CD109 Plays a Role in Osteoclastogenesis

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Faculty of Dentistry
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CD109 plays a role in OCG

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Abstract

Osteoclasts are large multinucleated cells that arise from the fusion of hematopoietic stem cells from the monocytes-macrophage lineage and play an important role in bone metabolism, specifically in bone resorption. To date, many of the factors involved in Osteoclast fusion remain unclear. The objective of this project was to identify novel factors that regulate Osteoclastogenesis. **Methods:** microarray, Rt-qPCR and Western blot analysis were performed to identify novel molecules involved in Osteoclastogenesis. A stable CD109 knockdown macrophage cell line was generated using a retroviral shRNA construct. **Results:** CD109 was identified as a novel molecule that varies its expression during RANKL induced OCG. CD109 KD cell lines showed a deficiency in their osteoclast fusion capacity. **Conclusions:** pre-osteoclasts express CD109 at different levels while undergoing OCG. CD109 might be an important regulator of OCG although further characterization of its activity is still required.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BMMs</td>
<td>Bone marrow monocytes</td>
</tr>
<tr>
<td>BMU</td>
<td>Basic multicellular unit</td>
</tr>
<tr>
<td>CFU-M</td>
<td>Macrophage colony forming units</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
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<tr>
<td>FI</td>
<td>Fusion index</td>
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<td>Fig</td>
<td>Figure</td>
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<tr>
<td>FOV</td>
<td>Fields of view</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl-phosphatidylinositol</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidise</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin like growth factor-1</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LIMMA</td>
<td>Linear models for microarray data</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCS-F</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteases</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NFATC1</td>
<td>Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor Kappa Beta</td>
</tr>
<tr>
<td>OCG</td>
<td>Osteoclastogenesis</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OC-STAMP</td>
<td>Osteoclast stimulatory transmembrane protein</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor Kappa Beta</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rt-qPCR</td>
<td>Real-Time quantitative PCR</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin RNA</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tween 20 buffer</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>TIMPS</td>
<td>Tissue inhibitors of matrix metalloproteinases</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α- Minimum essential medium</td>
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Chapter 1
Introduction

The focus of this chapter will be defining the primarily studied cell, the osteoclast, the processes related to these cells, i.e. osteoclastogenesis and osteoclast fusion, and molecules involved with those processes including CD109, the molecule of interest in this project.

1.1 Bone Physiology

Bone is a highly specialized type of connective tissue that serves of the support system of all vertebrates. In addition to protection and support, one of the important functions of bone is to provide inorganic ions that actively participate in calcium homeostasis in the body.

Bone consists of an organic matrix composed of 95% type I collagen. The remaining 5% is composed of noncollagenous proteins and proteoglycans. This matrix is strengthened by calcium and phosphate deposits in the form of hydroxyapatite. There are two forms of bone: cortical bone, which provides mechanical and protective functions and consists of condensed collagen fibrils forming concentric lamellae; and cancellous bone, which is responsible for the metabolic functions of bone (calcium and phosphate storage and mobilization), and has a loosely organized porous matrix[1].

1.1.1 Cellular Components of Bone

As regards the cellular components of bone, there are four distinct types of cell: osteoclasts, osteoblasts, osteocytes and bone lining cells. The osteoblasts produce the bone matrix and then regulate its mineralization, and the osteocyte, which is a mature osteoblast, is responsible for maintenance of bone and part of the response to metabolic challenges. These cells are actually embedded within bone matrix and are connected to one another by canaliculi, which provide channels for intercellular communication. Osteoblasts are derived from cells of mesenchymal origin[1]. In their structure, they are almost identical to fibroblasts but are probably more cuboidal in appearance. As well, they are of course associated with mineralized extracellular matrix. Osteoblasts express a number of molecules necessary for their differentiation and function such as Cbfa1 and osterix, osteonectin, osteopontin, osteocalcin and
bone sialoprotein [2, 3]. Also, members of the growth factor family of proteins have been related to the control of osteoblast differentiation during embryonic development. For instance, transforming growth factor beta (TGF-β) has been shown to regulate the amount of osteoblast differentiation in vivo and to inhibit the expression of Cbfa1 in cultured osteoblasts. Cbfa1 also regulates bone formation by modifying the expression of osteocalcin, which is only expressed by differentiated osteoblasts[4].

Bone lining cells have been thought to be inactive cells that merely cover bone surfaces [1]. However, recent studies report that bone-lining cells form a signaling network with osteocytes that controls bone remodelling by sensing mechanical loading [5]. Alternatively, osteoclasts are the only specialized cells that resorb mineralized bone and bone matrix. The coordination of the activities of these various cell-types is such that homeostasis is established whereby under normal circumstances (e.g. health), bone formation is balanced by bone resorption by a process known as coupling [1]. For the purposes of the proposed investigation, the following section will focus on describing osteoclast features such as differentiation, function and activity.

1.2 Osteoclasts

Osteoclasts are defined as large (50-100μm diameter) multinucleated cells derived from hematopoietic stem cells, themselves derived from the monocytes-macrophage lineage.[6-8] The primary physiological role played by osteoclasts is the extracellular resorption of mineral and organic bone matrix components of bone and regulation of calcium influx in the body[9]. These cells also play an integral role in the pathophysiological mechanisms underlying chronic osteolytic diseases such as periodontitis, osteoporosis and rheumatoid arthritis.[10, 11]

Structurally, osteoclasts contain several circumnuclear golgi stacks, a high density of mitochondria and multiple lysosomal vesicles. When active, these cells rest on the bone surface and have two plasma membrane specializations: a ruffled border, under which bone resorption takes place, and a clear zone which contains abundant microfilaments but has no organelles. The clear zone surrounds the ruffled border and works as a zone of attachment of the osteoclast to the bone matrix. Active osteoclasts present the majority of their nuclei located in the part of the cell away from the bone, and are considered polarized[1].
After differentiation and activation, osteoclasts undergo apoptosis. It was demonstrated that TGF-β and sex steroids such as estrogen and testosterone, factors that are known to inhibit osteoclast activity, can induce osteoclast apoptosis whereas different cytokines that promote osteoclast activity also increase osteoclast survival [7, 12, 13].

1.2.1 Osteoclastogenesis

Osteoclastogenesis (OCG) is a complex multistep process that involves the participation and interaction of a large number of molecules. Osteoclasts arise from the fusion of progenitor hematopoietic cells in the bone marrow after simulation by macrophage colony-stimulated factor (MCS-F) and receptor activator for nuclear factor-kB ligand (RANKL)[14]. Cell migration is also an important process necessary for OCG. It has been shown that osteoclasts migrate towards several bone matrix peptides as well as toward MCS-F and TGF-β [15-17]. Numerous other cytokines such as IL-1, IL-6, MCP-1, TNF-α, TGF-β, OPG, vitronectin, β integrins, CD44, CD200, DC-Stamp, Sirpa, Tetraspanins, ATP6V0D2, OC-stamp, TRAF6, cathepsin k, CIC/7 and CD27 have been reported to be involved in the process of OCG [6, 18-21]. Estrogen, sex steroids and aging have also been implicated in the regulation of osteoclast function[21]. Estrogen inhibits osteoblasts from producing MCS-F, RANKL, IL-1, IL-6 and TNF-α and stimulates the production of the RANKL inhibitor, osteoprotegerin (OPG), and TGF-β, and therefore reduces osteoclast formation and function. It has been shown that male and female sex steroids can affect bone metabolism by affecting bone cell function [22]. There is increasing evidence of age-related hormonal changes correlated with decreased bone mineral density [23, 24]. According to previous reports, marrow secretion of IL-11 and IL-6 in women increases with age and, when undergoing an estrogen replacement therapy, significantly lowers the expression of IL-6 and IL-11 are detected [25, 26]. Additionally, Szulc et al. (2001) showed that serum levels of OPG increase with age and that these levels correlated positively with levels of free estradiol and free testosterone indices but were negatively associated with the levels of PTH [21]. In contrast, although the serum levels of OPG increase with age, there is a marked decrease of bone marrow OPG expression in older subjects, permitting greater binding of RANKL to its receptor RANK, resulting in increased osteoclast formation and therefore, bone resorption [21, 27].
1.2.2 Macrophage colony stimulating factor

Macrophage colony stimulating factor (MCS-F) was first described as a regulator of macrophage formation but later studies found that mice with a mutation of this molecule lacked osteoclasts and demonstrated a severe osteopetrotic phenotype [28-30]. MCS-F binding to its cell surface receptor, c-fms, a tyrosine kinase, is regulated by stimulators of bone resorption and it causes activation of phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways, thereby causing pre-osteoclasts to differentiate into osteoclasts [31, 32]. In mature osteoclasts, MCS-F was found to increase c-src kinase activity, which induces cytoplasmic spreading of osteoclasts [33-35]. Together with RANKL, MCS-F is required for osteoclast fusion.

1.2.3 RANK, RANKL and OPG

As mentioned above, OCG can occur only when RANKL is present (note that RANKL is also known as TRANCE, ODF, TNFSF111 and OPGL). RANKL is a member of the TNF ligand family and is produced by osteoblasts, activated T-cells and stromal cells [21, 36]. It promotes osteoclast differentiation and activation and inhibits apoptosis of osteoclasts by binding to the transmembrane receptor RANK, which is expressed by osteoclast precursors and mature osteoclasts [21, 37]. The RANK molecule requires molecules such as the TNF receptor associated factor family of proteins (TRAF) in order to transduce signals in the intracellular domain. TRAF6 has been found to be the primary adaptor molecule that connects RANK to the differentiation and function of osteoclasts [37]. RANKL can also activate mature osteoclasts, increasing their survival time by the induction of changes in their cytoskeleton and stimulating multiple episodes of bone resorption [38, 39].

