THE ROLE OF TRPV4 IN OSTEOGENESIS AND CELL SIGNALING IN
MC3T3-E1 OSTEOBLAST-LIKE CELLS

by

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ABSTRACT

Bone is a living, dynamic tissue which is constantly being remodeled to maintain the integrity and strength of the skeleton. This process is carried out by three cells types that form the basic multicellular unit (BMU); Osteoclasts which resorb bone, osteoblasts which form new bone and osteocytes which are terminally differentiated osteoblasts that have become trapped in the bone matrix and promote remodeling. Mechanical loading promotes bone remodeling, however it is unclear how bone cells perceive this type of stimulation. Because the skeleton is sensitive to mechanical loads, it is apparent that bone cells must express a mechanism through which these cells can translate a mechanical stimulation into a biochemical response. One of the candidates for this “mechanosensor” is the transient receptor potential vanilloid 4 (TRPV4) channel, a member of the large TRP family of channels. This channel is a cation channel that has been shown to be osmotically sensitive in bone, but has been studied primarily using osteoclasts and chondrocytes.

The goal of this thesis is to define how activation of TRPV4 channels affects the normal function of MC3T3-E1 osteoblast-like cells. My overall hypothesis is that MC3T3-E1 osteoblasts express the TRPV4 channel and that this channel acts as the “mechanosensor” to enhance anabolic responses of the cell to mechanical stimuli.
I have shown that the TRPV2 and TRPV4 channels are present at both the mRNA and protein levels in MC3T3-E1 osteoblasts and MLO-Y4 osteocytes and performed functional studies to determine its role specifically in osteoblasts. MC3T3-E1 proliferation was significantly reduced by inhibition with the non-specific TRPV blocker ruthenium red, or the TRPV4 specific antagonist, RN-1734. After 96 hours, there was a 63% decrease in cells treated with ruthenium red and a 45% decrease in cellular proliferation in cells treated with RN-1734. A significant decrease in intracellular Ca^{2+} levels in response to hypotonic swelling was also observed in the presence of the inhibitors as well as a decrease in the number of responding cells, however baseline intracellular Ca^{2+} levels remained the same. Inhibition of the TRPV4 channel did not alter the response of these cells to fluid shear stress. There was no difference in ATP release or actin stress fiber formation detected when the cells were subjected to fluid shear stress. Finally, the mineralization data collected also did not provide any conclusive results as to if TRPV4 is involved in bone formation however did provide interesting information about the role of TRPV2 during this process.

Previous studies have shown conflicting data regarding TRPV4 as a “mechanosensor.” Several studies have shown that the channels are directly activated by mechanical stimuli and others showing they are not. The data I present here suggests that TRPV4 is not directly mechanically sensitive but may help to propagate the mechanotransduction signaling pathways in MC3T3-E1 osteoblasts.
Chapter 1

INTRODUCTION

1.1 Bone

Bone is a highly specialized connective tissue that functions to form a framework for the body, protect vital internal organs and maintain mineral homeostasis. The skeleton is divided into two distinct types, the axial skeleton which is composed of the skull, vertebrae, ribs and sternum and the appendicular skeleton made up of the limbs and pelvic girdle. The latter are the load bearing bones of the skeleton. All bones have an outer membrane known as the periosteum and an inner surface covering called the endosteum. The periosteum is a fibrous connective tissue made up of fibroblasts and some osteoprogenitor cells. The periosteum is anchored to the bone by Sharpie’s fibers and contains blood vessels and nerve endings that nourish the bone tissue. The function of the endosteum is to provide a barrier between the bone and the marrow cavity and also to provide nutrients and osteoprogenitor cells for fracture repair [1].

The primary load bearing bones of the skeleton are termed long bones that include; clavicles, humeri, ulnae, radii, metacarpals, femur, tibiae, fibulae, metatarsals and phalanges. Their general structure consists of a long cylindrical shaft referred to as the diaphysis, cone shaped metaphyses and rounded ends known as the epiphyses. Morphologically, there are two distinct types of bone: trabecular bone and cortical
bone. Trabecular bone, also known as spongy bone, is usually found at the ends of long or the ephphyses. Trabecular bone is porous and composed of rod and plate like structures. Cortical bone, also referred to as compact bone, is dense and surrounds the bone marrow space at the diaphysis or mid-shaft of long bones (Figure 1.1).

Approximately 80% of the skeleton is composed of cortical bone, with the remaining 20% being trabecular bone [2]. Cortical bone is stronger than trabecular and serves to provide support for the entire body and protection of internal organs. Trabecular bone is found in areas that experience high amounts of load and therefore are damaged more frequently. The increased surface area of trabecular bone allows for it to be repaired and remodeled at a faster rate than cortical bone [3].
Figure 1.1. **Long Bone Structure:** General structure of long bones with the diaphysis and epiphyses and the two types of bone, trabecular and cortical. This figure was reprinted with the permission of Dr. Paul Genever, Biomedical Tissue Research Group, University of York, UK.
1.1.1 Bone Cells

Bone is continuously turned over which is necessary for growth, fracture healing and mineral homeostasis. This remodeling process is carried out by three distinct types of bone cells, osteoblasts, osteocytes and osteoclasts. Osteoblasts are derived from pluripotent mesenchymal progenitor cells that have the potential to also become chondrocytes and adipocytes. The presence of growth factors, such as bone morphogenic proteins (BMP’s) and fibroblast growth factor (FGF) lead to an upregulation of runt-related transcription factor 2 (Runx2) and drive the stem cells into the osteoblastic lineage [4, 5]. During the early stages of osteoblast development osteoprogenitor and preosteoblast cells proliferate rapidly to expand the cell population. However, as these cells begin to differentiate into mature osteoblasts proliferation decreases. Mature or primary osteoblasts express differentiation markers like alkaline phosphatase, bone sialoprotein, osteopontin, osteocalcin that form the bone matrix and aid in bone mineralization. [6]. Osteoblasts secrete an osteoid, made up of specific extracellular matrix proteins, with type I collagen making up approximately 85%. Other minor forms of collagen and noncollagenous proteins such as osteopontin and osteonectin account for the remaining 15% [2]. Type I collagen is a triple helix molecule and serves as the framework for bone mineralization. It is secreted and forms a staggered arrangement so that there is a space in between the N-terminus of one molecule and the C-terminus of the next molecule [7]. Osteoblasts secrete vesicles containing calcium and phosphate ions along with enzymes that degrade mineralization inhibitors into these spaces between collagen molecules. These ions form a nucleation core that starts the formation of hydroxyapatite crystals which leads to complete mineralization [8].
Primary osteoblasts have two fates: 1) to become bone lining cells, or 2) to terminally differentiate into osteocytes. Bone lining cells are flat, elongated cells that line the surface of the bone at the endosteum and under the periosteum. They protect the surface of the bone and prevent osteoclasts from resorbing bone prematurely [9]. If the osteoblast becomes embedded in its own matrix then it can terminally differentiate into an osteocyte. Within mineralized bone, osteocytes keep the matrix from mineralizing around the cell body and dendritic-like processes of the cell. This unmineralized region is referred to as the lacunae-canicular system. The osteocyte’s cell body occupies the lacunae within bone tissue while the long, dendritic-like processes extend into the canaliculi. These processes connect to other osteocytes, osteoblasts, the bone surface as well as blood vessels which allows for easy and rapid communication [10]. The periosteocytic space found between the osteocyte and the mineralized bone is filled with extracellular fluid [11]. The fluid within this space along with the high amount of connectivity between osteocytes and the bone tissue has led people to believe that these cells are used to perceive and respond to mechanical load which the skeleton experiences [12].

The osteoclast is essential in bone remodeling and is responsible for resorbing bone. Osteoclasts are derived from hematopoietic precursors and begin as mononuclear macrophages [13]. Early in osteoclastogenesis, the cytokine, macrophage colony stimulating factor (M-CSF) is released from bone marrow stromal cells and is necessary for the proliferation of osteoclast precursors [14, 15]. Receptor activator of nuclear factor kappa-B (RANK) is a receptor located on the plasma membrane of osteoclast precursors and is activated by receptor activator of nuclear factor kappa-B ligand (RANKL) that is produced by osteoblasts. This interaction leads to the fusion of
osteoclast precursors to form large multinucleated osteoclasts which attach to the bone surface and resorb bone. Precursors fuse together to become one large multinucleated osteoclast which can then attach to the bone surface and become polarized.

1.2 Bone Remodeling

Remodeling is the process by which bone is renewed in order to ensure mineral homeostasis and maintain its strength. Importantly, the process of remodeling is tightly controlled by hormonal and mechanical regulations. Remodeling is broken down into four phases: activation, resorption, reversal and bone formation. The activation phase begins when osteoblasts are either hormonally or mechanically stimulated to signal mononucleated osteoclast precursors to begin osteoclastogenesis and to signal lining cells to expose the bone surface. These osteoclasts then bind to the surface of the bone via αVβ3 integrin to specific RGD (arginine, glycine and asparagines) sequences in extracellular matrix proteins. The central interface between osteoclasts and the bone surface is surrounded by a ring of contractile proteins which becomes the “sealing zone” and allows the cell to remain attached yet mobile [16-18].

