a gelsolin like domain (131,132).

The 85kDa gelsolin is the founding member of the gelsolin superfamily of actin binding proteins (132). Gelsolin is encoded by a single gene, but alternate splicing as well as alternate transcription initiation results in the production of three gelsolin isoforms: cytoplasmic gelsolin,
plasma gelsolin and gelsolin-3 (131,133). The plasma isoform differs from the cytoplasmic isoform by a twenty five amino acid intracellular peptide and a disulfide bond between amino acids positions 188 and 201 (134), and is believed to have protective effects in the lungs especially in the face of injury (135). Gelsolin-3, is characterized by the presence of an eleven amino acid peptide at the N-terminus and is expressed mainly in the brain, lungs and testis; however its specific function has yet to be determined (133). All isoforms of gelsolin have the ability to bind, sever and cap actin filaments thereby controlling actin polymerization at the barbed end. In addition, gelsolin can accelerate the formation of actin filaments by binding two G-actin molecules (131,136). Gelsolin’s actin severing activity is activated by calcium ions (µM levels) levels and intracellular pH of less than 6.5 (137). The exact mechanism of calcium activation of gelsolin and subsequent filament assembly are reviewed in detail by Silacci and colleagues (131). Unlike calcium, which activates gelsolin severing and capping activity, phosphoinositide-4,5-bis-phosphate (PIP₂), lysophosphatidic acid (LPA) as well as the Salmonella invasion protein a (SipA) inhibit gelsolin severing activity and promote the uncapping of gelsolin from the barbed end of actin filaments thus providing new sites for actin assembly (136-139). In addition, PIP₂ and LPA stimulate the tyrosine phosphorylation of a number of actin binding proteins, including gelsolin and fragminP (140). Tyrosine phosphorylation of gelsolin can induce conformational changes, which promotes its severing activity at submicromolar concentrations of calcium (141).

Gelsolin has multiple effects on actin filaments in vitro and serves as an important role in actin filament organization in vivo (136). For instance, gelsolin has been heavily implicated in cell motility as the over expression of gelsolin in fibroblasts resulted in substantially increased motility, while gelsolin null fibroblasts suffer from substantially decreased motility (142-144). Gelsolin has also been shown to regulate hematopoietic stem cell motility. In hematopoietic stem cell populations that exhibited differential motility, populations that expressed higher levels of gelsolin and adseverin were considerably more mobile (145). In vivo, gelsolin null mice experienced impaired regulation of neuronal growth where retraction of lamellipodia was impaired while the formation of filopodia was largely unaffected. This suggested that retraction is a gelsolin dependent process, whereas the formation of filopodia is likely independent of gelsolin, and most likely controlled by other actin binding proteins, including adseverin (146). In addition, gelsolin appears to be a downstream activator of the small GTPase Rac, as well as a
mediator of Fc receptor and integrin mediated phagocytosis (143,147). Furthermore, gelsolin behaves as a regulator of apoptosis. During apoptosis, gelsolin is cleaved nearly in half by the apoptotic and aspartate-specific cysteine protease, caspase-3. Upon cleavage, the N-terminal fragment of gelsolin, containing its actin-severing domain, rapidly depolymerizes the actin cytoskeleton due to the loss of calcium regulation normally provided by C-terminal fragment (148). Despite its numerous functional roles at maturity, gelsolin is not required for embryonic development. Gelsolin is highly expressed in many tissues during development, yet gelsolin null mice reproduce and develop normally, perhaps due to the redundancy in the function of the gelsolin family of actin binding proteins (136). This is supported by the complementary expression patterns of adseverin, gelsolin and capG during mouse development (136). In adult mice, gelsolin is mainly expressed in heart, lung and at lower levels in the skeletal muscle, kidney and testes. Further, adult mice lacking gelsolin do experience some alteration in processes involving cell motility and suffer from prolonged bleeding and poor wound healing suggesting that platelet function and the inflammatory response may be affected in the absence of gelsolin (131).

Adseverin is the closest homologue to gelsolin and will be discussed in substantially more detail below. Several other members of the gelsolin superfamily including villin, advillin, supervillin and flightless I have additional domains beyond the six gelsolin like domains (131). These other members serve diverse functions in a variety of biological systems. Villin has been demonstrated to participate in cytoskeletal remodeling in the intestine (149). Advillin who is closely related to villin (nearly 60% similarity at the amino-acid level) is highly expressed in the dorsal root and trigeminal ganglia during embryonic development, and at lower levels in adult uterine and intestinal epithelial cells (150). Supervillin also participates in interactions between actin filaments and the plasma membrane and has a nuclear targeting signal in its N-terminus half (151). Human flightless is a gelsolin homologue with an leucine-rich repeat (LRR) that also binds to actin as well as a flightless I LRR associated protein (FLAP). Flightless I is the only member of the gelsolin family of actin binding protein that is essential in murine embryonic development, as disruption of the flightless gene leads to the rapid embryonic degeneration (152,153). While villin, advillin, supervillin and flightless I have additional domains beyond the six gelsolin like domains, capG only has three gelsolin like domains. Like gelsolin, capG can bind and cap actin filaments but lacks actin severing capabilities. CapG, which also has a nuclear
localization signal, has been demonstrated to be an important mediator of endothelial cell response to mechanical stresses, wound healing response, regulation of macrophage membrane ruffling as well as endothelial and fibroblast motility (147,154,155)

D. Adseverin

Adseverin (also known as scinderin), initially identified as a calcium dependent actin binding was named after its isolation from the adrenal medulla and for its ability to sever actin filaments (156). The following discusses the structural and functional details of adseverin in addition to its localization in a number of biological systems.

i. Adseverin Domain Structure and Function

Adseverin is comprised of six gelsolin like domains (A1-A6) which share 60% similarity with the six domains found in gelsolin (157). Due to their overwhelming similarity, adseverin and gelsolin share many structural and functional properties. For instance, much like gelsolin, adseverin promotes actin filament depolymerization by severing actin filaments in a calcium dependent manner, while remaining bound to the barbed end of the newly formed filament. Similar to gelsolin, adseverin can also bind to actin monomers thereby promoting actin filament nucleation (158). Adseverin has three actin binding sites Ad-ABS₁ (77AAAIFTVQMDDYL89), Ad-ABS₂ (138RLLHVKGRR146) and Ad-ABS₃ (511RLFQVRRNLASIT523) in domains A1, A2 and A5 respectively (159). The first two actin binding sites found in adseverin are nearly identical to those found in gelsolin, in that they both differ by a single amino acid (160). Adseverin also has two PIP₂ binding sites, Ad-PIP₂BS₁ (112KGGLKYKAG120), and Ad-PIP₂BS₂ (sequence homologous to Ad-ABS₂), in domains A1 and A2, respectively (161). Once again the sequence of the second PIP₂ binding site in adseverin differs from that of gelsolin by a single amino acid (160). Both full length adseverin, and a truncated adseverin comprised of domains 1 and 2 (Ads₁₋₂₃₃) which contain both actin binding sites are fully capable of severing F-actin in a calcium dependent manner. Adseverin truncates containing the third actin-binding site (Ads₅₀₂₋₇₁₅ or Ads₅₀₂₋₆₀₆) are capable of nucleating/polymerizing actin filament assembly in a calcium independent manner but are devoid of the severing activity. Adseverin truncates lacking the third actin binding sites (Ads₅₁₉₋₇₁₅ or Ads₅₁₉₋₆₀₆) are incapable of severing actin or nucleating filament polymerization (162). Furthermore, a natural isoform of adseverin induced in T-lymphocytes by
Interleukin-9 which lacks the 5th domain and therefore the third actin binding site (adseverin D5) is also capable of severing and capping actin filaments but unable to nucleate actin assembly (163). Therefore, it appears that adseverin can both sever actin filaments using its first two actin binding sites in domains 1 and 2, and can nucleate actin assembly using its third actin-binding site in domain. Intracellular calcium levels control the regulation between these two functional states. Pene and colleagues (162) suggest that upon stimulation and subsequent elevation of intracellular calcium levels, adseverin depolymerizes actin filaments in a calcium dependent manner. Later, when stimulatory signals are absent or when intracellular calcium levels drop due to organelle sequestering or cell extrusion, adseverin promotes actin nucleation and polymerization in a calcium independent manner.

Despite all the similarities between adseverin and gelsolin, there are a number of critical differences between the structure and regulation of adseverin. Firstly, a wider range of membrane lipids than gelsolin inhibits adseverin’s severing activity. Where gelsolin severing activity is inhibited by phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol-4,5-bis-phosphate (PIP2), adseverin’s severing activity is inhibited by phosphatidylinositol (PI), PIP, PIP2 and phosphatidylserine (PS) (156,164). However, perhaps the most striking difference between adseverin and gelsolin is the presence of a C-terminal helix or “latch” in gelsolin that is missing in adseverin. This C-terminal helix in gelsolin is responsible for creating differential calcium regulation between adseverin and gelsolin. When examining the calcium dependent actin severing kinetics between gelsolin and adseverin it is clear that calcium controls one rate-limiting step in the activation of adseverin and two in the activation of gelsolin. This observation suggests that adseverin activation requires lower intracellular calcium concentrations than that of gelsolin (165).

**ii. Impact of Adseverin on Cellular Physiology**

Much of what is known about adseverin today has been discovered through detailed examination of stimulated and directed exocytosis in chromaffin cells of the adrenal medulla. Traditional immunofluorescence and total internal reflection fluorescent (TIRF) microscopy have shown that exocytotic vesicles suffer from restricted motion when approaching the plasma membrane (166). This phenomenon has been attributed to two factors; the first being the interaction of vesicles with docking or tethering molecules at the site of the plasma membrane (167) and the second
being a physical barrier created by cortical areas of polymerized actin (125,127,168). Immunocytochemistry analysis of chromaffin cells depicts a continuous cortical actin ring that co-localizes with staining for adseverin (169). Upon stimulation (e.g., nicotine, histamine, K+ ions) the F-actin and adseverin cortical rings are locally and transiently disrupted. Chromaffin cells accomplish this task through the action of two parallel pathways. Upon stimulation, chromaffin cells initially disrupt the cortical actin cytoskeleton through the Protein Kinase C (PKC) phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) that normally tethers actin filaments to the plasma membrane. Next, adseverin activated by a rise in intracellular calcium, severs and caps actin filaments leading to local and transient disruptions of the cortical actin network as well as a simultaneous increase in G-actin (125,168,170). The critical role of adseverin in depolymerizing the cortical actin ring was further demonstrated in a series of experiments using recombinant adseverin. Studies using permeabilized chromaffin cells showed that calcium induced F-actin disassembly and exocytosis was increased by recombinant scinderin. Similar effects could also be induced using actin-destabilizing factors such as cytochalasin D, DNase1 or latrunculin (171). Lastly, antisense oligonucleotides against adseverin not only decreased adseverin expression but also decreased cortical actin depolymerization and vesicular exocytosis in response to stimulation (172). Adseverin has also been implicated in a number of biological systems beyond chromaffin cells. For instance, a study designed to identify novel asthma mediators found adseverin to be over expressed upon allergen exposure in murine models of acute and/or chronic asthma (173). Adseverin has also been identified as a member of a multiprotein complex which generates the force and motion required for the trafficking of the water channel aquaporin-2 in rat renal collecting ducts, which is essential for the regulation of body water homeostasis (174). Furthermore, similar to the role of adseverin in chromaffin cells, adseverin has also been found to rearrange the apical actin caps that act as a physical barrier to secretion in airway goblet cells. Under the regulation of an apical phospholipase C G-coupled receptor that results in the activation of PKC and mobilization of calcium, airway goblet cells secrete mucin onto mucosal surfaces following the directed and regulated mucin granule exocytosis. Immunostaining of human lung tissues and mucin-secreting cells again reveal an apical distribution of actin that must be removed prior to exocytosis. Disruption of actin filaments with latrunculin A stimulated mucin, whereas stabilization with jasplakinolide, over-expression of β- or γ-actin as well as the over expression of synthetic peptides corresponding to
adseverin’s actin binding domains all inhibited mucin secretion. These results suggest that much like in the chromaffin cell, in airway goblet cells the actin filaments negatively regulate mucin secretion and therefore must be remodeled in stimulated cells to promote exocytosis (127). Similarly, adseverin might play role in regulating cortical actin dynamics and exocytosis of insulin secretion from murine pancreatic β-cell, as inclusion of adseverin derived actin binding peptides reduced the rate of calcium and GTPγS-induced exocytosis (175). However, this claim needs further investigation, as adseverin expression has not been confirmed in murine pancreatic β-cells. Furthermore, platelet activation requires platelet to undergo shape changes, formation of pseudopodia, aggregation and secretion, all of which require cycles of actin depolymerization polymerization. Studies found that recombinant scinderin induced calcium evoked serotonin release in platelets. This effect was inhibited in the presence of adseverin-derived actin binding peptides, γ-actin and PIP2. These factors also inhibited serotonin release in the absence of recombinant scinderin, suggesting that they also inhibited endogenous adseverin activity (176). All together, these results strongly indicate that adseverin is involved in the control of cortical F-actin networks during exocytosis.