RANKL function is inhibited primarily by OPG. OPG is a member of the tumor necrosis factor (TNF) receptor family that contains four homologous domains for binding RANKL. It is produced by several cells such as osteoblasts, endothelial and lymphoid cells, and others [36]. Simonet et al. (1997) demonstrated that mice with increased expression of OPG were osteopetrotic. In contrast others have shown that, mice with decreased expression of OPG were osteoporotic (Mizuno et al. 1998). Apart from OPG, many other factors such as Vitamin D, PTH, glucocorticoids, PGE₂, LPS, FGF, histamine, IGF-1 and TGF-β have been shown to regulate the expression of RANKL [21].
1.2.4 TRAF6

In order to transduce its signals to the nucleus, RANK requires the recruitment of proteins from the TRAF family in the cytoplasm due to a lack of intrinsic enzymatic activity in its intracellular domain. From the TRAF family molecules, it is known that TRAF6 is the principal molecule that adapts to RANK during OCG. TRAF6 binds to RANK and activates NF-κB and MAPKs. Its N-terminal RING finger domain is responsible for the formation of TRAP-positive multinucleated cells [6]. TRAF6 also binds to IL1-R intracellular domain, resulting in osteoclast activation [40]. It has been recently demonstrated that TRAF6 is essential for osteoclast differentiation and bone resorption activity and has been described as the key molecule responsible for linking RANK signaling in the cytoplasm to the nucleus[41].

1.2.5 TGF-β

TGF-β is a member of the TGF/activin subgroup of the TGF superfamily that plays an essential role in cellular differentiation. As a result of TGF-β binding to its receptors (type I and II) on the extracellular domain, phosphorylation of several serine and threonine residues occur, allowing SMAD2 binding and activation, which together with SMAD4 transmits signals through the cytoplasm to the nucleus and therefore regulates gene expression[42]. A number of cell types have been identified that also express TGF-β co-receptors such as betaglycan and endoglin. TGF-β receptors can be internalized via two different pathways: clathrin coated pits and caveolus-dependent pathways. The clathrin pathway of internalization has been associated with SMAD2/3 signaling and receptor recycling [43]. In contrast, TGF-β receptor localization in caveolae has been associated with down-regulation of SMAD2 and SMAD3 signaling [44].

TGF-β plays an important role in bone metabolism. Since 1990, several in vivo studies have shown that TGF-β increases bone turnover. Yan et al. (2001) found a dose dependent increase in OCG when RAW 264.7 cells were cultured with different concentrations of TGF-β. In this study, cells were co-cultured with RANKL (50ng/ml), MCS-F (20 ng/ml) for 7 days and then treated with different concentrations of TGF-β1 (0, 0.1, 0.5, 1, 5 and 10 ng/ml) which led to significant increases in the number of TRAP positive multinucleated cells (120 fold) using
concentrations of only 1 or 5 ng/ml of TGF-β [45]. Similarly, Quinn et al. (2001) stimulated RAW 264.7 cells with RANKL (50ng/ml) and different concentrations of TGF-β, demonstrating that 10 ng/ml of TGF-β increased osteoclast formation by 9-fold and that this stimulation only occurred during the first 3 days of culture [46]. When bone marrow monocytes and RAW 264.7 cells were cultured in the presence of RANKL, MCS-F and TGF-β, this caused a consistent increase in OCG (4.5-fold) (Koseki et al. 2002). The same author also reported that TGF-β suppressed cell growth without influencing RANKL’s growth inhibitory activity and suggested that increased OCG caused by TGF-β is due to TGF-β’s effect in the early stages of osteoclast differentiation and is mediated by activation of Smad2 and Smad3 signals thus causing macrophages to differentiate into pre-osteoclasts[47].

In contrast, other studies have demonstrated that TGF-β has an inhibitory effect on OCG. Using human monocytes, it was shown that when TGF-β was present during the entire culture period or during the preosteoclastic phase of culture (6-10 days), osteoclast formation and TRAP activity was inhibited by 75%, while the expression of cathepsin K and MMP-9 (key molecules for osteoclast bone resorption capacity) were also blocked, suggesting that TGF-β could control the amount of resorption by mature osteoclasts in more than one way (Karsdal et al. 2003). The same study showed that TGF-β down-regulates expression of RANK, which induces reductions in OCG due to a decrease in RANKL signaling but without affecting the expression of the receptor for MCS-F [48]. Other studies have shown that TGF-β reduces the levels of RANKL mRNA by stimulating the production of OPG by bone marrow stromal cells. This inhibitory effect appears to be dose dependant [46, 49].

1.2.6 Regulation of Osteoclast Activity

The primary function of the osteoclast cell, that being to resorb bone, can be achieved as a consequence of several critical characteristics that these cells possess as will be discussed below. First, osteoclasts have the capability of adhesion to the bone surface and migration in a direction to and within bone surfaces, both characteristics being facilitated by adhesion receptors (podosomes). Osteoclasts are also able to internalize the material produced following extracellular degradation of the bone matrix that depends on vesicular transport as well, but in the opposite direction. Later on in the process of resorption, osteoclasts are able to acidify the
sub-osteoclastic bone resorbing compartment when protons are generated by carbonic anhydrase. They are transported throughout the plasma membrane by a V-ATPase in combination with the extrusion of chloride ions across the CIC-7 chloride channel and homeostatic ion transport at the basolateral membrane. Finally, these cells have a secretion capacity which depends on vesicular transport from the endoplasmic reticulum to the Golgi and from the Golgi to the secretory pole of the cell [29].

For bone resorption to occur, a number of cellular activities are required. These are aimed towards first, dissolution of the mineral component of bone, and then degradation of the remaining organic matrix.

After migration of osteoclasts to a site that is about to undergo resorption, the osteoclasts attach to the bone after which a unique membrane domain called the sealing zone is formed[9]. Part of the sealing zone is formed by a ring of firmly packed actin filaments that are located at the ventral side of a polarized cell. Bone resorption starts with the dissolution of hydroxyapatite crystals, which occurs by the secretion of HCL through the ruffled border and therefore leads to the formation of resorption lacunae in which the pH is low [9, 50]. The dissolution of the mineral component of the bone is followed by the degradation of its organic matrix, a process accomplished by osteolytic enzymes such as cathepsins and the matrix metalloproteinases (MMPs), which will be discussed below in more detail [9, 29]. Once the bone matrix is degraded, the products have to be removed from the resorption lacunae. This happens by transcytosis through the cell from the ruffled border to the secretory domain, where the degradation products are released by exocytosis into the extracellular space [9, 51].

To elucidate more clearly, the events related to degradation of the organic matrix of bone, additional information is presented below. The interaction between integrins and the extracellular matrix proteins of the bone is one of the pathways responsible for the tight attachment of osteoclasts to bone surfaces [29] and therefore facilitates the formation of an acidic microenvironment abundant in acid-proteases that break down of the remaining organic matrix after demineralization has taken place [52]. It has been shown that osteoclasts express high levels of αvβ3 integrin that can be found at the plasma membrane and in various intracellular vacuoles [9, 53]. In the presence of αvβ3 blocking antibodies, bone resorption in vitro is inhibited [54]. Tyrosine kinases also play important roles in cell adhesion and migration. For instance, s-Src was found to be indispensable for osteoclast adhesion, resorption activity and
motility, and it has been shown that osteoclasts lacking c-Src were not able to form sealing zones and resorb bone [29, 52]. Pyk2, another protein from the tyrosine kinase family, is also an essential adhesion molecule in osteoclasts [29].

Compared to inactive or non-resorbing osteoclasts, active osteoclasts are highly polarized cells that contain, in addition to the sealing zone, three more specialized cellular domains: a ruffled border, a secretory domain and a basolateral membrane [9]. The ruffled border is formed by the fusion of intracellular acidic vesicles with the plasma membrane facing the bone surface, a process followed by secretion of hydrochloric acid vacuolar H+-ATPase, as well as the release of the proteases noted above. This series of events leads to degradation of bone matrix under the ruffled border. Subsequently, the degraded matrix is internalized and the functional secretory domain functions as a site of exocytosis of the degradation products [9, 51]. The third specialized membrane domain of active osteoclasts, the basolateral membrane, represents a homogeneous membrane area that is located away from the bone surface without being in contact with the mineralized bone matrix [9, 50].

1.2.7 Osteolytic enzymes

The main osteolytic enzymes secreted by osteoclasts are tartrate-resistant acid phosphatase (TRAP), cathepsins and the matrix metalloproteases (MMPs). These enzymes, in conjunction with a decrease in the pH, are responsible for degradation of bone matrix [29].

**Tartrate-Resistant Acid Phosphatase**

Tartrate-resistant acid phosphatase (TRAP) is an iron-containing enzyme found in bone as well as the immune system and is capable of hydrolyzing phosphoproteins, arylphosphates, ATP and other nucleotide triphosphates. It is essential for normal development of bone [29, 55]. It is expressed by cells of monocytic lineage such as macrophages and dendritic cells [55] and over the past years it has been used as a cytochemical marker of osteoclasts because it is expressed highly in these cells, although it has been also detected in osteoblasts and osteocytes [56, 57]. TRAP expression is also abundant in tissues other than bone such as the skin, thymus, spleen, liver, lung, linings of the gastrointestinal tract and in tissues of the nervous system[55].