Upon initial binding to the bone surface, the second phase, resorption begins. Osteoclasts form podosomes which become the primary cite of attachment to the bone surface causing subsequent polarization of the cell with a bone resorbing surface that develops a ruffled border and a sealing zone to enclose and isolate the resorption compartment. Osteoclasts secrete hydrogen ions through H⁺-ATPase pumps and Cl⁻ channels in their cell membranes to decrease the pH of the resorption compartment [19]. In addition, tartrate-resistant acid phosphatase (TRAP), matrix metalloproteinase 9 (MMP9) and cathepsin K are secreted to digest the organic matrix.
causing a “resorption pit” on the trabecular surface, and what is known as the “cutting cone” with osteoclasts tunneling through cortical bone and being followed by osteoblasts [20]. Resorption ends with osteoclast apoptosis [21].

The reversal phase occurs when there is a shift from resorption to bone formation. As osteoclasts degrade bone, reports suggest that growth factors such as transforming growth factor β (TGF-β), insulin-like growth factor’s (IGF-1 & II), bone morphogenic proteins (BMP’s), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are released from the bone to recruit osteoblasts to the remodeling site so the final phase of remodeling, bone formation, can begin [22-26]. During this final phase of bone remodeling osteoblasts that have been recruited to the site begin to synthesize new organic extracellular matrix. Mineralization follows when osteoblasts secrete vesicles which serve as nucleation cores at the ends of collagen fibrils. These vesicles serve as a protected microenvironment for Ca\(^{2+}\) and PO\(_4\)\(^{-}\) concentrations to increase enough to precipitate hydroxyapatite crystal formation (Figure 1.2) [27].

Several different events can trigger bone remodeling. Injuries to the bone itself such as bone fracture or microdamage causes an increase in inflammatory cytokines and prostaglandins including interleukin-1, 6 and 11 (IL-1, IL-6, IL-11), tumor necrosis factor-α and β (TNF-α, TNF-β), macrophage colony-stimulating factor (M-CSF), RANKL, osteoprotegrin and interferon-γ (IFNγ) [17, 18, 28, 29]. Calcitropic hormones such as parathyroid hormone (PTH) can also stimulate remodeling. PTH is released by the parathyroid gland in response to low serum Ca\(^{2+}\). PTH binds to receptors found on osteoblasts and increases RANKL production to lead to osteoclastogenesis [30]. PTH also can stimulate bone formation. One reason for this
paradox is the timing of PTH administration. Intermittent doses of PTH has been shown to increase bone formation where as continuous PTH administration favored bone resorption [31]. In addition to cytokine and hormonal regulation mechanical stimulation also regulates bone remodeling.
Figure 1.2: Bone Remodeling: Bone remodeling is composed of four distinct events. Activation begins the process by activating osteoblasts and osteoclasts. Osteoclasts then resorb old or damaged bone which is followed by the reversal phase when the process shifts to bone formation. Figure adapted from [32].
1.3 Mechanotransduction

In 1892, Julius Wolff proposed that the structure of bone was related to the physical forces acting upon it [33]. This became known as Wolff’s law. Later, in the 1960’s Harold Frost further refined Wolff’s law in which he proposed that bone growth and bone loss is stimulated by the local mechanical elastic deformation of bone, and not just static loading [34]. The mechanostat theory was further examined by Burr and Martin who related the adaptation of bone to mechanical strain levels. Within normal, physiologic levels, mechanical strain results in no net gain or loss of bone. However mechanical strain below physiological levels results in increased bone resorption. Mechanical strain above physiological levels can induce an anabolic response. [35].

Mechanotransduction is defined as the process of transforming a mechanical load into a biochemical response and can be divided into four distinct phases: mechanocoupling, biochemical coupling, signal transmission and the effector cell response [12]. During mechanocoupling, mechanical loads cause deformations in the cells matrix and cells stretch in response. Fluid movement is also created within the canaliculae of bone and causes shear stress against the cells. Biochemical coupling occurs when the mechanical signal is transmitted into a biochemical response by the loaded cell. During transmission of the signal, the cell that receives the mechanical signal can communicate the signal through several different mechanisms, including sharing of intracellular content through gap junctions, paracrine or autocrine signaling. Finally the effector cell response leads to the tissue-level response to mechanical loads [12]. The possible responses include an increase or decrease in density or bone
maintenance. In order for there to be an anabolic response however, the mechanical stimuli must be dynamic and reach a specific threshold. If the stimuli is continuous, then the cell becomes desensitized and no response is observed [36].

When a mechanical force is applied to bone several specific responses are seen; deformation of the extracellular matrix, defined as strain hydrostatic pressure and extracellular fluid flow. Deformations of the cells in bone however are minimal and are opposed by the mineralized matrix. As a result, the shear forces created by interstitial fluid flow in response to loading are more physiologically relevant. Fluid shear was modeled in the canaliculi of bone by Weinbaum and Cowin and suggest that force is a key component during mechanical loading [12, 37, 38]. In the early 1990’s fluid shear became a viable vector force on bone cells and studying its effects on osteoblasts began. Frangos et al., did extensive work looking at these responses and one of the first studies demonstrated that there was an increase in cAMP production in osteoblasts exposed to fluid shear [39]. Further studies demonstrated that there was an increase in nitric oxide (NO) release which is important for both osteoclast and osteoblast activation during remodeling and prostaglandin E₂ (PGE₂) production as a result of G protein activation [40, 41]. PGE₂ mediates load induced remodeling by increasing the number of progenitors and osteoblasts and inhibiting osteoclast activity in the remodeled area [42]. mRNA levels of osteopontin, a non-collagenous bone matrix protein, were shown to significantly increase when the murine clonal osteoblast-like cell line MC3T3-E1 cells were exposed to fluid shear, suggesting that fluid shear, not strain, is the primary mediator of osteogenic responses [43].

In vivo studies have shown that bone formation rate is directly affected by mechanical loading. A single brief episode of loading lead to a six-fold increase of
bone formation in rats [44]. On a larger scale, the bone mass of athletes who led intense and active lifestyles was significantly greater than their sedentary controls [45]. One of the most striking examples of this is seen in tennis players. The arm tissue mass and bone mineral content in their dominant arm can be as much as 20% greater when compared to the contralateral control in their non-dominant arm [46]. These mechanically induced increases in bone formation are mediated by both an increase in osteoblast proliferation and differentiation. Studies have shown that osteoblastic cells in culture respond to mechanical stimulation with an increase in DNA synthesis and proliferation [47, 48]. In contrast, other studies have shown that proliferation decreases when osteoblasts are mechanically stimulated. These studies report an increase in alkaline phosphatase levels which is an osteoblast differentiation marker, suggesting that mechanical stimulation plays a role in differentiation as well as proliferation [49].

Both an increase in osteoblast proliferation and differentiation is important for bone formation. An increase in proliferation results in a greater number of bone forming cells whereas increased differentiation enhances the capability of these cells to synthesize and secrete osteoid matrix.

Conversely the absence of loading has been shown to lead to decreased bone mass, seen in cases of disuse osteoporosis [50]. A critical consequence of extended spaceflight missions is the continuous loss of bone. Various space missions, including Skylab, the first United States space station, have reported a 2-4% decrease in bone mass, with the most dramatic losses seen in the hip and lumbar spine which normally experience high levels of loading [51]. Unloading in a 1G environment is also seen in patients who are placed on extended bed rest. In 1970, Donaldson et al., reported on three adult men who were placed on bed rest for 30-36 weeks. Throughout
this period there was an increase in Ca\(^{2+}\) excretion and an overall average loss of 4.2% of total body Ca\(^{2+}\). A significant decrease in bone mineral density was observed in the heel bone of these patients, which was able to be reversed once they regained mobility [52].

Osteocytes have been postulated to be the main “mechanosensor” in bone [53] however osteoblasts have equally responsive to mechanical stimulation [53]. The earliest response to shear in both osteoblasts and osteocytes is a rapid Ca\(^{2+}\) response that requires influx via a stretch activated or mechano-sensitive cation channel [54]. When these channels are activated they depolarize the cell membrane and trigger voltage-sensitive Ca\(^{2+}\) channels (VSCC’s). VSCC’s are key regulators of intracellular Ca\(^{2+}\) homeostasis and control the permeability of the plasma membrane in osteoblasts [13]. Two voltage-sensitive Ca\(^{2+}\) channels have been identified in osteogenic cells; L-type Ca\(_{\text{v}1.2}\) and T-type Ca\(_{\text{v}3.2}\). L-type VSCC’s are “long lasting” channels which require a high threshold for activation whereas T-types are “transient” and require a lower threshold for activation. Osteoblasts express both L-type and T-type VSCC’s, but once they differentiate to osteocytes they lose their L-VSCC expression [55]. The importance of the L-VSCC in bone remodeling can be seen in a study performed by Li et al., in 2002. They injected rats with vehicle, verapamil or nifedipine, which are both L-VSCC inhibitors. The rats were then subjected to one bout of mechanical loading and an increase in bone formation was seen in the control group, however this effect was inhibited in both L-VSCC inhibitor groups [56]. This suggests that Ca\(^{2+}\) entry through the L-VSCC is important in load-induced bone formation.