In addition to regulating actin dynamics leading to exocytosis, adseverin is also involved in the differentiation and activation of platelets as well as chondrocyte differentiation. Platelets are derived from long and thin cytoplasmic extensions of mature megakaryocytes, a process highly related to remodeling of the actin cytoskeleton. Forced expression of adseverin in megakaryoblastic cells (which normally express gelsolin but not adseverin) induced megakaryoblastic cell differentiation, maturation, polyploidization and apoptosis followed by the release of platelet-like particles. Expression of adseverin also caused down regulation of gelsolin expression, suggesting that gelsolin normally protects megakaryoblasts from apoptosis (177). Adseverin is also up regulated in hypertrophic chondrocytes, suggesting that it may be involved in regulating the actin organization during chondrocyte differentiation. Furthermore, the over expression of recombinant adseverin in non-hypertrophic chondrocytes caused dramatic changes in actin cytoskeleton leading increase in cell volume as well as the expression of two markers of chondrocyte maturation, Indian hedgehog (Ihh) and collagen type X (178).
iii. **Adseverin Localization**

Adseverin expression is significantly more restricted than that of gelsolin. In fact, the abundance of adseverin has been estimated to be only 10% of gelsolin’s. A number of studies have identified adseverin to be expressed in the salivary gland, pituitary, kidney, testes and platelets, albeit at low levels while no adseverin expression was detected in the heart, skeletal muscle or liver (136). During embryogenesis, adseverin expression is limited to endochondral bone primordial, renal tubules and intestinal microvilli. In adult mice, adseverin expression persisted in the kidneys and the intestine but disappeared from bone (136,179). Gelsolin on the other hand was expressed in intramembranous bone primordia, perichondrial and other tissues during embryogenesis, while gelsolin expression in the kidney and gut were complementary to that of adseverin. Therefore, in cells where both adseverin and gelsolin are expressed, they localized to different regions of the cells (179).
Statement of Problem

Osteoclasts and osteoblasts continuously remodel bone at over 1 million sites in transient structures called basic multicellular units (BMU) (9). In each BMU, osteoclasts resorb bone after which, osteoblasts move into the excavated areas and secrete the osteoid which will mineralize into new bone (11). This process is essential for proper bone development, growth and repair, and the regulation of calcium homeostasis (11,18). Under normal circumstances, bone degradation and formation are maintained in steady state equilibrium. However, there are a number of diseases such as osteopetrosis (13), osteoporosis (19), rheumatoid arthritis and periodontal disease (9,45) that result from an imbalance between bone resorption and formation. These diseases are associated with increased morbidity and shortened lifespan of afflicted individuals while placing a significant economic burden on the health care system (8). Current efforts aimed at treating bone disorders are primarily focused on developing therapeutics that reduce osteoclast formation (osteoclastogenesis, OCG) or osteoclast activation (9). OCG, mediated in part by Receptor Activator of NF-κB Ligand (RANKL) stimulated migration and fusion of osteoclast progenitors, requires significant actin remodeling (92,131). Microarray analyses have identified adseverin (Ads) as a gene of interest during OCG, as Ads is up regulated in osteoclast precursors (unpublished data) as well as mature osteoclasts (180). Ads is an actin binding protein first identified in the bovine adrenal medulla, which regulates F-actin dynamics by severing and capping actin filaments in a calcium dependent manner (156) . Ads has been found to be critical in various processes such as differentiation of chondrocytes (178), as well as cortical actin rearrangements during exocytosis (125,127,175). Surprisingly very little is known about the role of Ads and actin cytoskeleton remodeling during OCG.

Therefore the long-term objective of this project was to characterize the role(s) of adseverin during osteoclastogenesis. Considering that microarray data have found adseverin to be up regulated in osteoclast progenitors, this project focused on determining the role(s) of adseverin during the early phases of osteoclastogenesis.

**Hypothesis:** In this project we set out to test the hypothesis that adseverin expression is induced during osteoclastogenesis in response to RANKL. We further hypothesized that adseverin is playing a functional role during the early phases of osteoclastogenesis.
Introduction

Osteoclasts are highly specialized, terminally differentiated multinucleated cells that differentiate from hematopoietic precursors of the monocyte-macrophage lineage in a process known as OCG (56,57). OCG can be broken down into five major steps. Initially, osteoclasts progenitors are primed for OCG by the cytokine Macrophage Colony Stimulating Factor (M-CSF), which induces Receptor Activator of NF-κB (RANK) receptor expression in osteoclast progenitors while promoting the proliferation and survival of differentiated osteoclast precursors (65). Next, osteoclast precursors gain fusion competency through the transcription and cell surface expression of a number of fusion receptors such as DC-STAMP, CD44 and SIRPα/CD-47 (92,106,181). Fusion competent precursors then migrate towards each other in a chemotactic manner (92), followed then by cell-cell attachment (182) and finally cell membrane fusion (92). These processes are heavily dependent on the dynamic regulation of the actin cytoskeleton, and are primarily driven by signal cascades downstream of the RANK-RANKL axis (64,74). RANKL is regarded as one of, if not the most essential cytokine for osteoclastogenesis. Therefore, extensive effort has been devoted to deciphering the downstream signals of the RANK receptor. A recent microarray study aimed at characterizing the genes that are differentially modulated in osteoclast precursors during OCG, found that genes primarily involved in immune responses are down regulated in response to RANKL (180). The same study also noted that genes involved in osteoclast differentiation and function, oxidative phosphorylation, as well as cellular attachment, vesicular trafficking and migration are up regulated in pre osteoclasts in response to RANKL. Ads, a member of this latter group of genes, is of particular interest as its functions are heavily intertwined with the actin cytoskeleton and because preliminary microarray data from our lab also detected Ads to be differentially expressed gene during the early phases of osteoclastogenesis (unpublished data).

Ads, a member of the gelsolin superfamily of actin severing proteins, shares a great degree of homology with gelsolin (131). Therefore, much like gelsolin, Ads binds to, severs and caps actin filaments in a calcium dependent manner (136). Unlike gelsolin however, Ads is found to be active at lower intracellular calcium levels and is only expressed in cells and tissue with high secretory activity (157). Furthermore, Ads’s F-actin severing activity is inhibited by a wider range of membrane lipids than that of gelsolin (136,165). Previous studies have reported gelsolin
to be highly expressed in osteoclasts (183). Gelsolin has also been found to be functionally important during the formation of F-actin rings, V-ATPase transport to the plasma membrane and osteoclast motility (131,184-186). To our knowledge, the microarray analysis published by Yang et. al., (180) was the first report of Ads in osteoclasts. Despite the lack of knowledge regarding the role(s) of Ads in developing osteoclasts, studies have shown Ads to be up regulated during chondrocyte maturation. Further, over expression of exogenous Ads in non-hypertrophic chondrocytes causes cytoskeletal rearrangements, changes in cell morphology and cell volume and the transcript up-regulation of Indian hedgehog and collagen type X (178). Ads has also been shown to act as a molecular switch during exocytosis by serving as a cortical F-actin depolymerizing agent in bovine chromaffin cells (161,162), human airway goblet cells and murine pancreatic β-cells (127,175). Recently, Ads was also identified as a member of the multi-protein motor complex bound to the water channel, aquaporin-2 required for the delivery of the channel to the plasma membrane (174).

Based on these findings, we set out to investigate the potential role(s) of Ads during the early phases of OCG, particularly migration and/or fusion of pre-osteoclasts. OCG was induced in vitro using two previously established model systems: 1) using murine bone marrow derived monocytes precursors as previously described (187-189) and RAW 264.7 macrophage cell line (122,180,189). Here we have shown for the first time that Ads is up regulated during OCG in response to RANKL at both transcript and protein levels. Furthermore, we have generated an Ads knockdown cell line, called Ads KD, which failed to undergo OCG upon treatment with RANKL. Further investigation of the Ads KD cell line also identified diminished DC-STAMP expression in response to RANKL.
Chapter 2
Materials and Methods

i. Chemicals and Reagents

Ads Full Length (Ads) and Ads D5 (Ads-D5) cDNA in the bacterial expression vector pSE380 (Invitrogen, Carlsbad, CA) were a kind gift from Dr. J. Robbens (163). Cell culture medium: MEM alpha and D-MEM cell culture media (Invitrogen, Carlsbad, CA). Cytokines: M-CSF (M9170, Sigma, St. Louis, USA); sRANKL (315-11Peprotech, Rocky Hill, NJ). RNA Isolation: Qiagen RNeasy Mini Kit (74104); Qiagen RNase-Free DNase set (79254) (Qiagen, MD, USA).

Reverse Transcription: Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA); Oligo-dT18 VN primer (ACGT Corp; Toronto, ON). Western blotting: RIPA buffer (9806, Cell Signalling Technologies, Danvers, MA); 50x Protease Inhibitor Cocktail (51-21426Z, BD Bioscience Pharmingen, Franklin Lakes, NJ); BCA Protein Assay Kit (23225, Pierce, Rockford, IL); Nirocellulose Membrane (8549062, GE Healthcare, Piscataway, NJ); Amersham ECL Plus Western Blotting Detection System (RPN2132, GE Healthcare, Piscataway, NJ); Bioflex MSI film (CLMS810, Clonex Corporation, Markham, ON). Primary Antibodies: Rabbit polyclonal anti-murine-adseverin antibody was a kind gift from Dr. C. Svensson (190); Rabbit polyclonal anti-murine-gelsolin antibody was a kind gift from Dr. P. Arora; Mouse monoclonal anti-murine B-actin (A5316, Sigma); Mouse monoclonal anti-b-tubulin (E7) was a kind gift from Huib Croes (Nijmegen, the Netherlands). Secondary Antibodies: HRP-conjugated donkey anti-rabbit IgG (NA934V, GE Healthcare; 1:2000); sheep anti-mouse IgG-HRP (catalog no. NA931V; Amersham Pharmacia Biotech; 1: 8000). PCR Cloning: Pfx High Fidelity DNA Polymerase (Invitrogen, Carlsbad, CA); Taq DNA Polymerase (Qiagen, MD, USA); XhoI, MluI, BamHI and BstBI Restriction Endonucleases (New England Biolabs, Ipswich, MA); T4 DNA ligase (New England Biolabs, Ipswich, MA); Mammalian C-terminal GFP fusion protein construct pEGFP-c1 (Clonetech, Mountain View, CA); QIAquick Gel Extraction Kit (28704, Qiagen, MD,USA). QIAprep Spin Miniprep kit (27108, Qiagen, MD,USA). shRNA Transfections and Infections: GP-293 viral packaging cells (Clonetech, Mountain View, CA) as well as the pVSV-G envelope protein expressing construct were kind gifts from Dr. H. Saratis; FuGENE HD Transfection Reagent (Roche, Indianapolis, IN); Benzonase Nuclease (Sigma); Millex-HA, 0.45um syringe driven filter unit (Millipore, Carrigtwohill, Ireland).
**ii. Isolation of Murine Bone Marrow Derived Osteoclasts Progenitors and In Vitro Osteoclastogenesis**