TRAP is secreted by the osteoclast from lysosomes into the resorptive vacuole under the ruffled border during bone resorption and this secretion appears to correlate with its resorptive
capacity. TRAP activity in serum has been shown to be pathologically increased in conditions characterized by an increment in bone resorption, including osteoporosis, hyperparathyroidism, Gaucher’s disease and some types of cancer [29, 55].

**Cathepsin K**

Cathepsin K is an acid-activated cysteine proteinase that is only expressed by osteoclasts at sites of active cartilage and bone remodeling and plays an essential role in bone resorption due to its capacity for degrading collagen, elastin and gelatin [21, 58, 59]. During differentiation and fusion, osteoclasts regulated by RANKL produce large amounts of cathepsin K that increase as the process progresses[21]. As mentioned before, secreted proteases in the ruffled border have to be released from the cell at the secretory domain. The DAG-PKCδ pathway promotes exocytosis of cathepsin K vesicles in osteoclasts via the phosphorylation of the actin bundling protein, MARCKS. Diacylglycerol (DAG) regulates vesicle fusion with the membrane and it activates the serine/threonine kinase, PKCδ. This pathway was shown to modulate the secretion of cathepsin K independent of ruffled border formation or trafficking of v-ATPase[59].

Knocking out cathepsin K in mice results in an osteopetrotic phenotype, abnormal joint morphology, increased bone volume and greater trabecular thickness [29]. Additionally, studies have shown that human mutations affecting cathepsin K function lead to pycnodysostosis, a disease characterized by pathological increases in bone mass [59-61]. Moreover, Saftig et al. (1998) demonstrated that osteoclasts isolated from cathepsin K knockout mice exhibit impaired bone resorption activity in vitro [62].

**Matrix Metalloproteases**

Matrix metalloproteases (MMPs) are zinc-dependent endopeptidases that reside in bone in their latent form and can be activated by osteoclastic proteolytic activity and be involved in the bone resorption process. In order to inactivate and thereby regulate MMP activity, the expression of the tissue inhibitors of matrix metalloproteinases (TIMPs) is necessary and it has been shown that in non pathological human bone, TIMP1 is highly expressed in osteoclasts [29]. Four different groups of MMPs in mammals have been identified: collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10), and membrane-type metalloproteinases (MMPs 14–17) [63].
It is known that the gelatinases and MMP13 are produced by osteoblasts and osteoclasts [63]. Okada et al. (1995) showed that MMP-9 is produced by human osteoclasts and suggested that it can degrade bone collagen in conjunction with MMP-1 and several cysteine proteinases [64]. Moreover, Kusano et al. (1998) showed that IL-1 stimulates the expression of MMP-2, -3, and -13 mRNAs in mouse calvariae, inducing osteoclast formation, and therefore increasing the expression of MMP-9 mRNA which supports the concept that MMPs regulate matrix degradation during osteoclast-mediated resorption of bone [63].

1.3 Osteoporosis and other Bone Remodeling Disorders

As mentioned above, the osteoclast is the only specialized cell capable of resorbing bone as well as dentin and mineralized cartilage, and its function cannot be replaced by any other cell type. Consequently, a deficiency in osteoclast formation and/or function will lead to a broad scope of disorders in bone. An increase in osteoclast function that is not matched by a concomitant increase in osteoblast activity will lead to decreased bone mass density or disordered skeletal architecture; conditions that can lead to the development of osteoporosis. Osteoporosis is clinically defined as a condition in which low mineral density leads to a decreased skeletal strength and an increased risk of skeletal fracture [29]. The World Health Organization (WHO) defines osteoporosis as a bone mineral density of 2.5 SD or more below peak bone mass, when measuring with dual-energy X-ray absorptiometry [65]. Treatment with pharmacological agents (e.g. estrogen therapy), calcium and vitamins is usually recommended for all patients with an osteoporotic profile [66]. Recently, the WHO collected data from 9 large cohort studies in the UK and identified the risk factors for fracture: body mass index, prior history of fracture, smoking, use of oral glucocorticoids, rheumatoid arthritis. A new fracture prediction algorithm (FRAX) was developed in order to determine a patient’s absolute fracture risk in 10 years [66, 67]. A Committee form the National Osteoporosis Foundation together with the WHO later adapted for the U.S population. Additionally, this Committee analyzed the levels of fracture risk above which it is reasonable to consider treatment from cost-effectiveness prospective and reported that treatment would generally be cost-effective in patients with a 3% probability of hip fracture at 10 years [66, 68]. As eluded to above, osteoporosis might develop
as a consequence of increased activity of osteoclasts. However there are several cell-mediated scenarios under which osteoporosis might develop including: (a) an increase in osteoclast activity as indicated above, (b) decreased bone formation due to insufficient osteoblast formation and/or activity, or (c) uncoupling between the osteoclast and osteoblast activities. In addition, endocrine disorders such as estrogen deficiency during menopause or following oophorectomy, hyperparathyroidism, hyperthyroidism, and overproduction of cortisol by the adrenal glands can also lead to increased osteoclast formation and/or function [29]. The Canadian Multicentre Osteoporosis Study stated that by 2000, the prevalence of osteoporosis in Canadian women ≥50 years old was 15.8%, but the prevalence was lower for men with a 6.6% [65].

Other disorders can develop as a consequence of reduced osteoclast activity or osteoclast formation more specifically, one example being osteopetrosis also known as *marble bone disease*. Osteopetrosis characterized for presenting an increased bone mineral density with a phenotype of entirely marbled bones. The condition is actually fatal because the marrow spaces eventually close which then leads to a cessation of haematopoiesis[29]. Autosomal recessive severe (malignant) osteopetrosis presents in early infancy with macrocephaly, progressive deafness and blindness, extramedulary haematopoiesis, and pancytopenia [29] and its incidence ranges from 1 in 200,000 to 1 in 500,000 live births [69]. Autosomal dominant osteopetrosis is a milder form of osteopetrosis, is not lethal, and affects adults. This condition can also cause bone pain and hearing loss but there is less susceptibility to fracture in affected persons[29]. The prevalence of dominant autosomal osteopetrosis ranges from 1 in 100,000 to 1 in 500,000 adults[69].

Paget’s disease is a rare disorder of bone remodeling characterised by accelerated remodeling activity. Bones affected by Paget’s disease present with intense local bone resorption by multinucleated osteoclasts followed by the formation of large amounts of woven bone caused by increased recruitment of osteoblasts. The excessive formation of bone is thought to be related to the primary defect, that being increased bone resorption and this is supported by the fact that after treatment with bisphosphonates, drugs that inhibit osteoclast activity, bone formation also decreases[70] as measured by bone histomorphometry. Paget’s disease presents in middle-aged and older people. Patients affected can be asymptomatic, while others can present with bone pain due to nerve compression and microfractures (the most common
symptom) and also pathologic fractures, bowing of long bones [29, 70], and hearing impairment due to loss of bone mineral density in the cochlear capsule [71]. There is a genetic predisposition to this disease and it has been linked to mutations of RANKL in chromosome 18 and mutations in the gene for sequestosome 1 [29, 70].

Inflammatory disease can increase bone resorption and decrease bone formation[72]. In inflammatory osteolysis, which includes disorders such as rheumatoid and psoriatic arthritis, many inflammatory cytokines present in the synovial fluid such as RANKL, IL-1 and TNF-α induce osteoclast formation and activity resulting in the loss of bone surrounding the articulations, which leads to joint collapse and disfigurement [73, 74]. Another common inflammatory bone disorder is periodontal disease, which affects the teeth and surrounding tissues. Periodontitis is initiated by a bacterial infection followed by immune and inflammatory responses of the host that are essential for the pathogenesis of this disease [75]. These inflammatory responses activate the immune system, resulting in the release of cytokines such as IL-1, -6, -11 and -17, TNF-α, leukemia inhibitory factor, oncostatin M, and others that lead to the destruction of connective tissue and alveolar bone resorption [75, 76]. Moreover, it has been reported that expression of RANKL is induced in response to cytolethal distending toxin from Aggregatibacter actinomycetemcomitans, an abundant bacteria present in periodontal disease sites. It has been reported further that RANKL expression is affected in proportion to the rate of alveolar bone loss while its concentration in gingival crevicular fluid is higher in patients with periodontitis when compared to healthy controls [75].

1.3.1 Therapeutic management of osteoporosis

Numerous pharmacological substances have been developed to target osteoclasts, decreasing levels for biomarkers of bone turnover, increasing mineral density in bone and most importantly, preventing fractures. Bisphosphonates are pharmaceutical agents widely used for the treatment of osteoporosis. These drugs present two carbon phosphate bonds and various side chains. Differences in their structure determine potency, duration of action, side effects and efficacy with regard to inhibition of osteoclast-mediated resorption of bone by osteoclast apoptosis [29, 77]. Bisphosphonates are hydrophilic and therefore, their absorption from the gastrointestinal tract after oral administration is as low as 1%. Since they have a high affinity for
bone mineral, 50% of the absorbed drug is then retained in the skeleton, whereas the remaining is eliminated in via renal excretion without being metabolized [77].