It has been further demonstrated that when Ca\(^{2+}\) enters the cell through the L-VSCC there is an increase in bone formation makers including type I collagen,
alkaline phosphatase and cyclooxygenase-2 (COX-2) [56]. When there is a rise in intracellular Ca\(^{2+}\) from both extracellular and inositol 1,4,5-triphosphate (IP\(_3\)) mediated intracellular stores in the endoplasmic reticulum [57], ATP stored in vesicles is released and there is an increase in prostaglandin production [58]. ATP then binds to purinergic receptors and there is a cascade of intracellular signaling events eventually leading to bone formation. The downstream effects of fluid shear stress are NF-κB translocation and subsequent COX-2 production. Increase in COX-2 production has been shown to have an anabolic effect in bone that experiences mechanical loading [58].

Bone and osteogenic cells become rapidly desensitized to mechanical stimulation. A potential mediator of this desensitization is the formation of actin stress fibers. These are a key component of the cytoskeleton and are composed of g-actin monomers that have polymerized to form a double helix rope like structure that can span across the cell. Actin stress fiber formation increases in osteoblasts in response to fluid shear stress and other mechanical stimuli within one hour of the onset of the stimulus [58-61]. This is dependent on the release of intracellular Ca\(^{2+}\) stores from the endoplasmic reticulum which is regulated by the phospholipase C (PLC)/IP\(_3\) pathway, in addition to activation of the RhoA GTPase pathway [62]. This leads to downstream activation of rho kinases (ROCK) which activate LIM-kinase 2 (LIMK-2). LIMK-2 phosphorylates coflin, an actin binding molecule, allowing for actin polymerization and stress fiber formation (Figure 1.3) [63-66].
**Figure 1.3:** Hypothesized model of mechanotransduction in osteoblasts. Shear or another mechanical stimulation causes the initial influx of ions through the MSCC, depolarizing the membrane activating VSCCs. ATP release is stimulated, binding to P2X and P2Y receptors leading to downstream signaling cascade resulting in IP$_3$ induced Ca$^{2+}$ release from the ER and actin stress fiber formation. Figure was modified from an image courtesy of Dr. William Thompson.
1.4 TRP Channels

Studies of ion channels in osteoblasts have mainly focused on the voltage-sensitive family of channels, however voltage-insensitive channels have also been observed in osteoblasts. Initially described as “stretch-activated” these channels were first studied in rat osteosarcoma cells (UMR106) and found to be cation non-selective and carried an inward current of ions independent of the voltage across the membrane [67]. The molecular identity of these mechanically activated cation channels is still unknown. One candidate that has been proposed to be the mechano-sensitive cation channel (MSCC) in osteoblasts is a transient receptor potential channel.

Transient receptor potential (TRP) superfamily of channels were initially discovered in Drosophila [68, 69] and have been identified in a variety of organisms from yeast to mammals and virtually all tissue types. Four individual monomers assemble to form a tetrameric channel. Each monomer consists of six transmembrane regions with a pore forming loop between the 5\textsuperscript{th} and 6\textsuperscript{th} transmembrane segments. The amino (N) terminus has six ankyrin repeats and the carboxyl (C) terminus containing a calmodulin binding domain are located intracellularly and their lengths vary [70-72]. These variations allow for diverse activation and intracellular responses (Figure 1.4)

TRPs are a superfamily of ion channels split into two groups, each of which consisting of multiple subfamilies. Group one consists of five subfamilies with the strongest sequence homology to the first TRP identified in Drosophila. These include TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin) and TRPN (no mechanoreceptor potential C). The region of homology spans the six transmembrane segments including the pore loop between the fifth and sixth segments.
Group two consists of TRPP (polycystic) and TRPML (mucolipin subfamily) which are distantly related to the group one TRPs. They share a sequence homology over the transmembrane segments and have a large loop separating the first two transmembrane domains [73, 74].
Figure 1.4: TRP Superfamily. TRP channel subfamilies share the same general 6-transmembrane structure with intracellular N and C termini. Figure reprinted with the permission of Springerlink [75].
1.4.1 TRPV4

The TRPV subfamily of ion channels consist of six members and are involved in pain, thermal and mechanosensation and calcium homeostasis [76]. The ankyrin repeats are highly conserved in the TRPV family [77]. They consist of 30-34 amino acids folded into two α-helices that run antiparallel to each other [78-80]. The two helices are classified as the “inner” and “outer” surfaces and act as interfaces for specific protein-protein or protein-ligand interactions [75]. Evidence has show that TRPV4 has six ankyrin repeats which may be involved in the self-association of the N-termini into the tetrameric structure [81]. There is also a proline-rich domain near the first ankyrin repeat which has been implicated in the mechanosensitive properties of the channel [82]. The central proline residues make up an interaction site with protein kinase C and casein kinase substrate in neurons protein 3 (PACSIN3) which is a cytoskeletal protein involved in synaptic vesicular membrane trafficking and endocytosis [83]. The C-terminus is made up several calmodulin binding sites. One specifically has been found to be involved in Ca\(^{2+}\)-dependent activation of TRPV4 [84, 85]. This region is essential for constitutive opening of TRPV4 channels (Figure 1.5).
Figure 1.5: General TRPV4 structure. Image courtesy of Lauren Hurd, adapted from [86].
1.4.2 TRPV4 as a sensory channel

The first indication that TRPV4 was a sensory protein was in 2000 when Liedtke et al., observed that when Chinese hamster ovary (CHO) cells were transfected with TRPV4, they became osmosensitive. They also noticed that the response was temperature dependent [87]. When mechanical stretch was applied at room temperature no response was observed. However when mechanical stretch was applied at 37 °C cells responded with an increase in Ca\(^{2+}\) influx. Further studies were performed on TRPV4\(^{-/-}\) mice and they were found to be unable to regulate systemic tonicity effectively and also had a reduced response to mechanical stimulation after a high level of pressure was placed on their tails. [88]. Additional data that supports TRPV4 as a mechanically sensitive channel comes from work done in articular cartilage which expresses high levels of TPRV4. Chondrocytes are also highly sensitive to 4\(\alpha\)-phorbol 12,13 didecanoate (4\(\alpha\)PDD), a specific TRPV4 agonist. GSK205, a specific antagonist, decreases chondrocytes response to osmotic stress [89]. Studies performed in porcine articular cartilage have also suggested that TRPV4 is responsible for both the extracellular and intracellular Ca\(^{2+}\) release in response to hypotonic stress [89].

1.4.3 TRPV4 in skeletal development

Proper differentiation and function of chondrocytes is a key to skeletal development. Long bones undergo endochondral ossification which requires a cartilaginous framework followed by mineralization. In the metaphysis at each end of
a long bone, there is a secondary ossification center known as the growth plate which is responsible for their longitudinal growth. The chondrocytes nearest the epiphysis are considered to be the resting zone which is followed by the proliferation zone where resting chondrocytes undergo constant mitosis and increase in number. These cells then become hypertrophic, or increase in volume, and secrete extracellular matrix which elongates the bone. Hypertrophic chondrocytes undergo apoptosis allowing osteoblasts to move in and mineralize the newly deposited matrix [90, 91].

Data that supports the role of TRPV4 in promoting proper skeletal development comes from different TRPV4 mutations that are linked to a variety of skeletal dysplasias. They can range from a mild brachyolmia to neonatal- lethal metatropic dysplasia [92]. The characteristics of non-lethal metatropic dysplasia include kyphoscoliosis with platyspondyly, narrowing of the trunk and shortened long bones with widening at the metaphyses [86]. There is also a lack of mineralization seen at the secondary ossification sites in long bones. This suggests that TRPV4 may mediate normal chondrocyte and/or osteoblast function.

A functional gene screen was also performed in two mouse cells lines, ATDC5, a chondrogenic line, and C3H10T1/2, a mesenchymal line, to determine which genes in chondrocytes activated the transcription factor that regulates chondrogenesis, SOX9. It was found that TRPV4 activation was responsible for promoting chondrogenesis by inducing SOX9 transcription through a Ca\(^{2+}\)/calmodulin pathway [93].

Several studies have been conducted looking into the role of TRPV4 in bone cells. In 2008, Masuyama et al., performed several studies in TRPV4 \(^{-/-}\) mice and
found that prior to weaning there were no significant alterations in growth or bone formation compared to wild type mice. By 12 weeks of age, however, they noticed an increase in bone mass in the knockout mice. Both trabecular bone mineral density and cortical thickness were increased significantly however osteoblast number was not different between wild type and knockout. The number of multinucleated osteoclasts was identified using tartrate resistant acid phosphatase (TRAP) staining. There was no difference seen between the number of small osteoclasts (<10 nuclei) in wild type and knock out, however there was a significant decrease in the amount large osteoclasts (>10 nuclei) [94]. These observations suggest that osteoclast differentiation was altered. In normal osteoclasts, RANKL binds to RANK to initiate a Ca\(^{2+}\) signal which leads to nuclear factor activator of T-cells c1 (NFATc1) activation [95]. This occurs through a Ca\(^{2+}/\)Camodulin (CaM) dependent pathway in which c-fos expression is induced after binding to c-jun and there is an increase in NFATc1.