All procedures described were performed in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care Committee. Bone marrow derived osteoclast progenitors or bone marrow monocytes (BMMs) were isolated from 6-12 week old wild type (WT) mice (SV129/BL6). Mice were sacrificed via carbon dioxide asphyxiation, and the tibia and femora were aseptically removed and dissected free of adherent soft tissue inside a laminar air flow bio-safety cabinet. Bone ends were cut, and the marrow space was flushed out using a sterile 26-gauge needle with α-MEM culture media. The flushed marrow was passed through a 20-gauge needle three times to create a homogenous, single cell suspension. To remove stromal cells and fibroblasts present in the marrow cell suspension, cells were cultured overnight in α-MEM complete culture medium (supplemented with 10% FBS and 10% antibiotics (164 IU/ml penicillin G, 50 ug/ml gentamicin, and 0.25 ug/ml fungizone). Non-adherent cells were harvested and cultured overnight in α-MEM complete culture media supplemented with 20ng/ml M-CSF to obtain a BMM rich cell population. Adherent BMMs were harvested and counted by using a Z1 Coulter Particle Counter (Coulter Electronics, Hialeah, FL, USA). Osteoclastogenesis was induced by seeding 3x10^6 cells onto 60mm^2 diameter cell culture dishes in α-MEM complete culture media supplemented with 20ng/ml M-CSF for two days (unstimulated cells) or with 20ng/ml M-CSF and 30ng/ml of sRANKL for 2, 4 and 6 days. Fresh culture media supplemented with cytokines was added to the cells every two days. Cells were cultured at 37°C (5% CO₂). (Figure 1A-E).

**iii. RAW 264.7 Macrophage In vitro Osteoclastogenesis**

Murine RAW 264.7 macrophages (ATCC, Manassas, VA) were cultured in D-MEM culture medium supplemented with 10% FBS and antibiotics. For osteoclast differentiation, 2.5x10^5 cells were seeded in 6 well culture dishes and cultured with 30ng/ml recombinant murine sRANKL for up to 6 days. Unstimulated RAW 264.7 cells were used as controls. Cells were cultured at 37°C (5% CO₂). (Figure 1 A’ - E’).
iv. **TRACP Staining and Osteoclast Enumeration**

Prior to TRACP staining, cells were washed twice with PBS, fixed with 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRACP). Briefly, fixed cells were incubated in a solution of naphthol AS-BI phosphate and fast red TR salt (Sigma) in 0.2 M acetate buffer (pH 5.2) containing 100 mM sodium tartrate (Sigma) for a minimum of 10 min at 37°C and subsequently washed twice with sterile MilliQ water. Cells were viewed with a Leitz Wetzlar microscope at 200X magnification, and images were taken with a PixeLinK camera. The number of TRACP-positive osteoclasts and the number of nuclei within these osteoclasts were counted in 10 random fields of view (FOVs). The enumerated osteoclasts were characterized based on size, which correlated with the number of nuclei. TRACP positive (red stained) cells with 2-3 nuclei were considered to be small osteoclasts, whereas TRACP positive cells with 4-9, 11-29 and 30+ nuclei were considered to be medium, medium-large and large osteoclasts, respectively.

v. **Quantitative Real-Time PCR**

At the indicated time points (see results) total RNA was extracted from cultured monocytes/osteoclasts using the RNeasy Mini Kit. Residual genomic DNA was eliminated using Qiagen RNase-Free DNase treatment. Total RNA concentration was determined using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, Rockford, IL) and stored at -80°C until use. 10 random samples were analyzed for RNA quality (Bioanalyzer, Agilent technologies). 1ug of RAW 264.7 macrophage and 120ng BMM derived total RNA was reverse transcribed into cDNA using 200 units of Superscript II reverse transcriptase and 1 uM Oligo-dT18 VN primers. A 1:32 cDNA dilution was used for all primer pairs to yield optimal PCR efficiency. Quantitative real-time PCR was performed in triplicate using the BioRad CFX96 Real Time System. Each 20ul reactions contained: 5ul of diluted cDNA, 500nM of forward and reverse primers and 10ul of SsoFast EvaGreen Supermix (Bio-Rad; Hercules, CA) using. The PCR conditions were as follows: 95°C 30 sec, followed by 50 cycles of 95°C for 5sec and 60°C/65°C for 5 sec. A final extension at 72°C for 10 min was used to conclude the reaction. Melt curve analysis (60°C to 95°C) of the amplified product was used to determine the specificity of the PCR reaction. The CFX Manger Software (version 1.0) was used to analyze the PCR results, where mRNA expression of Ads, gelsolin, DC-STAMP, SIRPα, CD44 and RANK were
normalized to the control, internal housekeeping gene GAPDH. The following table depicts the primers used:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence F: Forward Primer Sequence</th>
<th>Amplicon Size</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adseverin</td>
<td>F: 5'-CCTATGGTGACTTTTACGTCGG - 3'</td>
<td>116 b.p</td>
<td>65°C</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTCATTCCGGGAACACTCTCTT - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelsolin</td>
<td>F: 5'-CCTACCGCACATCCCCCAG - 3'</td>
<td>286 b.p.</td>
<td>65°C</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GTGATGGGGGTCCCGCTG - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC-STAMP</td>
<td>F: 5'-TACGTGGAGAGAGCAAGGAA - 3'</td>
<td>100 b.p.</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ACACTGAGAGTGTTAGGAAT - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANK</td>
<td>F: 5'-CTAATCCAGCAGGGAAGCAAT - 3'</td>
<td>164 b.p.</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GACACGGGCATAGACTGATTC - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIRPα</td>
<td>F: 5'-TCGAGTGATCAGGAGGAGC - 3'</td>
<td>457 b.p.</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCTGGACACTAGCATGACTCTGAG - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDD44</td>
<td>F: 5'-TAGGAGAAGGTGTTGGGCAG - 3'</td>
<td>220 b.p.</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGGCACCTACACCAACTCCATC - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5'-CCTTCCGTGTCTCCTACCC - 3'</td>
<td>131 b.p.</td>
<td>60°C or 65°C</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCCGAAGATGGCCCTTCAGT - 3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### vi. Western Blot Analysis

At the indicated time points (see results), monocytes/osteoclasts in culture were washed three times with PBS and lysed in RIPA buffer supplemented with 1 mM PMSF and 1x protease inhibitor cocktail and collected by cell scraping. Cell lysates were centrifuged for 1 min at 15,000xg at 4°C to remove cellular debris. Total protein concentration was determined using the BCA protein assay kit. 5x Laemml sample buffer was added to 20ug of total cell lysates and boiled for 10 min. Samples were resolved via SDS-PAGE on 8-12% polyacrylamide gels. Following electrophoresis, samples were transferred onto a nitrocellulose membrane. Non-specific sites were blocked with 5% milk powder in TBST for 1 hour. Membranes were incubated with primary antibodies (see below) overnight at 4°C, washed three times with TBST, incubated with the corresponding secondary antibody for 1 hour and washed three times with TBST. Immuno-reactive proteins were detected using chemiluminescence with Amersham ECL Plus Western Blotting Detection System, upon exposure to Bioflex MSI film. Films were developed using the Kodak M35A X-OMAT Processor, scanned digitally using the Epson Perfection 1250 scanner and band intensities were quantified by densitometry using Image J 1.41 software. Band intensities were normalized against total actin or tubulin levels.

vii. **Adseverin Isoforms**

Primers were designed to flank the 5th domain of Ads (F: 5’-GTGCAGGTCCGTGTCTCTC-3’, R: 5’-GTGCAGGTCCGTGTCTCTC-3’). The design of the primers allowed for the specific detection of both two Ads isoforms. Specifically, if the larger, full length Ads isoform was expressed a 650 bp product was expected. However, if the smaller, Ads D5 isoform was expressed, a 350 bp product was expected. Likewise, if both isoforms were present, then both the 650 and 350 bp products were expected. Taq DNA polymerase was used to amplify the targeted sequences in cDNA from RAW 264.7 macrophage and BMM derived osteoclasts. 50pg of pSE380-Ads and pSE380-Ads D5 were used as positive controls. To check for expression of Ads isoforms during osteoclastogenesis, 20ug of TCL from unstimulated RAW 264.7 macrophages and BM derived BMMs (Days 0) as well as TCL from Day 6 RAW 264.7 and BM derived osteoclasts were run on an SDS-PAGE gel and immunoblotted against Ads (as previously described). Lysates from BL-21 *E. coli* transformed with pSE380-Ads or pSE380-Ads D5 and induced with 1mM IPTG to express either isoform of Ads were used as positive controls.

viii. **Adseverin and Luciferase Retroviral shRNA Knock-down Construct Preparation**

Ads specific single stranded oligonucleotides were designed in accordance to the guidelines described by the Clontech RNAi systems user’s manual (Top Strand: 5’ gatccAACAAATATGAGCGTCTGATTCAAGAGATCAGACGCTCATATTTGTTTTTTTTTA CGCGTg 3’, Bottom Strand: 5’aattcACGCGTAAAAAAAACAAATATGAGCGTCTGATCTTGAATCAGACGCTCAT ATTTGTTg 3’). The top and bottom strands were mixed together at a 1:1 ratio, heated at 95°C for 30sec, 72°C for 2min, 37°C for 2min and 25°C for 2min to generate 50uM Ads specific knock-down double stranded oligonucleotide. 0.5uM of the double stranded shRNA oligonucleotide was ligated into 50ng of the linearized pSIREN dsRed RetroQ expression vector using 200U of T4 DNA ligase via a one hour room temperature incubation. In addition, a
separate ligation was carried out using a Luciferase, control, shRNA-annealed oligonucleotides. The ligated vectors were used to chemically transform competent DH5a E. coli (as described under viii). The transformed E. coli were allowed to recover via a 6-hour 37°C incubation while shaking at 250rpm. The transformed cultures were plated onto Ampicillin (100ug/ml, source) selective media and incubated overnight at 37°C. Colonies were aseptically selected and used to inoculate 2ml of LB/Amp (100ug/ml), and incubated at 37°C for 8 hours while shaking at 250rpm. The shRNA construct plasmid was isolated using the QIAprep spin miniprep kit and DNA concentrations were determined using the Nanodrop spectrophotometer. 1 ug of each resulting construct was digested with 20U of MluI to confirm the presence of an shRNA oligo inside the vector, via a 4 hour incubation at 37°C The resulting digests were run on a 1% TBE gel and colonies with only a single band were determined to have an insert. 1ug of DNA from positive colonies (with insert) were selected and sequenced (ACGT Corp, Toronto, ON).

ix. **shRNA infections and Characterization of Luciferase and Adseverin Knockdown Macrophage Cell Lines**