Calcitonin is a hormone that has been also used for the treatment of osteoporosis as well as for other conditions such as Paget’s disease of bone and malignancy-associated hypercalcemia. It is secreted by the thyroid gland and regulates calcium homeostasis. Calcitonin binds to its receptor in the osteoclastic cell, changing its configuration and therefore, preventing it from attaching to the bone surface. It decreases the depth of the resorption lacunae but does not affect their number and does not induce osteoclast apoptosis. Administration of this agent for a long period of time can cause osteoclast resistance although this effect can be reversible by following a cyclical or intermittent dosing. It has been shown that calcitonin can improve the quality of osteoporotic bone and reduce the risk of fracture although in much less proportions than more potent antiresorptive agents such as bisphosphonates [78].

Many other compounds targeting specific molecules such as RANKL and cathepsin K, hormone replacement therapy, Parathyroid hormone (PTH) therapy and others have been also used for the treatment of osteoporosis. [29]. Denosumab is a human monoclonal antibody that binds to RANKL and inhibits RANK signaling. It has been recently approved in Europe for treating osteoporosis and several studies have shown that it reduces vertebral, hip, and non-vertebral fracture risk in postmenopausal women [79, 80]. It is also used for the treatment of rheumatoid arthritis. Inhibitors of cathepsin K such as ONO-5334 and Odanacatib, have been demonstrated to increase the bone mineral density in lumbar spine, femoral neck and hip. These inhibitors do not inhibit other osteoclast digestive enzymes and therefore their antiresorptive effect is less potent than drugs like bisphosphonates. Because of their short onset of action they need to be taken in conjunction with other antiresorptive agents[81]. It is known that levels of estrogen are decreased in post-menopausal women and therefore, hormone replacement therapy was widely used for symptoms related to menopause as well as for the management and prevention of chronic diseases such as osteoporosis due to its potential to reduce fracture risk at lower doses [82]. Because of its long term side effects such as breast cancer, coronary heart disease and thromboembolic events, hormone replacement therapy is not the first choice for the treatment of osteoporosis[83]. PTH therapy has also been demonstrated to be safe and effective for osteoporosis treatment. It has been demonstrated that, in contrast to antiresorptive agents, PTH stimulates bone formation at a greater rate than bone resorption. Stopping treatment with PTH
leads to rapid bone loss and therefore, patients need to be on a long-acting bisphosphonates therapy or other agents to maintain an adequate bone mineral density [81, 84]. Other novel targets including growth factors, such as BMPs, TGF-β and insulin-like growth factor-1 are currently being studied [81].
1.4 *In-vitro* experiments with Osteoclasts

**Osteoclast formation in vitro**

Takahashi et al. (1988) first suggested that TRAP-positive multinucleated cells formed in mouse marrow cultures in response to particular osteotropic hormones and that osteoblasts may play an important role in the process of osteoclastogenesis[85]. Later on, several authors concluded that the marrow microenvironment was indispensable for osteoclastogenesis to take place due to the presence of a variety of osteotropic factors that induce the resident stromal cells to produce RANKL [7]. Quinn et al. (1998) isolated and cultured mouse spleen cells and monocytes demonstrating that in the presence of MCS-F and a soluble form of murine RANKL, these cells formed large multinucleated osteoclast-like cells within 7 days of culture. These cells had very robust bone resorption activity. They also showed that the same protocol could be applied to peripheral blood human monocytes, but in this case the cells must be cultured for at least 11 days [86]. During the initial three days of culture, MCS-F-dependent proliferation of hematopoietic stem cells occurs, and after the third day, these mononuclear cells have completed differentiation into osteoclast precursors forming macrophage colony forming units (CFU-M) and now express TRAP, NF-κB, and NFATc1. From day 4, MCS-F-dependent proliferation slows down, and RANKL and its downstream molecules induce TRAP positive cells to fuse forming multinucleated giant cells and start to express more specific markers of the osteoclast lineage. In the maturation stage, multinucleated osteoclasts start expressing molecules such as cathepsin K and c-Src, obtaining their bone resorption capacity[6].

An alternative for culturing primary osteoclasts *in vitro* arose from the establishment of an osteoclastogenic cell line; RAW 264.7, as described by Raschke et al. (1978). This macrophage cell line was created from the ascites of a tumor induced in a male mouse by intraperitoneal injection of Abelson leukemia virus (it has been confirmed that subsequently, the cells do not secrete detectable virus particles) [87]. RAW 264.7 cells have been used for macrophage studies for over 30 years due to their capability to be stimulated by RANKL thereby leading to the development of multinucleated osteoclast-like cells that are able to resorb mineralized bone. RAW 264.7 cells express MCS-F and therefore do not require MCS-F for osteoclast formation[88]. Using RAW 264.7 cells may facilitate studies of OCG due to their ready availability, as well as the simplicity involved with their growth in culture. As well, these
cells are sensitive to biological manipulation and actively resorb bone matrix within a relatively short period of time, that being as early as 4 days following fusion of the precursors. Although not absolutely necessary RANKL can be used to accelerate fusion/differentiation of very active (i.e. in relation to bone resorption) osteoclast-like cells that also demonstrate a high degree of similarity to osteoclasts formed from primary precursor cells [88].

**Osteoclast function in vitro**

A model system for studying osteoclast-mediated bone resorption *in vitro* was first described by Boyde et al. in 1984. Osteoclasts were extracted from the femurs and tibias of New Zealand white rabbit foetuses and seeded for 24 or 48 hours on transverse slices of 100 to 200 µm thick dentin slices made from unfixed human roots or sperm whale dentin using a low speed diamond saw and cleaned by sonication in distilled water for at least 20 minutes. Dentin tissue, like bone, undergoes resorption *in vivo*. It was chosen due to its regular structure in that it is free of vascular canals and surfaces that might have been undergoing resorption *in vivo* which would confound experimental analyses later. The shape size and position of the osteoclasts as well as the more obscure areas corresponding to the resorption lacunae on the dentin slices were recorded. When toluidine blue stained slices were observed under light microscopy, more resorption lacunae, similar to those observed *in vivo*, were found in the 48 hour cultures when compared to the 24 hour ones. There was no evidence for the presence of other cell types. The authors concluded that this *in vitro* resorption method could be used to obtain greater knowledge of the factors influencing bone resorption mediated by osteoclasts [89].

Similarly, Chambers et al. (1984) created bone-slices of 0.5 cm² in area x 0.1 mm in thickness by cutting human femoral cortical bone with a carborundum wheel, and then cleaning them with sterile distilled water. After isolation of osteoclasts from rabbit femurs and tibias, osteoclasts were incubated on bone slices for 2, 6 and 24 hours. The number and depth of the resorption pits were counted in all slices and it was found that the bone surface after 2 or 6 hours of incubation with osteoclasts was not altered. By 24 hours, however, excavations in the bone slices were observed showing a fibrillar base (likely collagen fibres) with well-defined contours located under or close to an osteoclast. Regarding the shape and size of these concavities, three distinct patterns of resorption pits were distinguished: 1) Circular; small and deep concavities with well defined margins were the most common type observed. 2) Elongated, shallow and larger surface area pits. 3) Shallow pits with irregular contours [90].
Although these studies had their limitations, this technique was modified and adapted to study the direct and indirect effects of different molecules on the behaviour of osteoclasts during bone resorption and this system is still in wide use today [90].
1.5 CD109

CD109, the molecule of interest in this project, is a novel cell surface antigen that encodes a glycosyl-phosphatidylinositol (GPI)-linked protein of approximately 170 kd that belongs to the alpha (2) macroglobulin/C3,C4,C5 family of thioester containing proteins[91, 92]. It was originally identified as a cell surface antigen on a primitive acute myeloid leukemia cell line KG1a and first called 8A3 antigen[93]. CD109 has been found to be expressed in activated platelets and T cells, endothelial cells, leukemic megakaryoblasts, and a subpopulation of CD34 expressing cells [91, 92, 94-96].

Hashimoto et al. (2004) demonstrated that CD109 is overexpressed in human and mice cancer, and suggested that new therapeutics for malignant tumors, such as squamous cell carcinoma, could be developed by targeting the CD109 molecule [97]. Consequently, Hagiwara et al. (2008) suggested that CD109 plays a role in the development of oral cancers and that it could work as a prognostic marker of malignant transformation of premalignant lesions. In their study, they showed that, compared with control cells, oral squamous cell carcinoma cell lines overexpressing CD109 exhibited accelerated cell growth in vitro. Additionally, they demonstrated that overexpression of CD109 impaired the transforming growth factor beta1-mediated suppression of cell growth and therefore, cells overexpressing CD109 showed and increased proliferation rate when compared with control cells [98]. The same authors in 2010 found that CD109 is expressed in urothelial carcinoma. In this study, samples from 156 urothelial tissue carcinomas were collected and used to perform immunohistochemical and immunofluorescence staining for CD109, p-Smad2 and CD44. They found that 70% of urothelial carcinomas expressed CD109 compared to normal bladder epithelia where no CD109 expression was detected. These results suggest that CD109 might be involved in the development of bladder cancer. It was also observed that CD109 was higher in low-grade urothelial carcinomas when compared with high-grade lesions. These observations led to the conclusion that CD109 expression is one of the prognostic factors associated with better prognosis in urothelial carcinomas. It was also suggested that CD109 could be associated to the RTK-RAS pathway and that it may be involved in cell to cell adhesion in urothelial carcinoma [99].
Man et al. (2012) studied the expression of CD109 in scleroderma skin fibroblasts. Skin cells from systemic scleroderma patients were compared with those from healthy patients by immunohistochemistry and it was found that CD109 expression was increased in the skin tissue of scleroderma patients when compared with normal skin. It was also demonstrated by Western blot analysis that scleroderma skin fibroblasts and keratinocytes had a higher CD109 protein expression than normal cells. In this study, it was also shown that treatment with TGF-β, a cytokine known to play an essential role in the scleroderma pathogenesis, did not have any effect on CD109 protein levels in both normal and skin fibroblasts derived from subjects with scleroderma. This suggests that CD109 is not a direct target molecule of TGF-β in this cell type. Consequently, CD109 inhibits the phosphorylation of SMAD2 and SMAD3 as well as the production of extracellular matrix proteins such as collagen I and CCN2, although CD109 expression in sclerodermal fibroblasts was insufficient to inhibit the excess production of extracellular matrix[100].