Mature osteoclasts were treated with 4αPDD, a TRPV4, agonist in the absence of RANKL. They observed an increase in c-fos and c-jun mRNA levels in wild type cells however saw no difference in TRPV4 deficient osteoclasts. In addition, there was a clear induction of NFATc1 mRNA expression levels accompanied by increased levels of Calcr, a target gene of NFATc1. These results contrasted what was found in TRPV4\(^{-/-}\) osteoclasts where there was actually a decrease in NFATc1 and Calcr mRNA levels [94].

Mizoguchi et al., (2008), also used null mice to determine the role of these channels in skeletal unloading. TRPV4\(^{-/-}\) and wild type mice were unloaded for two weeks using the hindlimb suspension technique. In the wild type mice there was a normal decrease in both primary and secondary trabecular bone but this loss was
suppressed in the knockout mice. TRAP staining revealed that there was a 50% increase in osteoclast number in the wild type mice following unloading compared to the knockout mice in the primary trabecular bone. There was no significant difference seen in the secondary trabecular bone however. These findings indicate that TRPV4 is involved in the unloading-induced regulation of osteoclast activity in primary trabecular region [96].

Current understanding of TRPV4 in the skeletal system has been restricted to chondrocytes and osteoclasts. To further define the role of TRPV4 in osteogenic cells, I examined the role of this channel on the anabolic and loading response of MC3T3-E1 osteoblasts, an osteogenic cell line derived from mouse calvaria. I hypothesize that TRPV4 is present in osteogenic cells and activation enhances mechanosensitivity of the cells. To test this hypothesis I propose two specific aims:

Aim 1: Determine the expression and production of TRPV channels in MC3T3-E1 osteoblast-like and MLO-Y4 osteocyte-like cell lines. mRNA was isolated from MC3T3-E1 and MLO-Y4 cell lysates and RT-PCR was used to determine the presence of TRPV mRNA. Whole-cell lysates from both cell lines were then used to determine protein expression of the TRPV mRNA present using Western blot technique.

Aim 2: Define the role of TRPV4 channels in osteoblast function. The effects of TRPV4 activation and inhibition in proliferation and mineralization were determined as well as the effects of TRPV4 on Ca^{2+} response, ATP release and actin stress fiber formation in response to mechanical stimulation.
Chapter 2

TRPV4 IS EXPRESSED IN OSTEOGENIC CELLS

2.1 Introduction

The skeleton is a dynamic living tissue that is constantly remodeling. This process involves the resportion of old bone by osteoclasts and subsequent formation of new bone by osteoblasts. This is a very tightly regulated process which requires specific hormonal [97] and mechanical [12, 35, 98, 99] stimuli.

Mechanical stimulation is necessary for skeletal homeostasis and stimulates an anabolic response in osteogenic cells. The conversion of a mechanical stimulus into a biochemical response is known as mechanotransduction [12]. The earliest response seen in osteogenic cells to mechanical stimulation is a rapid raise in Ca\textsuperscript{2+} influx via a stretch activated or mechano-sensitive cation channel (MSCC) [54] followed by the activation of voltage-sensitive Ca\textsuperscript{2+} channels that initiates a cascade of intracellular signaling pathways. This ultimately leads to increased bone formation [56, 58]. The exact mechanism behind the detection of mechanical stimuli however is still poorly understood. A mechanosensitive cation-selective channel in osteoblasts has been characterized and may account for the [Ca\textsuperscript{2+}]\textsubscript{i} transients seen in these cells [67, 100]. Patch clamp and imaging studies indicate that this channel is activated by membrane deformation induced by multiple types of mechanical stimulation [67, 100, 101].
A potential candidate for the MSCC is the TPRV4 channel. This channel shares a strong homology to the mechanically activated osmotic avoidance abnormal family member 9 (Osm-9) gene found in *Caenorhabditis elegans* [102]. In addition, when CHO cells, which do not normally express TRPV4, were transfected with TRPV4 they became osmo-sensitive [87]. Studies performed in TRV4−/− mice have shown that these mice are unable to regulate systemic tonicity and exhibit a decreased response to mechanical stimulation [88].

TRPV4 is expressed in a variety of tissues and performs a number of different functions. One of the first mammalian tissues found to express TRPV4 was in the renal system. In the nephron TRPV4 is only expressed in the water-impermeable segments including the ascending loop of Henle and the distal convoluted tubule and moderate expression of TRPV4 has been noted in the collecting duct [103]. It is found on the basolateral side of these cells so it is believed to detect changes in interstitial osmolarity and signal for changes in reabsorption on the luminal side. TRPV4 is also abundant in epithelial cells located in the trachea of the lungs, cilia of the bronchi, bile ducts, and in the Fallopian tubes where it possibly regulates ciliary beating frequency, bile flow and oocyte transport [104-106].

TRPV4 is also expressed in the skeletal system. It is highly expressed in articular cartilage and has been shown to promote chondrogenesis in the ATDC5 cell line by inducing Sox-9 transcription, as well as regulates the Ca2+ response to hypo-osmotic stress [89, 93]. Osteoclasts and osteoblasts have also been shown to express TRPV4. In osteoclasts, TRPV4 is believed to play a role in osteoclast differentiation by initiating NFATc1 signaling [94]. TRPV4−/− mice even exhibit a decreased number of osteoclasts in trabecular bone following unloading. In addition, TRPV4−/− mice do
not show the characteristic signs of decreased bone formation and increased resorption during the unloading process [96]. The importance of TRPV4 in skeletal development can be seen clinically in patients that express a dominant mutation resulting in various skeletal dysplasias including autosomal dominant brachyolmia, spondylometaphyseal dysplasia Kozlowski type and nonlethal metatropic dysplasia [86]. A characteristic of these dysplasias is decreased mineralization at secondary ossification sites. This indicates there could be a problem with either chondrocytes function or osteoblast function.

To further assess a role for TRPV channels, specifically TRPV4, in bone, the expression of TRP channels needs to be evaluated. The current study was performed to determine TRPV protein presence in the murine osteoblast-like cell line MC3T3-E1 and murine osteocyte-like MLO-Y4 cell lines. I looked at expression at both the mRNA as well as protein level.
2.2 Materials and Methods

Cell Culture

The mouse calvarial osteoblasts-like cell line, MC3T3-E1 (subclone 14) were purchased from American Type Culture Collection (ATCC). Cells were grown in minimum essential medium, alpha modification (αMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Gemini, West Sacramento California), 100µg/ml streptomycin, 100 IU/ml penicillin (Mediatech, Manassas ,VA ) and 0.22% w/v sodium bicarbonate (Fisher Scientific, Pittsburgh Pennsylvania). Cells were passaged at 80% confluence and maintained at 37°C and aerated with 95% air, 5% CO₂.

RT-PCR

MC3T3-E1 and MLO-Y4 cells were grown to 80-90% confluence in T-75 flasks (Corning). Cells were trypsinized, spun down and RNA was isolated using the QIA shredder kit (Qiagen, Valencia, CA) with an average yield of 400-800 ng/μL. mRNA was reverse transcribed using Omniscript RT-PCR kit (Qiagen) according to the manufacturer’s protocol. PCR primers were designed using Primer 3 (v3.2) and purchased from IDT Integrated Technologies. HotStar Taq was used to perform the PCR and purified with QiaQuick PCR Purification kit (Qiagen). RT-PCR utilized cDNA that had been synthesized with the cDNA synthesis kit.
Table 2.1: Primers for expression of TRPV channels in osteogenic cell lines

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sequence</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV1 Left</td>
<td>5’-AGCTGAATAACACCGTTGGG-3’</td>
<td>215</td>
</tr>
<tr>
<td>TRPV1 Right</td>
<td>5’-CTTGCGATGGCTGAAGTACA-3’</td>
<td></td>
</tr>
<tr>
<td>TRPV2 Left</td>
<td>5’-CAGAAGGCTCCACTGGAAAG-3’</td>
<td>202</td>
</tr>
<tr>
<td>TRPV2 Right</td>
<td>5’-CACCACAGGCTCCTCTTCTC-3’</td>
<td></td>
</tr>
<tr>
<td>TRPV3 Left</td>
<td>5’-CCAAGGAACAGAGGCAG-3’</td>
<td>287</td>
</tr>
<tr>
<td>TRPV3 Right</td>
<td>5’-GAACCTGTCCAGGATGTCGT-3’</td>
<td></td>
</tr>
<tr>
<td>TRPV4 Left</td>
<td>5’-CTAGGGAACCCCAACTGTGA-3’</td>
<td>251</td>
</tr>
<tr>
<td>TRPV4 Right</td>
<td>5’-GCTGAAGGCAAAAAGTCTTGG-3’</td>
<td></td>
</tr>
<tr>
<td>TRPV5 Left</td>
<td>5’-TTGCTTCCTCTCGCTACTTTT-3’</td>
<td>249</td>
</tr>
<tr>
<td>TRPV5 Right</td>
<td>5’-AGCGCAGTAGGTCTCCAAAA-3’</td>
<td></td>
</tr>
<tr>
<td>TRPV6 Left</td>
<td>5’-CTTGGAGCAAAAGGAAGACG-3’</td>
<td>276</td>
</tr>
<tr>
<td>TRPV6 Right</td>
<td>5’-AGGTGGCAAGGCTCAGTCTA-3’</td>
<td></td>
</tr>
</tbody>
</table>
Western Blot

MC3T3-E1 osteoblasts and MLO-Y4 osteocytes were grown to 90% confluence in a 75 mM flask (Corning Inc., Corning, NY) and lysed with 500 μL RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCL and 150mM NaCl). Lysates were stored at -80°C until further analysis.