GP-293 pantrophic packaging cells were seeded onto 6 well tissue culture plates and cultured in DMEM culture media until 40% confluent. The GP-293 cells were co-transfected with 2ug of the previously described shRNA retroviral constructs (Ads-shRNA, Luc-shRNA) as well as 2ug of the pVSV-G envelope protein-packaging vector using FugeneHD transfection reagent. The transfected GP-293 cells were incubated at 37°C for three days, then the viral containing supernatant was harvested and spun free of cells. The viral supernatant was treated with 65U/ml of Benzonase to degrade residual cellular DNA for 30min at room temperature. The viral supernatant was passed through a Millex-HA, 0.45um syringe driven filter unit, and then used to infect RAW 264.7 macrophages. Infected RAW 264.7 macrophages were incubated for three days at 37°C and then sorted based on the dsRed signal by fluorescence-activated cell sorting (FACS). The sorted cells were subjected to limiting dilution to obtain a final dsRed positive cell concentration of approximately one cell per 100ul. 100ul of media was added to each well of 96-well plates and incubated for one week at 37°C. After one week, colonies were examined under an inverted fluorescent microscope at 100x magnification (through the plastic culture plate) and red colonies were identified and expanded. Ads expression levels were examined at the mRNA and protein levels. The Ads knockdown clone with lowest Ads protein level was used in the
subsequent experiments. Luciferase knockdown and control, uninfected wild type macrophages were used as controls.

x. Migration vs. Fusion Assay

Wild type and Ads knockdown cells were seeded onto 6-well tissue culture plates at three different densities; 10x10^5 cells/well (low density), 2.5x10^5 cells/well (normal/medium density) and 5.0x10^5 cells/well (high density). Osteoclastogenesis was induced with a 4-day incubation with 30ng/ml sRANKL. On day 4, cells were fixed, TRACP stained, and the number of osteoclasts enumerated as previously described.

xi. Statistical Analysis

For experiments where multiple observations were made per sample, numerical results were expressed as mean ± SEM or mean ± SD, as indicated. Each experiment had a sample size of n≥3, unless otherwise stated. Statistical analysis was performed using a student’s t-test and a p value of less than 0.05 was considered statistically significant. All statistical analyses were performed with the SPSS 12.0 statistics package (SPSS, Chicago, IL, USA).
Chapter 3
Results

Adseverin is up regulated during osteoclastogenesis in primary BMM derived osteoclast cultures.

Microarray gene studies have indicated that Ads is up regulated over 19 fold in mature, day 5, osteoclasts derived from RAW 264.7 macrophages in a RANKL dependent manner (180). Preliminary microarray data from our lab supports this finding, as a 5 fold up regulation of Ads was detected in maturing osteoclasts derived primary mouse BMMs upon a 2-day stimulation with M-CSF and sRANKL (unpublished data). These findings prompted us to closely examine the temporal expression pattern of Ads during osteoclastogenesis. BMM osteoclast progenitors were induced to undergo osteoclastogenesis (see materials and methods section ii) and Ads transcript and protein expression patterns were examined in day 0, 2, 4 and 6 cultures, by qRT-PCR and immunoblot analysis, respectively. Ads mRNA levels were up regulated in day 2 osteoclast cultures compared to basal Ads transcript expression levels in day 0, BMM cultures. Transcript levels in days 4 and 6 cultures were 23 and 22 fold higher (respectively) than those found in day 0, BMM cultures. The difference between the Ads transcript expression levels between day 2 and day 4 cultures was statistically significant (p<0.01), but there was no statistically significant difference between transcript levels between day 4 and day 6 cultures (Figure 2A). Because Ads is a member of a highly conserved family of gelsolin actin binding proteins, we needed to exclude the possibility that the qRT-adseverin PCR primers may amplify non-specific products. A melt curve analysis showed a single product peak at 84.5°C, thus suggesting that the primers amplified only a single product (Figure 2B). Because post-transcriptional processes and protein degradation also influence the final levels of any given protein, we also examined Ads protein levels during the various stages of osteoclastogenesis (Figure 2C). Statistically significant 6, 5 and 4 fold increases in Ads protein expression were detected in day 2, 4 and 6 cultures (respectively) compared to basal, day 0 Ads expression in BMM cultures (Figure 2D). There was no statistically significant difference between Ads protein levels between day 2, day 4 and day 6 cultures, although a trend towards decreased expression during the latter phases of osteoclastogenesis is observed. Thus, in line with increased mRNA levels, Ads protein levels increased significantly during OCG.
RAW 264.7 macrophage induced osteoclastogenesis is a suitable model system to examine the role of adseverin during osteoclastogenesis

Due to limitations associated with using primary BMMs as osteoclast progenitors (see discussion), RAW 264.7 macrophages (here forth RAW cells) were also used as the osteoclast precursor pool. To validate the use of RAW cells as progenitors for in vitro osteoclastogenesis, we wanted to ensure that Ads expression dynamics in RAW derived osteoclasts were similar to that of primary BMM derived osteoclasts. To this end, osteoclastogenesis was induced in RAW cells and adseverin transcript and protein expression patterns were examined in day 0, 2, 4 and 6 cultures. At the transcript level, significant 30, 60 and 27 fold up regulations of Ads was detected in day 2, 4 and 6 cultures (respectively) compare to un-stimulated, day 0 macrophage cultures (Figure 3A). The Ads transcript levels were significantly higher in day 4 cultures than in day 2 cultures (p < 0.05), but there was no statistically significant difference between transcript levels between day 4 and day 6 cultures. Once again, a melt curve analysis showed a single peak at 84.5°C, thus suggesting the primers amplified only a single product (Figure 2B). To confirm that up regulation in mRNA levels corresponded to increased Ads protein expression, immunoblot analyses were performed during the various stages of RAW derived osteoclastogenesis (Figure 2C/top panel & Figure 2D). Ads protein levels steadily increased during osteoclastogenesis such that significant 6, 16 and 24 fold increases in Ads protein expression were detected in day 2, 4 and 6 cultures (respectively) when compared to basal Ads protein expression levels in day 0 cultures. Gelsolin protein expression was also examined during RAW derived osteoclastogenesis (Figure 2C/middle panel & Figure 2E). Gelsolin levels remained constant and did not significantly change during osteoclastogenesis. Together, these results indicate that Ads expression in RAW derived osteoclasts is similar (albeit slightly divergent) to Ads expression in primary BMM derived osteoclasts. Therefore, RAW cells are a viable model system when studying OCG in vitro.

The Full Length Adseverin Isoform is the only Isoform up regulated during osteoclastogenesis

A report by Robbens and colleagues (163), suggested that the expression of a previously unknown isoform of Ads, Ads D5 could be induced in T-Helper Lymphocytes in response to IL-9 (163). Thus far, we have demonstrated that Ads is up regulated during osteoclastogenesis via the induction of RAW cells and primary BMMs with sRANKL. However, we did not
differentiate between the expression full length Ads or Ads-D5 during osteoclastogenesis. To determine if Ads-D5 is expressed during osteoclastogenesis, we designed primers flanking the 5th Ads domain (esD5 Forward and Reverse primers) (Figure 4A). PCR using the esD5 primers and pSE380-Ads or pSE380-Ads-D5 expression vectors as positive controls yielded 650bp and 350 bp products respectively, thus allowing us to differentiate the expression of the two isoforms (Figures 4 A and B). PCR analysis only detected the 650 bp product in day 0, 2, 4 and 6 RAW or primary BMM osteoclast cultures (Figure 4B). This suggests that only the full-length isoform of Ads is present during OCG, at least at the mRNA level. We validated this observation at the protein level using immunoblot analyses. Two distinct bands at 85 and 80 kDa are readily identifiable in lysates of BL-21 E. coli induced to express exogenous Ads or Ads-D5, respectively (Figure 5C, lanes 1 and 2). Similar to the expression pattern seen at the transcript level, only the 85 kDa full length Ads was detected in lysates of day 6 mature osteoclasts derived from RAW cells or primary BMMs. Collectively these results suggest that only full length Ads is expressed during osteoclastogenesis and that RAW cell and primary BMM derived osteoclasts share near identical patterns of Ads expression.

**Generation of Adseverin knockdown cell lines**

To determine the functional role of Ads, a stable RAW cell line with significantly reduced Ads expression was generated (see materials and methods sections viii and ix). To exclude the possibility that any observed phenotype would be due to pleiotropic effects of shRNA expression or retroviral transduction itself, a control, luciferase KD (Luc KD) cell line was also created. In addition, WT control macrophages were also used. The pSIREN RetroQ shRNA retroviral expression system used to infect our target cells which induced DsRed expression in all infected cells. After infection, DsRed positive cells were sorted using FACS. More than 8.5x10^5 and 1.0x10^6 of Luc KD and Ads KD macrophages (respectively) were sorted. After sorting, only 583 (0.07%) and 313 (0.03%) dsRed positive cells were obtained from the Luc KD and Ads KD populations. Cells were subjected to a limiting dilution and propagated. Since undifferentiated RAW cells do not express Ads (see Figure 3), the effectiveness of Ads knockdown was assessed after a 4-day incubation sRANKL. Ads expression in day 4 WT and day 4 Luc KD cultures was significantly higher than in their corresponding day 0 cultures, both at the transcript and protein levels (Figure 5). As anticipated, our Ads KD cell line failed to significantly up regulate Ads
expression after a 4-day incubation with sRANKL (Figure 5). When comparing day 4 WT cultures to day 4 Ads KD cultures, significant 94% and 65% reductions in Ads transcript and protein levels were detected (Figure 5A & B respectively).

**Osteoclastogenesis is impaired in Adseverin knockdown osteoclast progenitors**

After validating suppression of Ads expression in the Ads KD cell line, we assessed whether this had an impact on *in vitro* OCG. OCG was induced in WT, Luc KD and Ads KD macrophages via a 4 days stimulation with sRANKL at which point cells were fixed and TRACP stained for the presence of osteoclasts. Prior to fixation, WT, Luc KD and Ads KD macrophages were examined for DsRed signal using an inverted fluorescent microscope in order to ensure that the shRNA cassette was still present. As expected, WT macrophages were DsRed negative, whereas the majority of Ads KD and Luc KD cells were DsRed positive (Figure 6A). The DsRed signal was maintained throughout osteoclastogenesis in Luc KD and Ads KD cultures (data not shown). Staining for TRACP was reduced in day 4 Ads KD cultures in comparison to TRACP staining in WT and Luc KD day 4 cultures (Figure 6A). No difference in terms of osteoclast size, number of TRACP staining was noted between WT and Luc KD day 4. Quantification of the number of multinucleated osteoclasts showed that there were fewer osteoclasts (all sizes) in day 4 Ads KD cultures than in day 4 WT cultures. Specifically, in 10 random fields of WT cultures there were on average 3.85 ± 0.15 SD, 4.05 ± 0.95 SD, 1.2 ±0.5 SD and 0.4 ±0.1 SD osteoclasts with 2-3, 4-10, 11-29 and 30+ nuclei respectively. Conversely, in 10 random fields of Ads KD culture there were on average 0.45 ±0.25 SD, 0.05 ±0.05 SD osteoclasts with 2-3 nuclei and 4-10 nuclei respectively. More importantly osteoclasts with more than 10 nuclei were never observed in the Ads KD cultures. WT and Luciferase knockdown day 4 cultures shared similar osteoclastogenic properties in terms of the number and size of TRACP positive osteoclasts found in day 4 cultures. This experiment was repeated twice and similar results were obtained. Clearly, both WT and Luc KD macrophages were capable of differentiating into osteoclast when stimulated by sRANKL. Even though Ads KD macrophage did form some TRACP positive mononuclear osteoclasts, they were unable to form large multinucleated osteoclasts, suggesting that a reduction in endogenous Ads leads to a major defect in osteoclastogenesis.
**Adseverin knockdown osteoclast progenitors fail to fuse leading to a defect in osteoclastogenesis**