In addition, it has been shown that CD109 is mutated in colorectal cancer [101] and its expression is down-regulated in many other types of cancers [98, 102-106] and in psoriasis [107]. The specific function of CD109 in hematopoietic cells remains unknown, although it has been suggested that it is involved in antibody-inducing T helper function[108].

Recent studies have identified CD109 as a TGF-β co-receptor that binds to the TGF-β 1 subtype forming a heteromeric complex with its receptors [44, 109]. CD109’s high affinity for the TGF-β receptors results in an inhibition of the TGF-β signaling independently of ligand sequestration and may apply its effect on TGF-beta signaling by direct modulation of receptor activity [44, 102].

Bizet et al. (2011) studied CD109 on TGF-β receptors in a human keratinocyte cell line, showing that CD109 increases the rate of TGF-β proteosomal degradation by increasing TGF-β binding to its receptors and inducing TGF-β receptor compartmentalization and internalization into the caveolae and specifically inhibiting SMAD3 phosphorylation [44]. CD109 maintains its ability to bind to TGF-β1 and sequester it away from the TGF-β receptors [107]. The negative regulation of TGF-β signaling by CD109 has been demonstrated to result in an increased proliferation of keratinocytes by several authors [98, 102, 110]. In a more recent study, Bizet et al. (2012) demonstrated that CD109 enhances SMAD7/Smurf2-mediated degradation of TGF-β receptor 1 in a ligand-dependent manner and that it regulates the localization and the association
of SMAD7/Smurf2 with TGF-β receptor 1. This study concluded that CD109’s inhibitory effect on TGF-β signaling requires the expression of those two molecules (SMAD7 and Smurf2) [111]. TGF-β/SMAD signaling is involved tumor suppression and enhancement of tumor formation, depending on the stage of the cancer. During the early phases of tumourigenesis, signals from the TGF-β/SMAD pathway suppress tumor growth; conversely, in later stages of tumor formation, it is known that TGF-β enhances the growth, invasion and metastasis of the tumor. It has been reported that the level of phosphorylated SMAD2 molecule, a product of the TGF-β/SMAD signaling, is high in normal epithelia but relatively low in cells with high expression of CD109. This suggests that CD109 negatively regulates TGF-β/SMAD signaling in vivo [99].
1.6 Statement of the problem

Bone turnover is a continuous and complex process that occurs throughout life and it requires a balance of cellular activity and multiple biochemical and mechanical factors. It occurs at selected sites involving different type of cells called basic multicellular units (BMU). It starts with resorption of old bone by osteoclasts and continues with the formation of new bone matrix by the osteoblasts and a later mineralization[1]. An imbalance between bone resorption and formation can lead to several diseases such as osteoporosis, osteopetrosis[29] and periodontal disease[32]. To date, bone disease therapies target different molecules responsible for osteoclast formation and activation [29] but, since the process of OCG is complicated and not fully understood, there are still many therapeutic approaches to be developed to effectively treat and control bone disorders.

Data from the microarray analysis performed in the present study showed an up-regulation of CD109 expression in murine osteoclast forming cell lines undergoing OCG. CD109 is a novel molecule identified as a TGF-β co-receptor, which has only been found to be expressed in activated platelets and T cells, endothelial cells, leukemic megakaryoblasts, and a subpopulation of CD34 expressing cells [91, 92, 94-96]. CD109 is overexpressed in many types of cancer, being considered as a marker of tumourigenesis [97, 99]. However, to date there are no reports in the literature regarding the expression of CD109 in the monocyte/macrophage lineage or active osteoclasts and its role in osteoclast formation and/or function has not been identified.

Therefore, the long term goal of this project is to characterize the role of CD109 in osteoclast formation.

Hypothesis: CD109 is required for osteoclast formation
Chapter 2
Materials and Methods

2.1 Establishment of cell lines

Dr. Roland Wedlich-Soldner supplied our laboratory with pmEGFP-N1-Lifeact which was amplified by using a primer pair 5’-GCGCAGATCTATGGGTGTCGCAGATTTGATCAAGAAA-3’ and 5’GCGCGAATTCTATTACTTTGTACAGCTCGTCCATGCCGAG-3’. The resulting PCR product was digested with BglII and EcoRI and ligated into the corresponding sites of a retrovirus vector pMSCVpuro. The construct and the packaging plasmid pVSV-G were co-transfected into GP-293 cells using the Fugene HD transfection reagent. The resulting viruses were transfected into RAW 264.7 cells (ATCC no: TIB-71, Passage P6) and individual positive clones were picked up using a standard limiting dilution method. Two different cell lines were established and were named H10 and C8 according to their osteoclastogenic capacity.

2.2 Osteoclast differentiation

Osteoclastogenesis (OCG) was initiated by plating 0.5 x 106 H10 and C8 cells separately in two 60mm tissue culture dishes containing 8 ml of Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS) and 10% antibiotics (164 IU/mL of penicillin G, 50 mg/ml of gentamicin, and 0.25 mg/ml of fungizone). Purified recombinant RANKL (60 ng/ml) was added once the cells were placed in the dishes. Cells were counted using a Z1 Coulter counter (Coulter Electronics, Mississauga, ON, Canada). Cells were cultured for 4 days, with a change of cell culture medium and RANKL supplementation on the second day of incubation. On day 4, cells were washed twice with PBS, fixed with 4% paraformaldehyde (PFA) and stained for TRAP.
2.3 TRAP staining

Cultured osteoclasts (day 4) were washed once with pre-warmed PBS and fixed with 4% PFA for 15 minutes at room temperature. Fixed cells were washed with PBS and then incubated in a solution of naphthol AS-BI phosphate and fast red TR salt (Sigma) in 0.2M acetate buffer (pH 5.2) containing 100mM sodium tartrate (Sigma) for 30 minutes at 37°C. TRAP-stained cells were washed twice with PBS and viewed with a Leitz Wetzlar microscope at a magnification of 200 X, and images were taken with a PixeLink camera.

2.4 Microarray

H10 and C8 cells were plated and grown for two days in 60 mm tissue culture dishes at a density of 0.5 x 10^6 in a culture medium consisting of DMEM, supplemented with fetal bovine serum (FBS) and antibiotics with purified RANKL (60ng/ml). Following two days of culture, total RNA was extracted from the H10 and C8 cells (Qiagen RNeasy Minikit, Germantown, MD, USA) and the concentration of extracted RNA was measured (using a nanodrop method). Samples were sent to the Centre for Applied Genomics at Sick Children’s Hospital (Toronto, ON, Canada), where microarray analysis was performed using the Mouse Gene 1.0 ST array (Affymetrix).

2.4.1 Microarray Data Analysis

Raw data were normalized using a robust multi-array average (RMA) algorithm [112]. In order to identify genes that were expressed differentially in C8 and H10 cells when activated with RANKL, LIMMA (linear models for microarray data) was used [113]. This test is similar to an ANOVA except that the residual standard deviations are moderated across genes to ensure more stable inference for each gene. Genes with false discovery rate (FDR) equal to p <0.05 and fold change ≥5 were selected as having been up or down-regulated enough to be considered as representing true changes in gene-expression.
2.5 Validation experiments

2.5.1 Isolation of Murine Bone Marrow Monocytes and In-Vitro Osteoclastogenesis

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 removed aseptically 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 medium (Life Technologies, Grand Island, NY, USA). The flushed marrow was passed through a 20-gauge needle until a homogenous single cell suspension was created. To remove stromal cells and fibroblasts present in the marrow cell suspension, cells were cultured overnight in DMEM supplemented with 10% FBS and 10% antibiotics. Non-adherent cells were harvested and cultured overnight in DMEM complete culture media supplemented with 20ng/ml MCS-F. Adherent BMMs were harvested with a cell scraper and counted by using a Z1 Coulter Particle Counter (Coulter Electronics, Hialeah, FL, USA). Osteoclastogenesis was induced by seeding 60 mm culture dishes with 0.5x10^6 cells and incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

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.