Whole cell lysate were loaded and run on a 10% NuPAGE gel (Invitrogen, Carlsbad, CA) at 150 volts for 60 minutes. The separated proteins were transferred onto a 0.22μM nitrocellulose membrane (Bio-Rad, Hercules, CA) using a wet transfer method for 60 min at 160 volts. The membrane was blocked overnight with 5% non-fat dried milk in Tris-Buffered Saline Tween-20 (TBS-T). The membrane was treated with 1:500 dilution of antibody for 1 hour at room temperature on a rocker. Goat anti-mouse conjugated to horseradish peroxide (Jackson ImmunoResearch, Westgrove, PA) at 1:5000 concentration was used for a secondary antibody. The membrane was washed three times for 15 minutes with TBS-T between antibody treatments. Immunodetection was determined using the Super Signal West Pico Chemiluminescence kit (Pierce, Rockwood, IL).
2.3 Results

*MC3T3-E1 osteoblasts and MLO-Y4 osteocytes express TRPV mRNA transcripts*

Utilizing RT-PCR I found that there are two TRPV channels are expressed in MC3T3-E1 osteoblasts and MLO-Y4 osteocytes, TRPV2 and TRPV4 (Figure 2.1). The absence of reverse transcriptase (-RT) was used as a negative control.
Figure 2.1: TRPV expression in MC3T3-E1 osteoblasts and MLO-Y4 osteocytes. A. TRPV2 and TRPV4 mRNA transcript are present in MC3T3-E1 osteoblasts. B. TRPV2 and TRPV4 mRNA transcript are present in MLO-Y4 osteocytes. The light bands found in the +RT and −RT are primer dimers.
MC3T3-E1 preosteoblasts express TRPV2 and TRPV4 protein

To confirm that TRPV2 and TRPV4 were present at the protein level as well as mRNA, Western blotting was used to determine protein expression. Protein was collected from whole mouse brain and used as a positive control. β-actin was used as a loading control. Both TRPV2 and TRPV4 protein were found in MC3T3-E1 and MLO-Y4 whole cell lysates, with TRPV4 showing the characteristic double band (Figure 2.2), representing both the glycosylated and unglycosylated channel.
Figure 2.2: TRPV2 and TRPV4 protein expression in MC3T3-E1 osteoblasts and MLO-Y4 osteocytes. Whole mouse brain was used as a positive control to determine the presence of TRPV2 and TRPV4 protein was whole-cell MC3T3-E1 and MLO-Y4 lysate.
2.4 Discussion

Here I show that two TRPV channels are expressed in MC3T3-E1 osteoblasts and MLO-Y4 osteocytes. Using RT-PCR I was able to find that TRPV2 and TRPV4 are found at the mRNA levels in these particular cell lines. Further, I used Western Blot to determine that these two channels were found at the protein level as well.

TRPV4 expression has been examined in various types of tissue including cartilage. Gene expression in chondrocytes was examined in 2009 by Cameron et al., to determine specific genes that regulated the onset of chondrogenesis. They observed a significant increase in expression of 931 genes during chondrogenesis, one of which is TRPV4 [107]. The corresponding expression of differentiation markers suggests TRPV4 plays a role in development.

The results from the Western blot show a TRPV4 as a double band. This is consistent with previously published data [108, 109] done in mIMCD3 (mouse inner medullary collecting duct), mDCT (mouse distal convoluted tubule) and TRPV4 transfected HEK293 (human embryonic kidney) cells. This double band is reported to be the glycosylated form of TRPV4. An N-linked glycosylation motif was discovered between the 5th and 6th transmembrane segments of the channel and is thought to have a role in membrane trafficking. Mutations in this glycosylation site have been found to increase trafficking to the plasma membrane and therefore cause an increase in net Ca$^{2+}$ entry when stimulated with an agonist [109].
TRPV4 expression has been found to be important in chondrocyte development and behavior. Chondrocytes respond to their osmotic and mechanical environments and in 2009, Phan et al., used porcine articular cartilage to show that the Ca\(^{2+}\) response is mediated by TRPV4 channels [89]. Additionally, mechanical loading influences the physiology of articular cartilage. TRPV4 null mice exhibit severe osteoarthritic changes in their knee joints which are linked to a lack of osmotically induced Ca\(^{2+}\) signaling in articular chondrocytes. This suggests that TRPV4 plays a critical role in mediating Ca\(^{2+}\) signaling necessary for the maintenance of joint health [110].

The presence of TRPV4 in osteoblasts and osteocytes indicates that the channel is necessary for bone formation. For the purpose of my study I decided to focus specifically on TRPV4 function in osteoblasts. Previous studies have reported that TRPV4 is mechanically sensitive and is a potential candidate to be the MSCC in osteogenic cells. In addition, the primary functions of osteoblasts are to proliferate and mineralize bone, neither of which osteocytes do. I investigated the role TRPV4 channels have in cellular signaling and its overall effect on osteoblast function.
Chapter 3

THE ROLE OF TRPV4 IN CELLULAR SIGNALING AND OSTEOBLAST FUNCTION

3.1 Introduction

Bone is a dynamic tissue designed to withstand loads and is strengthened by mechanical stimulation such as exercise. During periods of disuse, such as prolonged bed rest or spaceflight, bone loss occurs due to a lack of mechanical stimulation at a rate of 1-2% per month [111, 112]. The loss of bone from disuse, referred to as disuse osteoporosis, results in an increased risk of bone fractures.

Bone cells, specifically osteoblasts and osteocytes, can perceive mechanical stimuli which initiates a signaling cascade that can eventually lead to a change in the architecture of the bone itself via remodeling [12]. The initial event in this process is an increase in $[\text{Ca}^{2+}]_i$ which activates voltage-sensitive Ca$^{2+}$ channels further propagating the signaling cascade leading to an anabolic response at the tissue level. It is clear that bone cells are sensitive to mechanical stimulation, however, the exact mechanism behind how it is perceived is still unknown.

The transient receptor potential channel, TRPV4 is a potential candidate for the “mechano-sensor” of bone cells. TRPV4 has been implicated in the detection of mechanical stimulation generated by hypotonic swelling and fluid shear stress [113-115]
The TRPV4 channel has been shown to be polymodally activated. The TRPV4 channel is agonized by several factors including acidic pH, citrate, phorbol esters, anadamide and several of its metabolites, and some herbal extracts [113, 116-119]. Temperature has also been shown to activate TRPV4 channels, however, physiologic temperatures are sufficient to activate the channel. TRPV1 and TRPV2 require higher temperatures to activate [117].

Despite being polymodally activated, there is evidence to suggest that TRPV4 is inherently mechanosensitive. TRPV4 has been implicated in the detection of mechanical stress generated by either cell swelling or fluid shear stress [114, 117-120]. Mice lacking TRPV4 display abnormal osmotic regulation and abnormal responses to injurious cutaneous pressure and acoustic stress implying mechanically sensitive defects [88, 121].

TRPV4 has also been found to be highly expressed in the urothelial cell layer of the bladder where it has been implicated in sensing the filling state of the bladder [122-125]. In TRPV4−/− mice they exhibited a lower voiding frequency and larger void volume when compared to wild type mice [126]. Using a potent and selective TRPV4 antagonist systemically on mice and rats with cystitis they also showed an increase in bladder capacity indicating TRPV4 is necessary for sensing pressure in the bladder.

Shear stress generated by blood flow is an important stimulus for the regulation of vascular tone. Increased shear forces induce the release of endothelial vasodilator factors that cause the relaxation of underlying smooth muscle [127] through Ca²⁺ signaling in the endothelial cells [128]. Mendoza et al. (2009), reported
that TRPV4 was highly expressed in mouse small mesenteric arteries and the activation of this channel resulted in Ca\textsuperscript{2+} entry into the endothelial cells and subsequent vasodilatation [129]. Pharmacological inhibition or siRNA suppression of TRPV4 expression blocked shear stress induced Ca\textsuperscript{2+} influx into the endothelial cells and vasodilatation [129].

The role of the TRPV4 channel in mechanotransduction in bone cells was initially studied in 2008 by Mizoguchi et al. TRPV4\textsuperscript{-/-} mice and wild type mice were subjected to a period of unloading. The characteristic bone loss was seen in the wild type mice, however the knockout mice did not show any significant difference in trabecular bone density [96]. This study suggests that TRPV4 is involved in the mechanosensitivity of bone however it does not point to the exact mechanism or cell type with which it works.