To further elucidate the role of adseverin during migration and/or fusion of osteoclast progenitors in response to sRANKL, we cultured WT and Ads KD osteoclast progenitors at low (10\(\times\)10\(^5\) cells/well), normal (2.5\(\times\)10\(^5\) cells/well) and high (5.0\(\times\)10\(^5\) cells/well) initial plating densities (Figure 7). The rationale for these experiments was as follows: If Ads KD osteoclast progenitors were unable to migrate, yet fusion mechanisms were unaffected, osteoclast formation could be rescued if the progenitors were initially close together (i.e. plated at high initial density), thus making the osteoclastogenesis defect in the Ads KD population predominantly a migration defect. On the other hand, if migration is largely unaffected, yet fusion mechanisms are compromised in Ads KD progenitors, then osteoclast formation could not be rescued regardless of the initial plating density, thus making the osteoclastogenesis defect in Ads KD population predominantly a fusion defect. OCG was induced and TRACP positive osteoclasts were visualized and counted in day 4 cultures. OCG in WT macrophages was not affected by differential seeding density, as mature osteoclasts of all sizes were seen in day 4 cultures (Figure 7A and B). Ads KD progenitors on the other hand, failed to undergo normal OCG and only formed small osteoclasts. At the lowest seeding density the number of small osteoclasts (2-3 nuclei/osteoclast) was not significantly different between the Ads KD (1.33 ± 1.81 SD) and WT (2.37 ± 1.07 SD) cultures. However, this difference became significant at the normal and high initial seeding densities (p< 0.05). At the normal seeding density we observed an average of 0.53 ± 0.67 SD and 2.17 ± 0.61 SD small osteoclasts in 10 random fields of Ads KD and WT cultures, respectively. Similarly, at the highest seeding density we observed an average of 0.27 ± 0.38 SD and 2.40 ± 0.40 SD, small osteoclast in 10 random of Ads KD and WT cultures, respectively (Figure 7B). In addition, Ads KD progenitors formed fewer medium sized osteoclasts (4-10 nuclei/osteoclasts) than the WT progenitors, regardless of the seeding density (Figure 7A). At the lowest seeding density we observed an average of 0.43 ± 0.67 SD, and 1.07 ± 0.23 SD, medium sized osteoclasts in 10 random fields of Ads KD and WT cultures, respectively. At the normal seeding density, we observed an average of 0.17 ± 0.23 SD, and 2.77 ± 1.46 SD, medium-sized osteoclast in 10 random fields of the Ads KD and WT cultures, respectively (Figure 7B). Similarly, at the highest seeding density, we observed an average of 0.17 ± 0.29 SD, and 2.07 ± 1.44SD medium sized osteoclasts in 10 random fields of the Ads KD and WT cultures,
respectively. The difference between the numbers of medium sized osteoclasts was only significant at the normal seeding density, perhaps due to the small sample size (n=3) and the variation between repeats. Most interestingly however, Ads KD progenitors failed to form any osteoclasts with more than 10 nuclei, regardless of the initial plating density. In fact, high seeding density did not rescue osteoclastogenesis and the numbers of multinucleated osteoclasts were similar to that observed when grown at low seeding density. Furthermore, Ads KD progenitors at day 2 of differentiation had a clear tendency to form clusters of cells, which suggests that migration was intact. However, this effect could be due to proliferation of RAW cells during differentiation. Taken together, these results show that Ads KD progenitors are unable to undergo normal osteoclastogenesis, most probably due to a fusion defect.

Adseverin Knockdown affects expression of the crucial fusion receptor DC-STAMP.

Expression of genes critical to osteoclastogenesis was examined by quantitative real-time PCR in day 0, 1, 2 and 4 cultures (Figure 8). These genes included the pre-osteoclast fusion receptors DC-STAMP, SIRPα and CD44, the cytokine receptor RANK as well as gelsolin and Ads. In unstimulated, day 0, macrophage osteoclast progenitors, transcript levels of all the aforementioned genes were similar between Ads KD and WT cells. In addition, the transcript levels of RANK, CD44, SIRPα and gelsolin during days 1, 2 and 4 of osteoclastogenesis were again similar between Ads KD and WT cultures. As before, Ads transcript expression levels was significantly lower in Ads K.D cells than WT cells upon stimulation with sRANKL (i.e. days 1, 2 and 4). Transcript levels of the crucial fusion receptor DC-STAMP nearly tripled in WT cultures between days 0 and day 4; however that of Ads KD cells was consistently lower than of WT levels and there was no significant difference between DC-STAMP transcript levels between day 0 and day 4 in Ads KD culture. Since DC-STAMP was shown to be indispensable for fusion (102) it is possible that decreased DC-STAMP levels in the Ads KD are responsible for the fusion defects.
Chapter 4
Discussion

Preliminary, unpublished microarray data from our lab, as well as a recent microarray by Yang et. al (180) reported 5.9 and 19.1 fold increases in Ads expression in maturing and mature osteoclasts compared to undifferentiated monocytes/macrophages, respectively. Ads, a member of the gelsolin superfamily of actin bind proteins has been heavily implicated in directed and controlled exocytosis and cytoskeletal rearrangements during chondrogenesis (125,127,169,178). However, to our knowledge, these microarray data are the first reports of Ads expression in the osteoclast biology system. To investigate the role(s) of Ads in maturing and mature osteoclasts we examined the temporal expression pattern of Ads during OCG, using two in vitro model systems. Firstly, OCG was induced via a well established in vitro OCG method where primary, murine bone marrow derived monocytes or BMMs, were stimulated with M-CSF and sRANKL for up to six days (189). This primary in vitro model system was ideal as it provided a true source of osteoclast precursors that most reliably and accurately mimicked in vivo OCG. Unfortunately, introducing exogenous gene products into primary hematopoietic cells, a major goal in this project, is challenging (191). Therefore, we sought a secondary in vitro model system that possessed three essential properties: 1) OCG could be induced by sRANKL 2) genetic manipulations, particularly retroviral infections is a viable and efficient method of introducing exogenous genetic material and 3) Ads expression was similar to that of primary, BMM derived osteoclasts. To this end we validated the used of RAW 264.7 macrophages (or RAW cells) as a model system to study Ads during OCG.

RAW 264.7 Macrophages as an in vitro Osteoclastogenesis Model System

RAW cells have the unique property of differentiating into osteoclasts when stimulated with sRANKL alone and are therefore commonly used when studying OCG in vitro (102,122,180,189,192). M-CSF, the second critical cytokine required for in vitro OCG in primary BMMs, induces the initial differentiation of hematopoietic monocytes into the osteoclast lineage, mediates the proliferation and survival of differentiated osteoclast precursors, and therefore increases the size of RANKL inducible pre-osteoclast population (65). RAW cells rapidly proliferate in the absence of M-CSF and as previously mentioned undergo OCG via
stimulation with sRANKL, and therefore met the first criterion of a suitable model system. To examine the functional role of Ads, we proposed to introduce shRNA sequences designed to knockdown endogenous Ads expression using a retroviral-mediated system (see results and below). Experience has shown efficient viral infections are extremely difficult and inefficient in primary cells. Conversely, viral transduction in RAW cells has been previously reported using various methods with efficiencies between 10-80% (102,193), thus meeting our second criterion of a suitable model system. The third and final criterion of the model system was similar Ads expression patterns when compared to primary BMM derived osteoclasts. In primary BMM derived osteoclasts, Ads expression was up regulated within 2 days of stimulation with sRANKL. Further, Ads expression remained elevated throughout OCG and in mature osteoclasts (Figure 2). Ads expression in RAW cells was similarly up regulated upon stimulation with sRANKL (Figure 3). The mRNA expression pattern was nearly identical between the two model systems (Figures 2A and Figure 3A), while protein expression patterns were similar (Figure 2D and Figure 3D). In both model systems, Ads protein levels were near maximal between days 2 and 4 of differentiation. These observations allowed us to conclude that RAW cells are a suitable model system that could be used instead of primary BMMs to study in vitro OCG. In addition, these results were suggestive of a role for Ads during the earliest phases of osteoclast formation: the fusion competency, migration and fusion of pre-osteoclasts. Elevated Ads expression in mature osteoclasts was also suggestive of a role for Ads in mature osteoclast function (e.g. polarization, formation of osteoclast specific structures such as podosomes, sealing zone and ruffled membranes) and/or mature osteoclast migration.

**RANKL Induces the Expression of Full Length Adseverin**

A report by Robbens et. al., (163) noted that when T-helper lymphocytes are stimulated with Interleukin-9 (IL-9), two gelsolin like proteins were up regulated. One of these two proteins was the murine homologue of bovine Ads. The other was a slightly smaller, previously un-identified isoform of Ads, Ads-D5, which was missing most of its 5th and a portion of its 6th gelsolin like domains. Ads-D5 is believed to be an alternate splice variant of full length Ads, with most of the typical characteristic common to all members of the gelsolin family of actin binding proteins, with the exception of nucleation of filament assembly in vitro (163). IL-9 is produced by activated T-helper type 2 lymphocytes and can stimulate the proliferation of T-cells and mast
cells (194). However, IL-9 is not considered to be an important cytokine in the progression and induction of OCG. We have shown that full length Ads is induced by RANKL; however the question of Ads-D5 expression in response to RANKL during OCG remains unanswered. To investigate the expression of Ads-D5 during OCG, we designed primers flanking the 5th domain of Ads that would amplify 650 bp and 350 bp products in the presence of full length or D5 Ads isoform templates (Figure 4 and B). Furthermore, we used immunoblot analysis to differentiate between the expression of the two Ads isoforms. Only the full-length isoform of Ads was up regulated during OCG in response to RANKL in both of our in vitro model systems. The symmetrical Ads expression in our model systems further validates the use of RAW cells as a precursor population to study OCG.

**Osteoclastogenesis is Impaired in the Absence of Adseverin**

One approach commonly used to determine the functional role of a target gene is to create “knockout” animal models, and examine phenotypic variances from wild type animals (195). In fact, much of what is known about the control of OCG through the RANKL:RANK signaling cascade was made possible through the generation of countless knockout animal models (reviewed in (12,64)). Although “knockout” animal models allow for somewhat definitive determination of the functional role of a gene, their generation requires an extensive commitment of both time and labor. Alternatively, gene silencing is possible via post-translational gene silencing, specifically through RNA interference (RNAi). RNAi is a valuable research tool used to silence target gene expression and is now widely used to determine gene function (196). The silencing principle of RNAi depends on the introduction of short double stranded 21-23 oligonucleotide sequences (siRNAs) into the host cell, which induces a well-conserved mechanism of dsRNA-mediated specific gene silencing (196). We took advantage of the RNAi gene silencing technique and generated an Ads knockdown (Ads KD) RAW cell line as well as a control luciferase knockdown (Luc KD) cell line. As a last line of control, we also used uninfected WT macrophages. The pSIREN DsRed RetroQ retroviral vector allowed us to stably integrate the shRNA cassette in frame with DsRed into the host cell’s genome. Consequently, all infected cells (Luc KD and Ads KD) and subsequent progeny were DsRed positive even after 5+ passages in vitro (Figure 6A). These cells maintained their DsRed signal throughout OCG (results not shown). When induced to undergo OCG, the WT and Luc KD macrophages were
capable of generating large osteoclasts with similar number of TRACP positive osteoclasts (Figure 6). WT and Luc KD osteoclasts also had near identical patterns of Ads expression (Figure 5). The similarities between the WT and Luc KD cultures suggest that the viral transduction and shRNA expression had no non-specific effects on OCG. Therefore, from this point on we compare the Ads KD cells to the WT cells. Unlike WT and Luc KD macrophages, Ads KD macrophages experienced a profound defect in OCG. Following induction with sRANKL, we detected 94% and 65% reductions in Ads mRNA and protein expression (respectively) in day 4 Ads KD cultures compared to the day 4 WT cultures. Furthermore, virtually no osteoclasts with 4 or more nuclei/osteoclast, and very few small (2-3 nuclei) TRACP positive osteoclasts were observed in day 4 Ads KD cultures (Figure 6). Therefore, our Ads shRNA knockdown cell line had significantly reduced Ads expression which resulted in the loss of multinucleated osteoclasts suggesting that Ads is involved during the early phases of OCG.