2.5.2 Rt-qPCR

On days 1 through 4 of OCG, RNA was extracted from RAW 264.7 and bone marrow cell cultures (Qiagen RNeasy Minikit, Germantown, MD, USA). Total RNA extracted from H10 and C8 cells on the second day of OCG used for microarray analysis was also used for this purpose. Total RNA (500 ng) was reverse transcribed into cDNA using Superscript II (Invitrogen Life Technologies, Carlsbad, CA, USA) and Oligo-dT18VN primer (ACGT Corp., Toronto, Ontario, Canada). Primer sequences were obtained from the Harvard Medical School
Primerbank (Table 1). A 1:10 cDNA dilution was used for all reactions. Quantitative realtime PCR (Rt-qPCR) was performed in 20 µl reactions containing 5 µl of diluted cDNA and 15 µl of SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) using the BioRad CFX96 Real Time System. Each reaction was done in triplicate. PCR conditions were as follows: initial denaturation was carried out at 95.8°C for 30 seconds, annealing temperature was set at 60.8°C for 1 minute, and extension was done at 72.8°C for 1 minute. For these procedures, 35 cycles were used. The reaction was concluded with a final extension at 72.8°C for 10 minutes. Data were normalized with internal GAPDH used as the control as described previously using the 2-∆∆Ct method [114]. CD109 expression in RAW 264.7 and bone marrow cells was expressed relative to the gene expression at day 0. Expression in H10 cells was expressed relative to the expression in C8 cells. As shown previously, expression of GAPDH did not change under these experimental conditions and could therefore be considered as being usable for normalization of RNA expression for other genes [115].

2.5.3 Western Blotting

To evaluate the CD109 expression in different stages of osteoclast fusion at the protein level, freshly isolated BMMs, RAW 264.7, H10 and C8 cells were cultured for 4 days in growth medium with RANKL (and MCS-F in the case of BMMs) as described previously. Subsequently, cells were lysed with 200 µl of ice-cold RIPA buffer (Sigma) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1x protease inhibitor (BD Pharmingen) for 5 minutes and then collected by cell scraping. Cell lysates were centrifuged for 1 minute at maximum speed to cause cellular debris to pellet at the bottom of the centrifuge tube. Protein concentrations were measured using the BCA protein assay kit (23225, Pierce, Rockford, IL, USA). Equal sample concentrations were added to 12 µL of 5 times Laemmli sample buffer, and reached a total volume of 60µl with RIPA Buffer (Sigma). Samples were boiled for 10 minutes and reserved for subsequent Western blotting. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 7.5% polyacrylamide gel, transferred onto nitrocellulose membrane (GE Healthcare), so that immunoblotting could be done. The following antibodies were used: goat polyclonal IgG anti-mouse CD109 (S-20, Santa Cruz Biotechnology, Inc, 1:200) followed by horseradish peroxidase (HRP)–conjugated donkey anti-goat IgG (Santa Cruz Biotechnology Inc, 1:2000). Primary and secondary antibodies used for Western blotting were diluted in Tris-buffered saline plus 0.05% v/v Tween 20 (TBS-T) with 5% milk.
Immunoreactive protein was detected using chemiluminescence with ECL Plus (GE Healthcare) on exposure to Bioflex MSI film (Clonex). Band intensities on scanned films were quantified by densitometry using Image J Version 1.41 software and normalized against β-actin levels used as internal loading controls.

2.6 shRNA Infections and Characterization of CD109 Knockdown Macrophage Cell Lines

A retroviral vector expressing four different small hairpin RNA (shRNA) reagents targeting mouse CD109 driven by the U6 promoter was purchased from OriGene (Rockville, MD). The shRNA sequences are shown in table 2. The same vector expressing a scrambled sequence (TR30013) was used as the control.

GP-293 pantrophic packaging cells were plated onto 6 well tissue culture plates and cultured in DMEM cell culture media until 40% confluence was reached. The cells were co-transfected separately with 2 µg of the previously described shRNA retroviral constructs (CD109 shRNA) as well as 2 µg of the pVSV-G envelope protein-packaging vector (kindly provided by Drs. Helen Sarantis and Scott D. Gray-Owen, Department of Molecular and Medical Genetics, University of Toronto) using FugeneHD transfection reagent (Roche, Mannheim). 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 30 min at room temperature. The viral supernatant was passed through a Millex-HA, 0.45 µm syringe driven filter unit, and then used to infect RAW 264.7 macrophages with the four different shRNA sequences. Infected RAW 264.7 macrophages were incubated for three days at 37°C. The medium in all dishes was replaced with new medium containing 7µg/ml of puromycin (Sigma) for cell selection and then cultured for another 2 days. CD109 expression in terms of protein level was examined by Western blot analysis. The two CD109 knockdown cell lines with the lowest CD109 protein level were used for further experiments. Cells expressing non-effective shRNA effects (i.e. no reduction in expression of CD109) and the original RAW 264.7 cell line was used for control purposes.
In-vitro assays of OCG were performed in the knockdown cells as described above. The number of fused nuclei was counted manually under a Leitz Wetzlar microscope with a 200X magnification, and images were taken with a PixeLink camera microscope in 10 fields of view (FOV). A fusion index (FI) was calculated for all cell lines:

\[
FI = \frac{\text{#fused nuclei}}{\text{total nuclei}} \times 100
\]

### 2.7 Statistical Analysis

Each experiment was performed in triplicates unless otherwise stated. Statistical analysis was performed using the non-parametric test, the independent-sample Kruskal-Wallis test, due to the small sample size, and a p-value of less than 0.05 was considered statistically significant. To compare different cell lines at different time lines, a comparison of means was performed and a p-value of less than 0.05 was also considered statistically significant. All statistical analyses were performed with the SPSS 12.0 statistics package (SPSS, Chicago, IL, USA).
Results

3.1 Difference in osteoclast fusion found in established cell lines

For the purpose of other ongoing research at our lab, two different cell lines were derived from the original RAW 264.7 cell line. The resulting cell lines developed from RAW 264.7 cells had different abilities to form osteoclasts in the presence of RANKL. After culturing these cells for 4 days with RANKL and under the same conditions, TRAP staining showed that one of the cell lines, (already referred to as H10), fused forming large multinucleated osteoclasts while the other cell line, C8, did not fuse, and so no OCG was observed (FI= 0%). Interestingly, despite the fact that both the H10 and C8 cell lines were derived from a highly osteoclastogenic cell line, RAW 264.7 cells, both did not retain the ability to form osteoclasts. This gave the opportunity to determine the basic molecular differences between these two new cells lines that might explain their disparate abilities to undergo OCG.

3.2 Microarray analysis results

Gene-expression microarray is an innovative technology that allows the examination and measurement of expression of thousands of genes at a time. [116-119]. It consists of a number of probes (single stranded cDNA oligonucleotides) containing small amounts of cDNA of a complementary sequence to the mRNA molecule of the gene that it is targeting. The fluorescence level of the dye shows the level at which tagged mRNA molecules hybridize to their complementary probes. When examining this with a scanner, relevant data such as the level of fluorescence of each spot and the level of background noise can be output in a text file and subsequently analyzed [116].

In order evaluate the genetic differences between C8 and H10 cells, both having been derived from RAW 264.7 cells, a microarray analysis was performed by extracting RNA from both derived cell lines. The RNA was obtained during on the early phases of RANKL-Induced OCG (day 2 of culture). After analyzing the microarray data under the parameters described above, 42 genes were found to be up-regulated in H10 cells as compared to gene expression demonstrated
with C8 cells in the second day of RANKL-induced OCG. Several genes involved in OCG and/or osteoclast function, such as cathepsin K, Prdm1, OC-STAMP and Oscar were overexpressed quite robustly with 11.87, 6.22, 6.02, and 4.48-fold increases respectively. Other genes heretofore not known to play a role in OCG were also found to be up-regulated, such as Usp18, Lgals9, serpin9, Irf7, trim30, Il7r and CD109 by 14.1, 13.8, 7.5, 13.3, 40.2, 5.5 and 17.62-fold respectively (Fig. 2).

3.3 CD109 expression is up-regulated throughout the days of OCG

After analyzing the microarray data, 8 genes not reported in the literature to be involved in OCG, were selected for confirmation of RNA expression by Rt-qPCR. Results from the Rt-qPCR analysis showed that, of all the tested proteins, CD109 was the only one that significantly increased its RNA expression over the phase of OCG in all cells including BMMs, RAW 264.7 cells and H10 but not in C8 cells (n=3): P<0.05 (Fig. 3). Confirmation of CD109 protein expression by Western blot analysis was performed in the same cell types and it was found that CD109 protein expression was also increased during de critical days of OCG in all tested cells (n=3): P<0.05 (Fig. 4). That CD109 RNA and protein expression were both demonstrated to be increased in the critical days of OCG, and further since CD109 was expressed and transcribed in all cell lines as well as in primary murine BMMs, this underscores the rationale for choosing this molecule as the focus of this investigation.

3.4 Fusion Capacity of CD109 Knockdown cells

Since CD109 was found to be up-regulated during the critical days of OCG, we wanted to characterize the role of this molecule in OCG. In order to do that, CD109 Knockdown cell lines were established by using four different shRNA sequences separately and named 94, 96, 97 and 99 according to the catalog number of each sequence. Once selection of KD cells was done by adding puromycin to the culture, we proceeded to confirm the percentage of CD109 protein knockdown within cells of each line by performing Western blot analysis to assess protein levels of CD109. This revealed 41.2% of CD109 knockdown in the 94 cell line, 49.54% of CD109 knockdown in the 96 cell line, 66.4% of CD109 knockdown in the 97 cell line, and 82.65% of CD109 knockdown in the 99 cell line (Fig 5). Because they had the lowest expression of CD109
CD109 KD cell lines were stimulated with RANKL for 4 days. The last day of culture cells were fixed and TRAP stained. There was a significant decrease noted for fusion efficiency in CD109 KD 97 and 99 cell lines (14% and 15.5%) when compared to the control cells containing the scrambled shRNA (48.6%) (n=3) p<0.05 (Fig 6). Since cells that underexpressed CD109 were less capable of fusing, it was concluded that CD109 might be an important regulator of OCG.
Chapter 4
Discussion

OCG is a complex process that can be characterized by several different stages of development; stages that rely on the expression of a large number of biomolecules as well as interactions with other cell-types that have not been identified fully [120]. As shown by microarray analysis of pre-osteoclast cell-lines during the early phases of OCG, I showed that CD109 was up-regulated in the osteoclastogenic cell-line H10. This protein has only been identified recently and has been shown to have characteristics consistent with its being a TGF-β co-receptor. It has been demonstrated that CD109 is expressed and produced in activated platelets and T cells, endothelial cells, leukemic megakaryoblasts, and a subpopulation of CD34 expressing cells [91, 92, 94-96]. It is considered a marker of tumourigenesis since it has been found that it is overexpressed in several types of malignant cells [97, 99]. However, the expression of CD109 in osteoclasts has not been identified to date and represents a novel finding.