In my first aim, I demonstrated that TRPV 2 and TRPV4 channels are present in both MC3T3-E1 osteoblasts and MLO-Y4 osteocytes. Based on previous research I narrowed my focus studying TRPV4 in osteoblasts. The overall aim of this study was to determine the role of TRPV4 in normal osteoblast function. The effects of TRPV4 activation and inhibition on proliferation and mineralization were determined as well as the intracellular Ca\textsuperscript{2+} response, ATP release and actin stress fiber formation in response to mechanical stimulation.
3.2 Materials and Methods

Cell Culture

The mouse calvarial osteoblasts-like cell line, MC3T3-E1 (subclone 14) were purchased from American Type Culture Collection. Cells were grown in minimum essential medium, alpha modification (αMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 100μg/ml streptomycin, 100 IU/ml penicillin and 0.22% w/v sodium bicarbonate. Cells were passaged at 80% confluence and maintained at 37°C with 95% air/5% CO₂. Studies using plates that were not pretreated were coated with 50μg/ml type I collagen (BD Biosciences, San Jose, CA), reconstituted in 0.02M acetic acid and placed under UV light for 1 hour.

Pharmacological Agents

Ruthenium red, a general TRPV inhibitor and RN-1734, a specific TRPV4 inhibitor, were purchased from Sigma. Pharmacologic agents were used at the following concentrations: 10μM ruthenium red (from a 10mM stock in DMSO) and 10μM RN17-34 (from a 10mM stock in DMSO) [130, 131].

Calcium Imaging

MC3T3-E1 cells were seeded onto collagen coated 35-mm Mat-Tek (Ashland, MA) glass bottom dishes at a density of 1.0 x 10⁴ cells per dish and allowed to grow for 72 hours. On the day of the experiment, cells were rinsed with Hank’s Buffered Saline Solution (HBSS, Sigma). Cells were then incubated for 30 minutes at 37°C for 30 minutes in HBSS containing 3μM Fura-2 AM (Molecular Probes,
Carlsbad, CA). Fura-2 stock was originally dissolved in a 5% w/v pluronic acid F-127 DMSO solution. After the 30 minute incubation the cells were rinsed with HBSS twice and returned to 37°C for an additional 15 minutes before imaging.

Changes in intracellular calcium ([Ca^{2+}],) were measured using a ratio-metric video-image analysis technique (Intracellular Imaging, Cincinnati, OH). Using a Xenon lamp with quartz collector lenses, Fura-2 fluorescence was viewed on an inverted Nikon microscope using a 10x fluorescence objective. Fura-2 has a high affinity to Ca^{2+}. It is retained within the cell by non-specific esterases cleaving acetoxymethyl esters. To determine Ca^{2+} concentration, filters for fluorescence at 340 nm and 380 nm were used. Fura-2 is excited by UV light and the excitation ratio is measured to determine the amount of free Fura-2 to bound Fura-2 in the cell. InCytIm3™ imaging software was used to analyze single cells. A calibration curve was generated using Fura-2 free acid and this curve was used to convert the fluorescence ratio between 340 nm and 380 nm into values for [Ca^{2+}], [132].

For each treatment, 7-12 cells were chosen in each field of view. A baseline was obtained for 60 seconds before hypotonically swelling with the addition of 1 mL of diH2O. All inhibitors were placed on the cells at least 10 minutes before imaging and maintained throughout the experiment.

**Fluid Shear Stress (FSS)**

A parallel plate flow chamber, with a closed flow loop as previously described [39] was used for all fluid shear experiments. MC3T3-E1 cells were seeded onto collagen coated glass slides and allowed to grow for 72-96 hours. The cells were
serum starved for 12-16 hours prior the experiment. Fluid shear was applied at a rate of 12 dynes/cm$^2$ while the environmental temperature was held at 37°C.

*ATP Measurement*

MC3T3-E1 cells were subjected to fluid shear stress as previously described. Inhibitors were added into the media 10 minutes prior to shear and maintained in the flow media. Media aliquots (1 mL) were collected prior to the onset of shear and after 15 minutes of shear and placed immediately on ice. Following shear, cells were washed twice with ice cold PBS and lysed with 100μL of RIPA buffer and placed on ice before being stored at -80°C along with the media samples.

ATP concentrations present in media aliquots were determined using a bioluminescence based assay kit (ATP Bioluminescence Assay Kit HS II, Roche, Indianapolis, IN). This assay uses the conversion of D-luciferin by luciferase into oxyluciferin and light that requires ATP as a cofactor. The resultant luminescence was measured using Polar star optima plate reader (BMG Labtech, Cary, NC) and compared against a standard curve of known concentrations. To normalize the measured ATP release to total cell protein, cell lysates that were analyzed using a BCA assay (BCA assay protein kit, Pierce) and a 96-well micro-injector plate reader.

*Phalloidin Immunostaining*

The effect of fluid shear on actin stress fiber formation was determined using fluorescent phalloidin immunostaining. Cells were subjected to fluid shear stress for one hour. The inhibitors ruthenium red and RN-1734 were added to the media 10 minutes prior to shear and maintained in the flow media. GdCl$_3$, a known MSCC
inhibitor, was also used as a positive control. Cells were pretreated with 10 µM GdCl₃ for 10 minutes and the inhibitor was maintained in the flow media. Following fluid shear, cells were washed twice with PBS and fixed with 4% paraformaldehyde and permeabilized with 0.1% TritonX-100 in PBS for 15 minutes at room temperature. Following fixation, cells were blocked with 5% BSA (Gibco, New York, NY) overnight at 4°C. F-actin was then labeled green by incubating cells with Alexa Fluor 488 phalloidin conjugate (Invitrogen) overnight at 4°C. Cells were then washed with 5% BSA three times for 10 minutes each before mounting the slides with polyvinyl alcohol mounting medium (Electron Microscopy Sciences, Hatfield PA). F-actin stress fibers were then imaged using a 63x oil immersion lens on a Zeiss 510 Multiphoton confocal microscope at 488nm excitation.

**Proliferation assay**

MC3T3-E1 cells were seeded onto 6-well tissue culture plates (Corning Inc.) that had been coated with type I collagen. After 4-12 hours from plating media was collected from each well and any floating cells were counted to determine attachment. Inhibitors were added to the growth media and replaced daily. Plated cells were divided into groups corresponding to 24, 48, 72, and 96 hour time points. At each time point, the media was removed from the well and cells were rinsed with PBS before the addition of 200 µl trypsin. Cells were allowed to completely detach before adding 800 µl of media. Cells were counted using a hemacytometer.

**Live/Dead Cell Assay**

MC3T3-E1 cells were seeded onto 35-mm Mat-Tek (Ashland, MA) glass bottom dishes coated with type I collagen as previously described, at a density of
25,000 per dish. Cells were treated with 10μM ruthenium red or 10μM RN-1734 in growth media for 48 hours. The inhibitors were replaced daily in fresh media. The day of the experiment cells were gently washed once with Dulbecco’s phosphate-buffered saline (DPBS) and then incubated for 30 minutes with 2 μM calcein green AM and 4μM ethidium bromide-1 in PBS from the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Carlsbad, CA). Calcein was used to label viable cells in culture while ethidum bromide was used to identify dead or dying cells. For a positive control, 0.1% Tritonx-100 (LabChem, Pittsburgh, PA) was added to cells in order to induce apoptosis. Staining media was gently removed and replaced with DPBS and the cells were imaged on an inverted confocal microscope (Zeiss, Thornwood, NY). The calcein was excited at 488nm diode laser and ethidium bromide fluorescence was excited at 543nm. Images were taken using a 10x water lens.

**Von Kossa Staining**

MC3T3-E1 cells were plated in four 12-well tissue culture plates (Corning Inc.) coated with 5μg/ml type I collagen and grown to confluence. Upon confluence, the cells were differentiated with 50 μg/ml ascorbic acid and 10mM β-glycerol phosphate (βGP) was added as a phosphate donor for mineralization. Media was changed every 48 hours. After 21 days, cells were washed with PBS and fixed using 4% w/v paraformaldehyde for 20 minutes in 4°C. Cells were washed 3 times with dH2O to remove remnants of the fixative. von Koss stain (5% w/v silver nitrate in dH2O) was applied to each well and exposed to high intensity UV light for 30-45 minutes. Cells were washed with dH2O until dH2O washed clear.
Data was quantified using ImageJ software (NIH, Bethesda, MD), where images were converted to 8 bit and threshold was set to positive control. The same threshold was used for all treatments in an individual experiment. The intensity density of each well was quantified to quantify mineralization.