**Adseverin is a Pro-Fusion Marker and Regulator of Osteoclastogenesis**

The earliest phases of OCG can be subdivided into three major steps. Initially pre-osteoclasts must migrate towards each other in a chemotactic manner (94). Close proximity of cells (92) along with the activation of RANK receptor with RANKL (50), is thought to induce fusion competency via the transcription and surface expression of fusion receptors including, DC-STAMP, SIRPα, CD44, CD47 and E-cahderin (102,106,111,182). Next, fusion competent cells recognize and adhere to one another, perhaps mediated via E-cahderin, followed by membrane attachment and fusion (92). By seeding Ads KD and WT at low, normal and high seeding densities we were able to tease out the role of Ads in terms of migration or fusion (102,187). Regardless of the seeding density, Ads KD macrophages formed fewer TRACP positive multinucleated osteoclasts when compared to the WT macrophages. Interestingly, the Ads KD cells formed the greatest number of multinucleated cells when seeded at the lowest density (10x10^5 cells/well), but even under these conditions, they formed virtually no osteoclasts with more than 4 nuclei (Figure 7). Although indirect, these results also suggest that Ads is not involved in the migration of pre-osteoclasts, as increased seeding density could not rescue the phenotype associated with Ads KD cultures. Further evidence against the involvement of Ads during pre-osteoclast migration came from the observation of pronounced cellular clumps or colonies in day 2 Ads KD cultures seeded at low and normal densities. Formation of cellular
clumps in Ads KD cultures suggests that migration was unaffected in the Ads KD macrophages. However, this clustering effect could also be caused by constant proliferation of RAW cells during differentiation. This uncertainty could be resolved if this experiment is repeated in the presence of a proliferation inhibitor such as Actinomycin D. Lastly, gelsolin which was highly expressed in undifferentiated macrophages as well as maturing and mature osteoclasts (Figure 3E), has been implicated in osteoclast matrix adhesion and migration (118,120,183). Therefore, it is possible that Ads is not required for osteoclast migration. It is also possible that any migratory defects caused by a lack of Ads are masked by gelsolin, perhaps due to a redundancy in gelsolin and Ads function. However, the most likely cause of the OCG defect present in the Ads KD is a fusion defect. It is worth noting that a fusion defect does not necessarily equate to a defect in osteoclastogenesis (102). As noted by Yagi et.al (102), DC-STAMP null macrophages also suffer from a fusion defect leading to a lack of multinucleated osteoclasts in vitro. However, the DC-STAMP null macrophages did undergo normal OCG, as mononuclear, TRACP positive osteoclasts were capable of resorbing bone-like substrates, albeit less efficiently than WT multinucleated osteoclasts. Therefore, without bone resorption assays we cannot differentiate between fusion and OCG defects in our Ads KD cell line. Ads KD macrophages appear to suffer from a fusion defect as both mononuclear and small multinuclear TRACP positive osteoclasts can be seen in day 4 Ads KD cultures (Figures 6 and 7). Unfortunately, the phenotype of large multinucleated osteoclasts was lost in our Ads KD cultures. At this point we cannot comment on the functional role of Ads in mature osteoclasts, although a potential involvement of Ads in mature osteoclasts should not be overlooked particularly since gelsolin is known to be involved in mature osteoclast podosome formation and migration.

**DC-STAMP Expression is Diminished in Ads KD Osteoclasts**

The introduction of siRNAs can induce complex signaling pathways in target cells leading to undesired effects beyond the specific silencing of the gene of interest (196). Although the Luc shRNA cell line suggests that this is not the case, there can be clonal effects caused by the genomic integration of the shRNA cassette. To exclude the possibility that the fusion defect in Ads KD macrophages was caused by reduced expression of other factors, we analyzed the expression of two cell fusion receptors SIRPα and CD44, which were expressed at similar levels between the WT and Ads KD cells. We also examined the expression of the RANK receptor.
RANK expression was not significantly different between our WT and Ads KD cells throughout OCG. In addition, gelsolin expression levels were unaffected in the Ads KD population indicating that our knockdown was in fact specific to Ads (Figure 8). There was no significant difference in DC-STAMP mRNA levels between undifferentiated, day 0, WT and Ads KD macrophages. Surprisingly, DC-STAMP expression was significantly diminished in our Ads KD population upon stimulation with sRANKL (Figure 8). This observation can be explained in a number of ways. First, DC-STAMP expression could be downstream of Ads expression. Ads severing of actin filaments and actin monomer sequestering can significantly impact actin dynamics and cytosolic monomeric G-actin concentrations, which in turn can regulate gene expression (197). G-actin concentrations are known to regulate the shuttling of the MAL transcription factor into and out of the nucleus. In the absence of F-actin filaments monomeric actin concentrations are high and MAL is bound to G-actin. The MAL-G-actin interaction sequesters MAL in the cytoplasm. Upon actin filament assembly, G-actin levels in the cytoplasm drop, MAL disassociates from monomeric actin and translocates to the nucleus where it functions as an SRF co-activator and induces SRE-mediated gene expression (e.g., VE-cadherin and β-actin) (197,198). Although, there is no literature supporting the regulation of DC-STAMP expression by MAL/SRF, it is a possibility worth investigating. Another example of Ads regulated gene expression is illustrated during chondrocyte differentiation. In hypertrophic chondrocytes, elevation in cytoplasmic calcium concentration activates Ads’s F-actin severing activity. Activated Ads then induces changes in cell shape brought upon by cytoskeletal rearrangements that in turn activate Erk1/2 and p38 pathways which lead to the expression of Indian Hedgehog (Ihh) and Collagen X (178). Alternatively, Ads expression could be downstream of DC-STAMP. Over-expressing exogenous DC-STAMP in unstimulated RAW cells could test this hypothesis. If the over expression recombinant DC-STAMP in WT undifferentiated macrophages leads to the up regulation of Ads message or protein expression, then we can conclude that Ads expression is downstream of that of DC-STAMP. A third and final scenario explaining the lack of both Ads and DC-STAMP expression in our knockout system could be that there are two entirely unrelated genetic defects in our cell line. The only way to differentiate between any of these scenarios would be to rescue Ads and/or DC-STAMP expression in our Ads KD cell line (see Future Experiments).
Adseverin Mediated Preosteoclast Fusion

Although the relationship between Ads and DC-STAMP is currently unclear, it appears that RANKL induced Ads is critical during the early stages of osteoclastogenesis, most notably during fusion of pre-osteoclasts. Upon stimulation by RANKL and activation by calcium, Ads may be regulating fusion on a number of fronts including surface expression of fusion receptors and membrane fusion. Ads could potentially be involved in the directed and controlled surface expression of fusion receptors including DC-STAMP, CD44 and SIRPα. Unlike DC-STAMP, the transcript levels of CD44 and SIRPα are not elevated during fusion or OCG (Figure 8), an observation supported by the literature (106,111). However, the surface expression of both SIRPα and CD44 are induced strongly and transiently at the onset of fusion by a mechanism that most likely involves post-transcriptional control (100,105,106). Blocking of SIRPα with antibodies and the genetic deletion of CD47 and DC-STAMP all inhibit the formation of multinucleated TRACP positive osteoclasts in vitro (100,102,107). A failure of cells to deliver fusion receptors to the cell surface would be analogous to genetic deletions, as these proteins would not be present in their native functional environment. The mechanisms involved in the delivery and cell-surface expression of transmembrane fusion receptors, such as DC-STAMP, SIRPα and CD44 is similar to exocytosis (10). As previously mentioned, Ads has been heavily implicated in controlled exocytosis. In fact, the targeted down regulation of Ads expression in chromaffin cells not only inhibited cortical actin disassembly, not exocytosis as well (172). Therefore, lack of Ads could disrupt normal delivery of important fusion receptors including but not limited to DC-STAMP, SIRPα and CD44.

Ads may also be mediating the membrane fusion among two fusing cells, particularly when considering the regulation of Ads severing activity by phosphatidylserine (PS). The lipid composition of the inner and outer leaflets of the plasma membrane is an important fusogenic signal (92,116,199). Specifically, exposure and recognition of PS is required for successful fusion events between macrophages, myoblasts and trophoblast (o-5). For instance, PS a lipid normally confined to the inner leaflet of the plasma membrane bilayer was found on the outer plasma membrane leaflet in contact areas between fusing myoblasts (92). It is possible that macrophages show similar changes in PS localization during fusion. Studies have shown that during macrophage-macrophage fusion leading to the formation of foreign body giant
macrophages, a process similar to osteoclastogenesis, the CD36 fusion receptor on one fusion partner mediates the capture of PS on the other fusion partner. A similar process may be involved during osteoclastogenesis, even though CD36 is not a contributing fusion receptor in osteoclasts (116). Furthermore, PS has been implicated in phagocytosis as PS exposure on the surface of apoptotic cells leads to their engulfment. At this point in time, both the regulation of PS exposure and how cells can differentiate in cellular fusion or “cellocytosis” and phagocytosis is unclear. One way that cells may be differentiating between these two mechanisms could be via PS interactions with CD47 and SIRPα which influence macrophage fusion but down regulates phagocytosis (105). Regardless of the regulation of PS during macrophage fusion, PS is known to act as a unique inhibitor of Ads severing activity (200). Therefore, Ads may be primary regulator of F-actin dynamics at the fusing cell boundary due to its intimate relationship with PS.
Figure Legend

Figure 1. Bone marrow derived monocytes and RAW 264.7 macrophages can be induced to undergo osteoclastogenesis in response to sRANKL stimulation. 3.0x10^6 bone marrow derived monocytes/MOPs were seeded onto 6-well tissue culture plates and stimulated for 2 days with 20ng/ml M-CSF (A) or for up to 6 days with 20ng/ml M-CSF and 30ng/ml sRANKL to induce osteoclastogenesis (B-D). 2.5x10^5 RAW 264.7 macrophages were seeded onto a 6-well tissue culture plate and culture overnight (A’) or cultured for up to six days with 30ng/ml sRANKL to induce osteoclastogenesis (B’-D’). Representative images of TRACP-stained mature osteoclasts taken after 6 days in culture (E and E’). All images were taken at 200x magnification.

Figure 2. Adseverin expression is up regulated during osteoclastogenesis in response to sRANKL. Osteoclastogenesis was induced using primary, bone marrow derived monocytes as previously described. A) Quantitative real-time PCR analysis was used to quantify gene expression in days 0, 2, 4 and 6 of osteoclast cultures. Results are expressed as mean fold expression vs. GADPH (+SEM), which was used as the internal control. Adseverin transcript levels were significantly up regulated by a factor of 10.1, 23.4 and 22.8 on days 2, 4 and 6 of osteoclastogenesis (respectively) when compared to basal adseverin expression in day 0 monocyte cultures (n>3). B) Melt curve analysis shows a single peak at 84.5°C suggesting that the adseverin qRT-PCR primers used amplified a single, specific product. C) Western blot analysis was used to quantify adseverin protein expression in days 0, 2,4 and 6 of osteoclast cultures. Results are expressed as fold expression vs. b-actin (+SD), which was used as the loading control. D) Quantification of the Western blot. Adseverin protein expression was significantly up regulated by a factor of 6.1, 4.6 and 3.8 on days 2, 4 and 6 of osteoclastogenesis (respectively) when compared to basal adseverin protein expression in day 0 primary monocyte cultures (n=3). ** p <0.01. *** p < 0.001.