As described in the introduction, osteoclasts arise from the fusion of their progenitor hematopoietic cells in the bone marrow after simulation by MCS-F and RANKL followed by activation of other signalling proteins such as cathepsin K, c-Src and Pyk2 [14, 121]. Previous studies have used large scale screening methods such as microarray to identify molecules expressed by RAW 264.7 cells and several have focused on proteins participating in RANKL-induced OCG [122]. In order to identify molecules regulated by RANKL, others, (Yang et al. 2008) performed microarray analysis in RAW 264.7 cells after stimulation with RANKL for up to five days. A number of molecules were identified that appeared to be regulated quite substantially by RANKL induction. The investigators then divided these proteins into 5 different categories depending on their primary molecular activity [123]. The same group of researchers used high-density microarrays to examine gene expression changes in BMMs and RAW 264.7 cells after stimulation, at different time-points (2 and 4 days), with RANKL while using non-stimulated cells as controls. After microarray data analysis and confirmation by RT-qPCR, it was found that a specific sequence, named OC-STAMP by the authors, is expressed at a very low level at baseline, while its expression was increased dramatically at both time points during OCG [124]. In the present study, microarray was used in order to determine whether there might be differences in gene-expression between 3 cell types; RAW 264.7, H10 and C8
cell-lines, during early stages of OCG. After two days of treatment with RANKL (which would ordinarily induce OCG) it was found that the C8 cell-line was incapable of fusing. In this manner it was shown that OCG does not occur in the C8 cell line even though it was derived from the osteoclastogenic RAW 264.7 cell line and that its absence was associated with an inability of these cells to fuse into new osteoclasts. Alternatively, OCG was induced in the 264.7 and H10 cell-lines. Subsequently, the C8 cells were used for control purposes (i.e. non-osteoclastogenic) in subsequent experiments that were designed to determine whether there were any unique gene-expression patterns that might define cells with the ability to produce osteoclasts. Consistent with other studies, our data showed that genes involved in OCG such as cathepsin K, Oscar, GHR, TGF-βr1 and TNF receptor, were up-regulated greatly in the cell-line with the highest fusion efficiency, H10. Additional molecules were up-regulated including Trim30 Usp18 and CD109 (the protein of interest in this particular case), which have, as with CD109, also never been reported to be involved in OCG.

Given that microarray analysis can lead to false positive outcomes, other investigative tools were used to confirm findings demonstrated with this method of assessment. In this regard, measurement of mRNA and protein levels can be performed by Rt-qPCR and Western blot respectively [125]. Rt-qPCR is commonly used as the primary validation tool to confirm changes in gene expression demonstrated initially with microarray analysis. Findings obtained by use of Rt-qPCR analysis may differ from those produced by microarray analysis due to several factors including biological variability, the quality of RNA harvested from the target tissues or cells, efficiencies of reverse transcriptases and differences in normalization methods for the data produced with various methods of assessment [126, 127]. In the study mentioned above, Yang et al. used Rt-qPCR to confirm results obtained with microarray and found that, although OC-STAMP was only up-regulated by 8-fold when measured using microarray the actual amount of up-regulation was in the range of 300-fold when the RNA was analysed using Rt-qPCR. It is also known that measures of gene expression alone do not necessarily mean that there will be concomitant changes in actual synthesis of the protein that is encoded by the mRNA of interest and so protein identification and quantitation is then required; often by use of Western blot analysis [124]. Hence others (Kim et al. 2011) used Rt-qPCR and Western blot analysis to evaluate mRNA levels and protein levels of OC-STAMP in RANKL-stimulated BMMs and RAW 264.7 cells [18]. In this study, Rt-qPCR analysis was carried out for 8 of the genes that were found to be up-regulated in H10 cells, using microarray analysis. Using Rt-
qPCR analysis it was shown that mRNA for CD109 was expressed in increasingly greater amounts throughout the process of OCG in BMMs, RAW 264.7 cells and the H10 cell line. In parallel to the findings demonstrated with mRNA it was also shown, using Western blot analysis, that the relevant proteins, and especially CD109 were produced in increasing amounts over the period of OCG in all of the cell lines that were tested (i.e. RAW 264.7, BMM and H10). Taken together the findings are consistent with the notion that the production of CD109 during OCG requires the presence and activity of RANKL.

In order to determine the role of specific proteins that participate in cellular function, a method using shRNA can be used whereby uptake of this RNA analogue can be used to reduce or eliminate (i.e. silence) expression of the target mRNA and protein in vitro in a manner similar to what is done using knockout mouse models. These are known as ‘knockdown cell-lines’[122]. As an example of this, others have demonstrated that when four lentiviral constructs expressing a specific shRNA sequence were introduced to BMMs, expression of the protein PARP-1 could be reduced substantially [128]. In light of these previous findings, it was thought that a similar approach could be used to elucidate, at least in part, the biological functions of CD109 in OCG by introducing, shRNA sequences for this mRNA in order to develop stable CD109 knockdown cell lines. These cell lines were cultured with RANKL and it was clear that even after 4 days of culture OCG was reduced/inhibited. Even though CD109 KD cell lines 97 and 99 had a difference in KD percentage of almost 20%, both had similar fusion potential. This can be explained by factors that affect ShRNA infections such as cell stage in culture and growth potential. Ultimately, these results suggest that CD109 may be an important regulator of osteoclast formation and that monocytes unable to express CD109 are less likely to fuse and form large multinucleated (or functional) osteoclasts.

More research needs to be conducted in order to determine the mechanisms by which CD109 affects OCG and whether expression of this molecule is affected by or has effects on expression of TGF-β in osteoclasts given the latter’s important role in OCG. Notwithstanding the relative lack of knowledge about this recently discovered protein, it is possible to infer the function of CD109 by using data reported from other published studies. In relation to this, for example, it has been suggested that CD109 might be a co-receptor or decoy receptor for TGF-β that would otherwise bind to cell-associated TGF- β receptors [44, 102, 109]. Studies in human keratinocytes have demonstrated that CD109 promotes TGF-β binding to its receptors located in
caveolae with further internalization of this complex, causing down-regulation of TGF-β signaling at the same time. Bizet et al. (2011) demonstrated that CD109 overexpression inhibits phosphorylation of SMAD3 in a caveolin-dependent manner. It was suggested that inhibition of SMAD3 by CD109 was due to the putative ability of CD109 to direct the TGF-β ligands to a compartment where their signaling is not effective [44]. TGF-β plays an essential role in cellular differentiation as well as in OC formation [42, 45-47]. Its receptors (type I, II and co-receptors) can be internalized in two different ways; clathrin-coated pits and via the caveolae mentioned above. The clathrin-mediated pathway of internalization has been associated with SMAD2/3 signaling and receptor recycling [43] while TGF-β receptor localization in caveolae has been associated with down-regulation of SMAD2 and SMAD3 signaling [44]. TGF-β signaling has been implicated in both, stimulation [45-48, 129] and inhibition [48] of OCG. A pilot study was performed in this investigation to confirm the effect of culturing RAW 264.7 cell lines in the presence of TGF-β and 60ng/ml of RANKL for 4 days. Consistently with several studies, after stimulation with TGF-β and RANKL, OCG in vitro was increased when compared to those cells only stimulated with RANKL. To explain the mechanism by which TGF-β regulates OCG, Yasui et al. (2011) reported a direct association between SMAD2/3 and the TRAF6-TAB1-TAK1 molecular complex which is generated in response to RANKL stimulation and that, when TGF-β signaling was blocked, the TRAF6-TAB1-TAK1 complex cannot form. Moreover, when eliminating SMAD3 signaling in osteoclast precursors using gene silencing (i.e. shRNA transfection), a significant decrease in RANKL-induced osteoclast differentiation was observed. These authors concluded that the binding of SMAD3 to the TRAF6-TAB1-TAK1 complex is crucial for RANKL-induced osteoclastogenic signaling [130]. When TGF-β signaling is down-regulated by CD109, subsequent inhibition of SMAD3 signaling occurs which might lead to a decrease in the formation of TRAF6-TAB1-TAK1 complexes. This could then lead to reductions in the formation of active osteoclasts (Fig 7). According to our results, cells undergoing OCG, in a TGF-β free environment, express more CD109 than those that are inherently unable to form osteoclasts. The same reduction in OCG occurs when the expression of CD109 is reduced using gene silencing. These findings suggest strongly that CD109 plays a critical role in OCG and further that this is not likely related to or reliant upon the TGF-β signaling pathway.