Statistical Analysis

Each experiment was done in triplicate from at least three separate passages of cells unless otherwise noted. One-way analysis of variance (ANOVA) was used to compare means in order to determine statistical significance when making multiple comparisons. $P>0.05$ was considered statistically significant.
3.3 Results

TRPV4 mediates intracellular Ca\textsuperscript{2+} levels in response to hypotonic swelling in osteoblasts

To determine if TRPV4 is necessary for a Ca\textsuperscript{2+} response in MC3T3-E1 preosteoblasts, cells were treated with ruthenium red and RN-1734 and then subjected to hypotonic swelling. Ruthenium red and RN-1734 significantly reduced the intracellular Ca\textsuperscript{2+} response (Figure 3.1a). The representative traces show a diminished response when treated with the inhibitors (Figure 3.1b). The percent of responding cells was significantly decreased by RN-1734 and with ruthenium red (Figure 3.1c).
Figure 3.1: Inhibition of TRPV4 reduces Ca^{2+} response to hypotonic swelling.
A. Graph depicting the effect of TRPV inhibition with 10 μM ruthenium red and 10 μM RN-1734 on the peak change in [Ca^{2+}]_i (nM) over baseline in response to hypotonic swelling. B. Representative trace of [Ca^{2+}]_i in response to hypotonic swelling in the presence of 10 μM ruthenium red and 10 μM RN-1734. C. Percent responding cells. **p<.026
To determine if TRPV4 plays a role in ATP release from MC3T3-E1 preosteoblasts, cells were treated with either ruthenium red or RN-1734 and then subjected to 15 minutes of FSS. Media was collected before the onset of FSS and then again after 15 minutes of treatment. ATP release increased in all groups when compared to their static controls however there was no difference seen between treatment groups (Figure 3.2). This data was unable to be repeated due to problems with the assay kit.
Figure 3.2: ATP release in response to FSS in osteoblasts. ATP assay of conditioned media shows an increase in ATP release in response to FSS. When treated with either 10 μM ruthenium red or 10μM RN-1734 there was no difference in ATP release seen between groups.
**TRPV4 does not affect the formation of actin stress fibers in osteoblasts stimulated with FSS**

It has been previously found that FSS causes a significant increase in actin polymerization in osteoblasts [61]. In order to find if TRPV4 plays a role in this process, MC3T3-E1 preosteoblasts were stimulated by FSS and treated with either 10 μM ruthenium red or 10 μM RN-1734. The static control shows a random organization of f-actin which can be seen as the diffuse staining throughout the cell (Figure 3.3A). There was an increase in f-actin stress fiber organization and alignment after one hour of FSS as predicted and this served as a positive control (Figure 3.3B). When cells were treated with 10μM GdCl₃, a broad spectrum channel inhibitor, there was a dramatic decrease in f-actin staining and no clear stress fiber formation even after one hour of FSS (Figure 3.3C). This served as a negative control. Finally when cells were treated with either 10 μM ruthenium red or 10 μM RN-1734 you can still see actin stress fiber formation and organization when stimulated by FSS (Figure 3.3D,E).
Figure 3.3: Inhibiting TRPV4 does not affect actin stress fiber formation in osteoblasts stimulated by FSS. A. Static controls show minimal stress fiber formation and random f-actin staining. B. When stimulated by FSS for one hour there is a distinct alignment of actin stress fibers. C. Treatment with 10μM GdCl₃ blocked stress fiber formation and there is minimal f-actin staining seen within the cell. D, E. Ruthenium red (10μM) treatment and RN-1734 (10μM) prior to and throughout the one hour of fluid shear stress shows actin stress fiber formation and in an organized, parallel alignment.
TRPV4 is important for osteoblast proliferation

MC3T3-E1 cells were grown on type I collagen coated plates and treated with 10 μM ruthenium red or 10 μM RN-1734 and cell growth was measured over four days. Daily treatment with both ruthenium red and RN-1734 inhibited cell growth (Figure 3.4). After 96 hours of initial treatment with ruthenium red, there was a 63% reduction in cell number and a 45% reduction in cells treated with RN-1734 compared to untreated cells. In order to determine if this decrease was due to apoptosis induced by the inhibitors a Live/Dead cell viability assay was performed. Viable cells were stained with calcein and apoptotic cells were stained using ethidium bromide, followed by imaging using a confocal microscope. It was determined the inhibitors did not induce apoptosis in cells treated for 48 hours with the two specific inhibitors (Figure 3.5).
Figure 3.4: TRPV4 mediates the proliferation of MC3T3-E1 osteoblasts. Growth curve depicting proliferation of MC3T3-E1 cells in the presence of 10 μM ruthenium red and 10 μM RN-1734. *p<.0001
Figure 3.5: Treatment with TRPV inhibitors does not induce apoptosis in MC3T3-E1 osteoblasts. MC3T3-E1 cells were treated with 0.1% TritonX-100 for 10 minutes to induce apoptosis (positive control). Cells 48 hours after seeding treated with 10 μM ruthenium red or 10 μM RN-1734.
TRPV4 does not mediate osteoblast mineralization

MC3T3-E1 preosteoblasts were grown to confluency and then treated with ascorbic acid and βGP to induce differentiation and mineralization. A group was treated with ruthenium red and another group was treated with RN-1734. There was no significant difference in mineralization in the group treated with RN-1734 when compared to control, however there was a significant difference in the cells treated with ruthenium red (Figure 3.6) suggesting TRPV2 may play a role in mineralization.
Figure 3.6. Mineralization of MC3T3-E1 osteoblasts in the presence of TRPV inhibitors. A. Graph depicting mineralization intensity compared to the control. ImageJ was used to analyze the intensity density of each image. B. Representative images of mineralization.*p<.001
3.4 Discussion

TRPV4 channels have been associated with the development and remodeling of the skeleton. The focus of this study was to determine the functional role of TRPV4 in MC3T3-E1 osteoblasts. To directly define the role of this channel in the function of osteoblasts, I examined the effects of general inhibition of TRPV channels and specific inhibition of TRPV4 channels on proliferation, mineralization and cell signaling in response to mechanical stimuli. I found that inhibition of TRPV4 using RN-1734, a specific blocker, and ruthenium red a general TRPV blocker, was able to significantly reduce the proliferation rate of MC3T3-E1 cells. One of the key signaling molecules during the cell cycle is Ca^{2+}. Transient rises in intracellular Ca^{2+} initiate a Ca^{2+}/CaM signaling cascade which is necessary for cells in the G_0 phase to reenter the cell cycle, and is also necessary for the beginning stages of mitosis [133]. Since proliferation is dependent on intracellular Ca^{2+}, I further extended this study to look at Ca^{2+} responses when stimulated by hypotonic swelling. I found a diminished response to the stimulus when cells were in the presence of the inhibitors as well as a decrease in the percent of cells that actually responded.

This data is supported by Ducret et al., who studied the proliferation of pulmonary arterial muscle cells (PASMC) when activated by serotonin. They found that when PASMCs were stimulated with serotonin there was an increase in proliferation due to a biphasic rise in intracellular Ca^{2+} concentrations. When they inhibited TRPV4 however, proliferation was slowed and there was a decrease in the Ca^{2+} response [134].

It is not possible to draw conclusions on ATP release in response to fluid shear because this experiment was not able to be repeated. Technical problems
occurred with the assay kit which prompted inaccurate readings in subsequent trials. As an alternative, I looked into actin stress fiber formation in response to fluid shear stress. Cytoskeletal reorganization is a common cellular response to mechanical stimulation [59, 61, 62, 135, 136] and if TRPV4 is necessary for sensing mechanical loads then it would be expected to see a lack of stress fiber formation when this channel is inhibited. When I treated my cells with ruthenium red and RN-1734 I did not see a difference in actin stress fibers when compared to the untreated control.

Since ATP is necessary for actin stress fiber formation, this suggests that TRPV4 does not play a role in ATP release. There is conflicting data from previously published reports with regard to this topic. Several studies have shown that TRPV4 is necessary for ATP release [137, 138] while others have shown that ATP may be the stimulus for TRPV activation [139, 140]. My data is in line with the previous studies that suggest TRPV4 is downstream of ATP release. To confirm this hypothesis, studies utilizing ATP as a stimulus instead of mechanical load while inhibiting or knocking down TRPV4 using siRNA could be performed.

One of the hallmarks of osteoblast differentiation and mature osteoblast function is mineralization. My data indicates that when TRPV4 is inhibited there is little difference in the amount of mineralization when compared to control, however treatment with ruthenium red appears to have completely abolished mineralization. Ruthenium red is a general TRPV inhibitor; my studies show that only TRPV2 and TRPV4 are present in MC3T3-E1 cells, suggesting that TRPV2 may be vital for proper mineralization. Little is known about TRPV2 in bone physiology, with the only studies being performed in osteoclasts [141]. It has been demonstrated however that TRPV2 is stored in intracellular pools within cells and only translocates to the plasma
membrane when stimulated with insulin-like growth factor (IGF) [142]. This coincides with data showing that IGF receptor knockout mice have decreased rate of osteoid mineralization [143] suggesting TRPV2 may play a vital role in the final phase of bone formation.
Chapter 4

CONCLUSION

4.1 Summary and Prospectus

The purpose of this study was to determine to role of TRPV4 in MC3T3-E1 osteoblasts behavior and cellular signaling. My hypothesis was that TRPV4 channels act as the “mechanosensors” in osteoblasts and enhances the anabolic responses seen after the cells experience mechanical load. Based on my studies, I was able to make several observations:

1. MC3T3-E1 osteoblasts and MLO-Y4 osteocytes express TRPV2 and TRPV4 at the mRNA and protein levels.

2. TRPV4 is necessary for MC3T3-E1 osteoblast proliferation and mediates the Ca\(^{2+}\) response to hypotonic swelling.

3. Actin stress fiber formation in response to fluid shear stress is not mediated by TRPV4.

4. TRPV4 inhibition does not affect mineralization however TRPV2 may play a role in this process.

Overall, my data does not support my hypothesis that TRPV4 is the “mechanosensor” in osteoblasts.
One of the key factors that regulate bone remodeling is mechanotransduction. This process is carried out by three cell types, osteoclasts degrade and resorb old or damaged bone, osteoblasts form new bone and osteocytes are terminally differentiated osteoblasts which have become embedded in their own mineralized matrix. The exact mechanism of how these cells recognize and respond to mechanical stimuli is still unknown. In 1989, Duncan et al., described a voltage-insensitive, “stretch-activated” non selective cation channel in the rat osteosarcoma cell line UMR106 [67]. The addition of gadolinium (Gd\(^{3+}\)) significantly decreased the Ca\(^{2+}\) response in osteoblasts to mechanical strain and fluid shear indicating that any type of membrane deformation can activate the MSCC [100]. The MSCC was also found to be sensitive to parathyroid hormone by increasing the kinetics of the channel. PTH may prime the channel to respond to lower levels of mechanical stimulation. This data indicates that the MSCC may be a point of convergence for PTH activity and membrane deformation [144, 145]. This MSCC has yet to be identified; however it has been proposed that it could be a member of the transient receptor potential (TRP) channel family.