Figure 3. Adseverin expression pattern in osteoclasts derived from RAW 264.7 macrophages mimics that of primary, bone marrow monocyte derived osteoclasts. Osteoclastogenesis was induced in vitro using RAW 264.7 macrophages as previously described. A) Quantitative real-time PCR analysis was used to quantify gene expression in days 0, 2, 4 and 6 of osteoclast cultures. Results are expressed as mean fold expression vs. GADPH (+SEM),
which was used as the internal control. Adseverin transcript levels were significantly upregulated by a factor of 29.6, 60.2 and 26.5 on days 2, 4 and 6 of osteoclastogenesis (respectively) when compared to basal adseverin expression in day 0 RAW 264.7 macrophage cultures (n≥3). B) Melt curve analysis depicts a single peak at 84.5°C suggesting that the adseverin qRT-PCR primers used amplified a single, specific product. C) Western blot analysis was used to quantify adseverin and gelsolin protein expression in days 0, 2, 4 and 6 of osteoclast cultures. Results are expressed as fold expression vs. b-actin (+SD), which was used as the loading control. D) Quantification of the Western blot. Adseverin protein expression was significantly upregulated by a factor of 5.8, 15.6 and 23.8 on days 2, 4 and 6 of osteoclastogenesis (respectively) when compared to basal adseverin protein expression in day 0 RAW 264.7 macrophage cultures. Gelsolin protein levels did not significantly change throughout osteoclastogenesis, although a trend towards increased expression was observed during the latter stages of osteoclastogenesis (n=3). ** p <0.01. *** p < 0.001.

Figure 4. Full-length adseverin is the only adseverin isoform expressed during osteoclastogenesis. A) A schematic representation of the pSE380 bacterial expression vector with the full length (pSE380-A-FL) and D5 (pSE380-A-D5) adseverin isoforms within its Nco I and Pst I sites. Primers flanking the 5th gelsolin like domain of adseverin (esD5 forward and esD5 reverse) allow for the amplification of a 650 bp amplicon from the full length adseverin isoform and a 350 bp amplicon of the D5 adseverin isoform. B) PCR analysis shows that only the full-length adseverin isoform mRNA was expressed throughout the various stages of osteoclastogenesis in both primary monocyte and RAW 264.7 macrophage derived osteoclasts. 10pg of pSE380-A-FL and pSE380-A-D5 were used as positive controls (lanes 1 and 2). 1:10 diluted cDNA from day 0, day 2, day 4 and day 6 RAW 264.7 macrophage derived osteoclasts (lanes 3-7), and cDNA from day 0, day 2, day 4 and day 6 primary monocyte derived osteoclasts (lanes 8-11) were used as templates. C) Immunoblot analysis showing that only the full length adseverin isoform was up regulated during osteoclastogenesis. Lysate from E. coli (Strain BL-21) induced to express the exogenous full length or D5 adseverin isoforms were used as positive controls (lanes 1 and 2 respectively). Only the 85 kDa full length isoform of adseverin was detected in 20ug of total cell lysates from day 6 mature osteoclasts derived from primary monocyte and RAW 264.7 macrophages (lanes 4 and 6 respectively). Virtually no adseverin
signal was detected in 20ug of total cell lysates from unstimulated, day 0 primary monocytes and RAW 264.7 macrophages.

**Figure 5.** shRNA directed gene silencing provides an effective means to knockdown adseverin expression during osteoclastogenesis. WT RAW 264.7 macrophages and a Luciferase knockdown macrophage cell line (clone A8) express adseverin in response to sRANKL stimulation. The adseverin knockdown macrophage cell line (clone F1) failed to up regulate adseverin in response to sRANKL. A) Quantitative real-time PCR results analysis was used to quantify gene expression in day 0 and day 4 culture in WT, Luciferase and adseverin knockdown cell lines. Results are expressed as mean fold expression vs. GAPDH expression (+SEM), which was used as the internal control. Adseverin transcripts are significantly up regulated in wild type and luciferase knockdown day 4 cultures compared to their corresponding day 0 cultures. No such up regulation was detected in the adseverin knockdown day 4 cultures. Adseverin day 4 osteoclasts experienced a significant 94% reduction in adseverin transcript levels when compared to WT day 4 osteoclasts (n≥3). B) Immunoblot analysis was used to examine the adseverin protein expression levels in the WT, Luciferase and adseverin knockdown cell lines. C) Quantification of Western blots. WT and Luciferase Day 4 cultures experienced a significant up regulation in protein levels compared to the corresponding day 0 cultures. Adseverin day 4 osteoclasts experienced a significant 65% reduction in adseverin transcript levels when compared to WT day 4 osteoclasts (n=3). ** p <0.01, *** p < 0.001.

**Figure 6.** Adseverin knockdown RAW 264.7 macrophages display a profound defect in osteoclastogenesis. 2.5x10^5 WT, Luciferase and adseverin knockdown RAW 264.7 macrophages were seeded onto 6-well tissue culture plates and stimulated for 4 days with 30ng/ml sRANKL for 4 days to undergo osteoclastogenesis. Cells were examined under fluorescent microscopy to examine the percentage of dsRed expressing cells (Far left panel). Images of TRACP-stained osteoclasts derived from WT and adseverin knockdown were taken at 200x magnification (far right panel), osteoclasts were classified based on size, counted and quantified (B).

**Figure 7.** Fusion defect in Adseverin knockdown RAW 264.7 macrophages cannot be rescued by high seeding density. Wild type and adseverin knockdown cell line RAW 264.7 macrophages were seeded at three initial densities in 6 well tissue culture plates; low density (1x10^5 cell/well), normal density (2x10^5 cell/well) and high density (5x10^5 cells/well). Cells
were induced to undergo osteoclastogenesis via 4-day stimulation with sRANKL. A) Representative images of day 0, day 2 and TRACP-stained day 4 cultures (taken at 200x magnification). B) Quantification of the average number of osteoclasts in ten random fields, classified based on size (+SEM). At low densities there is no significant difference between the number of osteoclasts formed between WT and adseverin knockdown cell lines. At normal and high densities, significantly fewer osteoclasts are formed by the adseverin knockdown cell lines. As density is increased WT macrophages form larger osteoclasts whereas adseverin knockdown macrophages never form osteoclasts with more than 5 nuclei. This is suggestive of a fusion defect. (n=3). * p<0.05, ** p < 0.01, ***p<0.001.

**Figure 8. Adseverin knockdown osteoclasts fail to up regulated DC-STAMP in response to RANKL.** Quantitative real-time PCR was used to quantify gene expression on days 0, 1 2 and 4 of osteoclastogenesis. Results are expressed as mean fold expression vs. GAPDH (+SEM), adseverin and DC-STAMP transcript levels following stimulation with sRANKL (day 1), which persist throughout osteoclastogenesis. Expression of other important osteoclastogenesis gene such as RANK, SIRPα, CD44 and Gelsolin are unaffected by adseverin knockdown. n=3. *p < 0.05, ** p < 0.01.
Supplemental Figure Legend

Figure S1: Adseverin is diffusely expressed in the cytoplasm and has modest co-localization with actin rich areas of maturing osteoclasts. In order to visualize the sub-cellular localization of adseverin, we transiently cotransfected wild type RAW 264.7 macrophages with GFP-Ads and RFP-Lifeact using the Amaza nucleofection transfection system. The pEGFP-c1-adseverin construct allowed us to visualize the localization of the exogenous adseverin tagged at its c-terminus with GFP. Likewise, the RFP tagged lifeact, a 17 amino acid peptide that binds specifically to F-actin without interfering with actin dynamics allowed us to visualize the localization of actin structures in the osteoclast progenitors and maturing osteoclast. 2.0x10^5 double transfected RAW 264.7 macrophages were seeded onto 35mm, glass bottom tissue culture plates and incubated with or without sRANKL at 37°C. Live cell images were taken using confocal fluorescent microscopy (40x objective). Adseverin was diffusely expressed in the cytoplasm of undifferentiated macropages (- sRANKL panels). However, upon stimulation with sRANKL, osteoclast progenitors displayed typical actin rich structures where adseverin intensity was increased (+ sRANKL panels).
Figures

FIGURE 1.

Day 0  Day 2  Day 4  Day 6  Day6 (TRACP)

Primary BMMs

RAW 264.7 Macrophages

A A' B B' C C' D D' E E'
FIGURE 2.

A

![Bar graph showing normalized expression (vs. GAPDH) over days 0, 2, 4, and 6.](image)

Day 0: (10.1) **
Day 2: (23.4) **
Day 4: (22.8) ***
Day 6: 

B

![Melt curve graph showing temperature (Celsius) vs. dR(dT)/dT.](image)

C

![Western blot images of Adseverin and β-actin](image)

Day 0: Adseverin, Day 2: Adseverin, Day 4: Adseverin, Day 6: Adseverin

D

![Bar graph showing normalized expression (vs. B-actin) over days 0, 2, 4, and 6.](image)

Day 0: (6.1) **
Day 2: (4.6) **
Day 4: (3.8) ***
Day 6: 

Legend:
- **: p < 0.01
- ***: p < 0.001
FIGURE 3.

A

Normalized Exp. (vs. GAPDH)

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
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<tbody>
<tr>
<td>0.1</td>
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<td>**</td>
<td>***</td>
</tr>
<tr>
<td>(29.6)</td>
<td>(60.2)</td>
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B

Melt Peak

-40[FU]/[MR]

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<tr>
<td>80</td>
</tr>
<tr>
<td>90</td>
</tr>
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<td>95</td>
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</table>

C

Adseverin

Gelsolin

β-actin

D

Normalized Exp. (vs. B-actin)

<table>
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<tr>
<td>0</td>
<td>0.5</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>(5.8)</td>
<td>(15.6)</td>
<td></td>
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</tr>
<tr>
<td>(23.8)</td>
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</table>

E

Normalized Exp. (vs. B-actin)

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<th>Day 6</th>
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</tbody>
</table>
FIGURE 4.

A

B

C

pSE380

RAW 264.7, WT

Primary, WT

5E380

A-FL  A- D5  Day 0  Day 2  Day 4  Day 6

Day 0  Day 2  Day 4  Day 6

650 bp

350 bp

BL-21

Primary, WT

RAW 264.7, WT

AdS-FL  AdS-D5  Day 0  Day 6

Day 0  Day 6

Ads (Full Length 85kDa)

Ads (D5 80kDa)
FIGURE 5.

A

B & C

TABLE 5.

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<thead>
<tr>
<th></th>
<th>WT</th>
<th>Luc KD</th>
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<tr>
<td>Ads</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td>Day 4</td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
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</table>
FIGURE 6.

WT

Luc K.D.

Ads K.D.

dsRed | Day 0 | Day 2 | Day 4 | Day 4 (TRACP)

Avg. # Osteoclasts/F.O.V.

0 1 2 3 4 5 6

2-3 Nuc. 4-10 Nuc. 11-29 Nuc. 30+ Nuc.

Osteoclast Size

WT N.I.C. | Luc K.D. | Ads K.D.
FIGURE 7.

<table>
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<td>![Image]</td>
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<tr>
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</tr>
</tbody>
</table>

![Graph](Image)
FIGURE 8.
FIGURE S1.

RAW 264.7 Macrophages
(- sRANKL)

RAW 264.7 Macrophages
(+ sRANKL)
Chapter 5
Future Directions

In this project we have already taken major strides towards determining the role of Ads in the osteoclast biological system. We have described the temporal expression pattern of Ads during OCG, shown Ads to be specifically up regulated in response to RANKL, have shown how attenuated Ads expression has deleterious impacts on the fusogenic ability of pre-osteoclasts. However, there are a number of important questions that still remain unanswered. This section is devoted to describing necessary future experiments needed to resolve these remaining issues.