In summary, the current study demonstrated that CD109 is expressed in monocytes undergoing RANKL-mediated OCG in vitro. Moreover, when CD109 expression is suppressed, osteoclast
formation in vitro decreases. This suggests that CD109 might be an important regulator of OCG and it appears that it affects this process independent of its proven relationship with TGF-β receptors. Further research is needed in order to characterize CD109 activity in the monocyte/macrophage lineage as well as how the expression of this molecule could affect bone metabolism. In developing new treatments for management of diseases characterized by increases in osteoclast formation and/or activity, it is conceivable that, on the basis of the studies reported here, CD109 might represent a novel biotherapeutic target for the creation of new drugs designed to inhibit pathological osteoclast-mediated resorption of bone or other hard tissues. Indeed, regulation of the production of CD109 could be used to reduce OCG and pathological loss of bone, in a more effective and less problematic (i.e. side effects) manner than is available currently.
In this project we have shown that CD109 is expressed in osteoclasts and that CD109 KD cell lines present a degree of fusion deficiency. However, there are many important questions that need further research to be answered. In this section, a number of future experiments that might be helpful in resolving the remaining problems related to the understanding and harnessing of CD109 are suggested.

According to our results, CD109 acts during the early phases of OCG in the process by which pre-osteoclasts fuse to form large multinucleated cells. However, it has not been demonstrated whether CD109 KD cell lines also have a defect in osteoclast maturation that translates to changes in osteoclast function/activity. To assess whether CD109 KD cell lines have a decreased bone resorption capacity, the following previously described experiment is suggested [89, 90]. Osteoclasts will be generated on 4 dentine slices (OsteoSite-dentin discs, IDS Ltd, Boldon Colliery, UK), by plating 50,000 CD109 knockdown cells (97 and 99) as well as the shRNA control on a 24 well plate containing Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS) and 10% antibiotics (164 IU/ml of penicillin G, 50 mg/ml of gentamicin, and 0.25 mg/ml of fungizone). Purified recombinant RANKL (60 ng/ml) will be added once the cells are seeded. Cells will cultured for 4 days, with a change of cell culture medium and RANKL supplementation on the second day. On day 4, all dentin slices will be washed 3 times with warm PBS and placed in 6% bleach for 10 minutes on a shaker. Dentin slices will then be washed 3 times with distilled water to remove the bleach and stained with 0.1% of toluidine blue and sodium borate for 30 seconds. Excess staining solution will be removed by washing the slices 3 times with distilled water. The number and size of resorption pits can then be used to assess the extent and efficiency of bone resorption of CD109 KD cell lines when compared to the scramble control. Osteoclast viability assay and TACS assay, which detects the number of cells undergoing apoptosis by staining nucleus in blue and which results correlate with caspase-3 activity (a regulator of osteoclast apoptosis) [131] can also be performed to define other osteoclast mechanisms that might be affected by CD109´s expression.
The results obtained in the OCG and osteoclast functional assays will be confirmed by establishing a cell line overexpressing CD109. In this cell line, osteoclast formation and resorption capacity should be significantly higher than controls.

In order to establish the mechanisms by which CD109 affects OCG, it is important to define the molecules that relate to it and therefore find a possible pathway to explain the role of CD109 in osteoclasts. To rule out the relationship between CD109 and TGF-β receptors, CD109 KD cell lines and controls will be cultured with recombinant TGF-β and the effect in osteoclast fusion will be evaluated after 4 days of culture. Another approach to detect other molecules related to CD109 in osteoclasts could be performing immunoprecipitation and mass spectrometry.

Finally, it would be interesting to study the effect that CD109 has in bone structure in vivo. For this purpose, CD109 knockout mice will be generated for comparison to CD109 heterozygous knockouts and Wild Type mice. At 6 to 7 weeks of life, growth rates and major organ functions will be determined. Bone static histomorphometry will be performed on mouse long bones (femur) of 4-5 mice per group (control and CD109 knockout). Wild-type and CD109 knockout mice will then be sacrificed and the femurs from each mouse will be removed and fixed in 70% ethanol at 4°C, dehydrated in progressive concentrations of ethanol, cleared in xylene and embedded in paraffin. Bone sections will be stained for TRAP, and quantification of osteoclasts will be performed. Osteoclasts will be identified as TRAP+ cells that were adjacent to bone. Osteoclast surface and osteoclast number will be measured as previously described. Trabecular morphometry within the metaphyseal region of the distal femur will be quantified using micro-CT analysis. Three-dimensional images will be reconstructed and volumetric regions for trabecular analysis will be selected within the endosteal borders to include the central 80% of vertebral height and secondary spongiosa of femoral metaphyses. Trabecular morphometry will be characterized by measuring the bone volume fraction, trabecular thickness, trabecular number and trabecular spacing [132].
Table and Figures Legend

Table 1. **Rt-qPCR Primer sequences.** Primer sequences from the Harvard Medical School Primerbank for Rt-qPCR analysis, used for Rt-qPCR analysis of 8 selected genes not involved in OCG found up-regulated in H10 cells by microarray analysis.

Table 2. **CD109 shRNA constructs.** Four different shRNA sequences targeting mouse CD109 expressed in the retroviral vector driven by U6 promoter purchased from OriGene (Rockville, MD). According to the percentage of CD109 knockdown, each sequence will represent a different cell line.

**Figure 1. Osteoclast formation potential of H10 cells versus C8 cells.** Photomicrograph of TRAP-stained osteoclasts derived from (A) H10 cells and (B) C8 cells after 4 days in culture with RANKL (Leitz Wetzlar microscope, original magnification 200X). H10 cells were able to fuse formed large multinucleated cells (FI=100%) while C8 cells did not fuse at all (FI= 0%).

**Figure 2. Heat map of microarray analysis.** RNA was extracted from H10 and C8 cells undergoing day 2 of RANKL-mediated OCG. Microarray analysis was performed with an Affymetrix array and genes with a FDR of 0.01 and fold change over 5 were selected. Heat map showing 8 randomly selected genes for Rt-qPCR confirmation that have not been reported to be involved in OCG.

**Figure 3. Rt-qPCR confirming microarray results.** Quantitative real-time PCR analysis was used to quantify gene expression of osteoclast cultures in days 2,4,6,8 for BBMs; days 1, 2, 3 and 4 for RAW 264.7 cells and day 2 for H10 and C8 cells. Day 0 was used as internal control in BBMs and RAW264.7 Rt-qPCR and C8 cells were used as an internal control in the H10 Vs C8 Rt-qPCR. Results are expressed as mean fold expression, normalized with GAPDH as a reference gene. There was a statistically significant increase of CD109 RNA expression over the days of OCG in RAW 264.7 and bone marrow monocytes as well as when comparing RNA expression of CD109 in H10 cells versus H10 cells in the second day of OCG (n=3): P < 0.05.

**Figure 4. CD109 Protein expression over the days of OCG.** Western blot analysis showing that CD109 protein expression is increased in the critical days of RANKL-induced OCG in H10.
cells, RAW264.7 cells and BBMs (n=3): P<0.05. Results are expressed as fold expression vs. β-actin (+SD), which was used as the internal loading control.

**Figure 5. CD109 Knockdown cell lines.** Western blot analysis was used to reveal the percentage of protein knockdown per construct used. Results are expressed as fold expression vs. β-actin (+SD), which was used as the internal loading control. Quantification of the Western blot showed that the higher knockdown efficiency was observed in constructs 97 and 99 when compared to scramble control (cell line 13). Note that cell line 7 represents the infection of RAW 264.7 with an empty vector.

**Figure 6. In vitro OCG with CD109 knockdown cell lines.** RANKL-induced OCG *in vitro* experiments showed decreased fusion efficiency in CD109 KD cell lines (14% and 15.5%) when compared to shRNA control (48.6%). (Leitz Wetzlar microscope, original magnification 200). n=3, P < 0.05

**Figure 7. Schematic model of CD109 down-regulation of TGF-β.** CD109 down-regulation of TGF-β signaling and inhibition SMAD3 signaling, would cause a decrease in the TRAF6-TAB1-TAK1 complex formation resulting in a decrease in osteoclast formation. Up-regulation of CD109 showed an increase in OC formation suggesting that CD109 plays a role in OCG and it might be independent of TGF-β in osteoclasts.
Table 1

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Figure 1
Figure 2
Figure 3

- **BMMs**
  - Fold change expression over days 2, 4, 6, and 8.

- **Raw 264.7 cells**
  - Fold change expression over days 1, 2, 3, and 4.

- **Day 2**
  - Fold change expression for C8 and H10.
Figure 4

**H10 Vs C8 cells**

**Raw 264.7 Cells**

**BMMs**
Figure 5

CD109 Knockdown %

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Anti CD109 (S-20)
Figure 6

Fusion Efficiency

Control | CD109 KD (97) | CD109 KD (99)

Fusion index

Cell line

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47
Figure 7
In the current study, it was demonstrated that CD109 is expressed in monocytes undergoing RANKL-mediated osteoclast formation \textit{in vitro}. Moreover, when CD109 expression is suppressed, osteoclast formation \textit{in vitro} decreases. This suggests that CD109 might be an important regulator of OCG. Further research is needed in order to characterize CD109 activity in the monocyte/macrophage lineage and to determine the mechanisms by which CD109 affects OCG, as well as how the expression of this molecule could affect bone metabolism.
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