First and foremost, we have yet to fully answer the question: What is the role of Ads during the early phases of OCG. Based on the phenotype of the differentiated Ads KD osteoclasts, it is clear that the depression of Ads expression caused a defect in pre-osteoclast fusion. However, this arouses yet another question; does the observed fusion defect in the Ads KD cell line also lead to a defect in osteoclast maturation? As Yagi et. al., (102) described, osteoclasts derived from DC-Stamp null mice failed to fuse thus leading to an absence of large, multinucleated osteoclasts. However, DC-Stamp null macrophage, still undergo normal OCG, such that mononuclear, TRACP positive osteoclasts capable of resorbing bone-like substrates were observed in in vitro cultures (102). Therefore, in order to definitively understand the extent of the defect caused by the loss of Ads expression, we must assess the functional, bone resorbing activity of Ads KD osteoclasts. Therefore, we propose an experiment that will measure and compare resorbing capacity of WT and Ads KD osteoclasts using an in vitro assay, as previously described (187).

Briefly, WT and Ads KD macrophages will be seeded onto 0.25-0.5mm thick dentin sections and differentiated into mature osteoclasts via a 4 day stimulation with sRANKL. Cells will then be fixed and osteoclast formation will be confirmed by TRACP staining. In order to view the resorption pits, cells will be chemically removed from the dentin slices using a 6% bleach solution and the resorption pits will be stained using 1% toluidine blue and 1% sodium borate. The number and size of resorption pits can then be used to assess the extent and efficiency of bone resorption in the Ads KD osteoclasts in comparison to WT osteoclasts.

Secondly, to confirm that the phenotype observed in the Ads KD cells was not caused by non-specific effects (e.g. unfavorable genome integration), we must reintroduce Ads in an attempt to
rescue pre-osteoclast fusion. If Ads KD macrophages regain the ability to fuse following the reintroduction of Ads, then that would reaffirm our theory on the importance of Ads during pre-osteoclast fusion. Furthermore, a rescue experiment can also shed light onto the relationship between Ads and DC-STAMP expression. As shown in Figure 8, DC-STAMP expression is diminished rather than up regulated upon RANKL stimulation, in our Ads KD cell line. The reintroduction of Ads can help us to assess whether up regulation of DC-STAMP is a downstream event of Ads. For instance, if the reintroduction of Ads can rescue fusion and DC-STAMP expression, DC-STAMP up regulation takes place downstream of Ads. Conversely, if the reintroduction of Ads can rescue fusion but not DC-STAMP expression, then we can say that Ads is critical for osteoclast formation in a pathway independent of DC-STAMP. On the other hand, if the reintroduction Ads cannot rescue fusion or DC-STAMP expression, then we are left to conclude that the fusion defect seen in our Ads KD cell line, was in fact caused by an unrelated mutation to DC-STAMP expression. To this end, we have proposed a series of rescue experiments where we will attempt to reintroduce Ads in our KD cells using either TAT-mediated protein transductions or retroviral introduction of a mutated yet functional Ads, that would be safe from RNAi mediated silencing in the KD cell line.

We have been working on rescuing Ads expression using TAT-mediated protein transduction, which has the advantage of not being affected by the shRNA in our KD cells. TAT-mediated protein transductions of mammalian cells was first described over 20 years ago and has recently been developed into a broadly applicable method that allows for the rapid introduction of full-length proteins into mammalian cells (201). A bacterial expression vector coding for HIV-TAT-Ads fusion protein (pTAT-HA-Ads) was cloned by in-frame insertion of full-length murine Ads into XhoI/BstBI sites of the pTAT-HA vector (a kind gift from Dr. G. Bokoch (The Scripps Research Institute, CA)). The accuracy was confirmed using DNA sequencing (ACGT Corp. Toronto, ON). TAT-Ads was isolated using Qiagen Ni-NTA columns and purified using dialysis as previously described (201,202). The yield and the purity was determined by blotting against anti-HIS (Primary antibody: Mouse monoclonal anti-HIS (NI44/14, UC Davis/NIH NeuroMab Facility, CA). Unfortunately, much of the TAT-Ads (~95 kDa) was lost during the preliminary washes of the purification and very little was TAT-Ads was left following dialysis (data not shown). Currently, we have no explanation for the low yield of TAT-Ads fusion protein, since we have successfully isolated other TAT-chimeras in our laboratory (202). Although, our yield
was less than ideal, we have added purified TAT-Ads to undifferentiated WT macrophage to see if we can deliver exogenous Ads to our RAW macrophages. In a preliminary experiment, we cultured RAW macrophages overnight in DMEM culture media and subsequently added 500nM of TAT-Ads to the culture media for a total of 60min. Both the concentration and exposure times were determined based on reports of osteoclast transduction using TAT-gelsolin constructs (183). Following the 60min incubation, the cells were extensively washed with PBS, and cultured for an additional 60min, 24hours or 48hours. Total cell lyates were harvested and probed for Ads expression. Ads protein was detected after a 60min incubation and Ads levels gradually dropped but persisted for up to 48 hours after the initial incubation with TAT-Ads (data not shown). It is important to note that any Ads signal was exogenously introduced, as undifferentiated macrophages do not express endogenous Ads (Figure 3). Based on these preliminary results and reports in the literature on TAT-transductions of osteoclasts with TAT-gelsolin constructs, there is clearly a potential for the use of TAT-Ads to rescue Ads expression. By differentiating Ads KD cells in the presence of 500nM TAT-Ads, we can determine if introducing exogenous Ads can rescue either fusion or DC-STAMP expression in our Ads KD cell line in response to RANKL. However some technical issues in terms of purification and isolation of the TAT-Ads still remain. With some effort and more time these hurdles can be overcome and we can apply that TAT-Ads approach in trying to rescue Ads expression in our KD system.

As an alternative approach, we will attempt to rescue Ads by introducing a mutated, siRNA resistant and functional Ads construct to our KD cell line. As previously discussed, we targeted a 19 bp region of the Ads open reading frame (5’ nt. 295 AACAAATATGAGCGTCTGA nt. 313 3’) when designing the Ads-specific shRNA. Using site directed mutagenesis we mutated Ads open reading frame where the same 19 bp region reads as follows: 5’ nt. 295 AACAAAGTACGAACGGCCTCA nt. 313 3’. These nucleotide substitutions (A300-G, T303-C, G306-A, T309-G, G312-C) lead to five silent mutations such that the amino acid sequence of both the original and mutated 19 bp regions reads as follows, N-K-Y-E-R-L. We used the previously described pTAT-HA-Ads vector as the starting material for our site directed mutagenesis. Therefore, in addition to the mutations we also had an HA tag added to the N-terminus of mutated Ads (HA-mut-Ads). We cloned the newly generated HA-mut-Ads into the BamHI and BstBI restrictions site of pIRESpuro3 vector (Clontech, Mountain View,CA). The unique feature
of this vector is that it contains an internal ribosomal entry site or IRES, which permits the translation of two open reading frames (in this case HA-mut-Ads and Puromycin) from a single transcript under the control of a single promoter. Consequently, following transfection and puromycin selection all surviving clones will be expressing HA-mut-Ads. We will generate a stable Ads-rescued cell line by transiently transfecting the Ads KD cell line with the pIRESpuro3-HA-mut-TAT construct, using the FugeneHD transfection reagent. Next, we will select a stable colony by culturing cells under puromycin selection for one week. Any surviving colonies expressing the mutated Ads mRNA, will be selected, propagated and assessed for their ability to differentiate into mature, multinucleated osteoclasts, as well as for their ability to induce DC-STAMP expression in response to sRANKL.

Another important question that still remains unanswered concerns the exact sub-cellular localization of Ads in maturing and mature osteoclasts. Unfortunately the exact sub-cellular localization of Ads has eluded us thus far, primarily due to the lack of a specific primary antibody. Our current, anti-murine Ads primary antibody recognizes 4 non-specific bands at 30kDa, 55kDa, 70kDa and 15kDa, and is therefore not an ideal antibody for immunofluorescence experiments. To circumvent this problem, we have proposed a number of experiments. First and foremost, we plan to develop a new rabbit, anti-murine Ads primary antibody, raised against a peptide sequence (AQELQHPEFARAGQC) derived from the N-terminus of Ads. A similar antibody was previously raised against this very same peptide sequence by Lueck and colleagues (179). This peptide derived antibody was shown to have specific affinity for Ads, did not cross react with gelsolin, and was used for immunofluorescence localization studies in the murine kidney and intestine (179). Therefore, we feel confident that this antibody will be instrumental in determining the localization of Ads throughout the various stages of osteoclastogenesis. Unfortunately, the development of this antibody will take a minimum of 33 weeks (Pacific Immunology, CA). In the meantime however, we propose a number of secondary experiments that would also answer questions regarding Ads sub-cellular localization in maturing and mature osteoclasts. We have preliminary data regarding Ads localization in undifferentiated, macrophages or osteoclast precursors and maturing RANKL stimulated pre-osteoclasts. We cloned the full length Ads ORF cDNA (from pSE380-Ads) into the XhoI and BamHI restriction sites of the Enhanced Green Fluorescence Protein expression vector, pEFGP-c1 (GFP-Ads). We transiently co-transfected wild type RAW macrophages with
GFP-Ads and pRFPruby-N1-Lifeact (RFP-Lifeact) using the Amaxa Biosystems Nucleofector II transfection system (Nucleofector Kit V, program D-032). The pRFPruby-N1-Lifeact vector was a kind gift from Dr. R. Wedlich-Soldner (Cellular Dynamics and Cell Patterning, Max Plank Institute of Biochemistry, Martinsried, Germany). The pEGFP-c1-adseverin construct allowed us to visualize exogenous Ads tagged at its c-terminus with GFP. Likewise, RFP tagged lifeact, a 17 amino acid peptide that binds specifically to F-actin without interfering with actin dynamics (203), allowed us to visualize the localization of actin structures. 2.0x10^5 double transfected RAW 264.7 macrophages were seeded onto 35mm, glass bottom tissue culture plates (Mat Trek Corp) and incubated with or without sRANKL at 37°C. Live cell images were taken using confocal fluorescent microscopy. Ads was diffusely expressed in the cytoplasm of undifferentiated macrophages (Figure S1, - sRANKL panels). However, upon stimulation with sRANKL, osteoclast progenitors displayed typical actin rich structures where adseverin intensity was increased (Figure S1, + sRANKL panels). The images shown in Figure S1, are 102uM sections of live cell images of macrophage taken using the confocal microscope. Because the width of the section is quite large, the co-localization of Ads with F-actin seen in response to RANKL stimulation could be due to cellular thickness around the cellular periphery rather than actual co-localization between Ads and F-actin. Further, GFP-Ads was over expressed in the transfected cells. Therefore, GFP-Ads was most likely not contained to its native site within the cell but more likely was simply diffusely located throughout the cell. A more accurate localization of Ads would be achieved by examining Ads localization in weakly GFP expressing macrophages. By optimizing our transfection conditions we can increase better use this GFP-Ads system to examine the localization of Ads during the OCG.
Chapter 6
Conclusions

This was the first report that examined the expression and function of the actin binding protein, Ads, in the osteoclast biology system. We have shown for the first time that ads message and protein expression are up regulated in response to RANKL during the early phases of osteoclastogenesis. We have also shown Ads to be highly expressed in mature, multinucleated, TRACP positive osteoclasts. In terms of function, we have shown that a targeted knock down of Ads expression leads to a major fusion defect leading to the loss of multinucleated osteoclasts. However, a number of questions regarding the role of Ads in osteoclast maturation, the interplay between Ads and DC-STAMP expression and the sub-cellular localization of Ads during osteoclastogenesis still remain. All in all, we have made significant advancement in the field of osteoclast biology and have identified a novel protein that can serve as a specific marker for both mature osteoclasts as well as a pro-fusion marker during osteoclastogenesis. Despite the great progress made thus far, this project has just taken first important and necessary steps towards fully understanding the role of Ads during OCG. There is great potential in Ads and much is left to be unraveled when it comes to Ads and osteoclasts.
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