TRPV4 specifically is osmosensitive and studies indicate it is also mechanically sensitive. TRPV4\(^{-/-}\) mice are unable to regulate their systemic tonicity and show a decreased reaction when pressure is placed on their tail [88]. Further studies done in TRPV4\(^{-/-}\) mice have shown that following hind-limb suspension they do not exhibit the typical loss of primary trabecular bone as seen in wild-type mice, indicating it plays a role in mechanotransduction [96].

In the first section of this project focused on determining the expression of TRPV channels in MC3T3-E1 osteoblasts. My results have shown that this cell line expresses TRPV2 and TRPV4 at both the mRNA and protein level (Figure 2.1 and
Figure 2.2). This is supported by previous research by Abed et al., who looked into the gene expression of the TRPC, TRPV and TRPM families in MC3T3-E1 osteoblasts [146]. TRPV4 gene expression has also been found to be up regulated during chondrogenesis indicating it plays a key role in skeletal development [107]. The double band that appears in the western blot of TRPV4 is also consistent with previously published data. It is indicative of a glycosylated version of the channel. Between the 5th and 6th transmembrane segments there is an N-linked glycosylation motif. Studies have found that mutations in this site have increased trafficking of the channel to the plasma membrane causing an increase in Ca\textsuperscript{2+} entry when stimulated [109].

The second half of my project focused on the functional role TRPV4 plays in MC3T3-E1 osteoblasts. Based on previous reports on the role TRPV4 has in skeletal development and mechanosensation, I decided to focus my research strictly to this channel. One of the first responses seen to mechanical stimulation is osteoblasts is a rapid rise in intracellular Ca\textsuperscript{2+} and ATP release. To investigate the role of the TRPV4 channel in osteoblast signaling, I looked into the Ca\textsuperscript{2+} response and ATP release of MC3T3-E1 cells when mechanically stimulated. I utilized two different forms of mechanical stimulation when performing these experiments; hypotonic swelling and fluid shear stress (FSS). FSS is believed to be more physiologically relevant however hypotonic swelling of a cell results in stretching of the membrane and the initiation of signaling pathways including a rise in intracellular Ca\textsuperscript{2+} concentration [147, 148].

A significant increase in intracellular Ca\textsuperscript{2+} concentration was seen in untreated cells when swelled with a hypotonic solution. This effect was blocked however when
cells were treated with ruthenium red and RN-1734 (Figure 3.1a). This data can be explained by either a decrease in the response of the cell to hypotonic swelling or if fewer cells responded as a whole to the stimulus. The representative traces indicate that there is a diminished response to hypotonic swelling when treated with both inhibitors (Figure 3.1b) however the percent responding cells suggests that ruthenium red and RN-1734 also prevent cells from responding to the stimulus (Figure 3.1c). My data suggests that TRPV4 channels offer a significant Ca\textsuperscript{2+} influx pathway which enhances Ca\textsuperscript{2+}-dependent responses in MC3T3-E1 osteoblasts.

One effect of an increased intracellular Ca\textsuperscript{2+} is the release of ATP. When stimulated with FSS there was a significant increase in ATP release, however using the inhibitors ruthenium red and RN-1734 did not show a marked difference on this increase (Figure 3.2). Conclusions cannot be drawn from this data however because I was unable to repeat it due to variability with the assay. Due to these problems, I decided to look at actin stress fiber formation in the presence of FSS as an alternative. When FSS is applied to MC3T3-E1 cells the PLC/IP\textsubscript{3} pathway is initiated which causes the release of intracellular Ca\textsuperscript{2+} stores in the ER and RhoA GTPase activation. This leads to activation of rho kinases (ROCK) and LIM-kinase 2 (LIMK-2). This facilitates reorganization of the actin cytoskeleton by phosphorylating the actin binding molecule cofilin. Actin monomers can then polymerize and form organized stress fibers [48, 58, 60, 61, 63-66]. This pathway suggests that purinergic signaling is required for regulation of the cytoskeleton. The P2Y\textsubscript{2} receptor, a G-protein coupled receptor (GPCR) found in MC3T3-E1 osteoblasts plays a role in activating the PLC/IP\textsubscript{3} pathway. If TRPV4 is required for ATP release, then inhibiting this
channel would block activation of the P2Y$_2$ and therefore prevent actin stress fiber formation.

When FSS was applied to MC3T3-E1 cells in the presence of ruthenium red and RN-1734 however there was no difference seen in actin stress fiber formation when compared to the load control (Figure 3.3). This finding indicates that TRPV4 may not be mechanosensor of osteoblasts and that it is not required for ATP release from the cell. Several studies have shown that TRPV4 may actually be down stream of ATP release in mechanotransduction. Research done in rat submandibular salivary gland cells has shown that inhibition of TRPV4 impairs ATP-induced Ca$^{2+}$ signals while another group studying aldosterone-sensitive distal nephron (ASDN) cells found that ATP stimulates TRPV4 activity [139, 140]. Additional data from the ASDN cells indicates that inhibition of PLC attenuates changes in intracellular Ca$^{2+}$ mediated by ATP treatments. This would suggest that TRPV4 activation is dependent on PLC signaling. If TRPV4 is determined to be down stream of ATP signaling then that would implicate it in mechanotransduction signaling but not the actual “mechanosensor”.

Extensive research regarding mechanotransduction has been performed in endothelium due to the virtually constant fluid shear stress it experiences. One of the main functions the arterial endothelium is to regulate the contractile state of the smooth muscle of the vasculature which can control system blood pressure. To date TRPV4 is the only member of the TRPV subfamily to be identified in this tissue [119]. Since TRPV4 is gated by diverse stimuli, it has been referred to as the mechano- and osmo-sensitive TRP channel [149]. This, along with its relatively high selectivity for
Ca$^{2+}$ [119, 150], which provides a significant Ca$^{2+}$ influx pathway, has made it a focus of mechanotransduction research in the endothelium.

Shear stress was increased in rat carotid arteries by increasing the viscosity of the profusion medium and treated with ruthenium red. Shear stress is known to induce vasodilatation in arteries; however the ruthenium red was able to block this effect. Vasodilatation was also inhibited by blocking phospholipases A2 (PLA2) which is involved in releasing fatty acids from the plasma membrane. One of these fatty acids is arachadonic acid which has been shown to mediate TRPV4 activation by hypotonic swelling [151, 152]. When applied exogenously, arachadonic acid was shown to activate TRPV4. Endogenous arachadonic acid may not directly activate the channel however. It has been suggested that the conversion of arachadonic acid to 5,6 epoxyeicosatrienoic acid (5,6 EET) and 8,9 epoxyeicosatrienoic acid (8,9 EET) may be the direct stimulus [118]. This proposes that TRPV4 may not be a “mechanosensor” and that PLA2 mediated release of arachadonic acid initiated by fluid shear stress is what mediates the channels activation. These findings are in line with the data I collected indicating that TRPV4 plays a role in mechanotransduction but does not directly respond to a mechanical stimulus.

To determine the role TRPV4 has on osteoblast function I looked into the effect inhibiting the channel would have on proliferation. Using both a general TRPV inhibitor and a specific TRPV4 inhibitor I saw a significant decrease in the proliferation of preosteoblasts over the course of 96 hours (Figure 3.4). This data is similar to that found by Patricia Jones in our lab who investigated the role of the L-VSCC in osteoblast proliferation. She found that when treating MC3T3-E1 cells with nifedipine, a specific L-VSCC inhibitor she able to significantly block proliferation in
a manner similar to my results using ruthenium red. Taken together, our data could suggest that the L-VSCC and TRPV4 interact or are scaffolded together since both play such a vital role in osteoblast proliferation. Calmodulin (CaM) could serve as a link between L-VSCC and TRPV4 activity. CaM is a ubiquitous Ca\(^{2+}\) dependent signaling molecule that can link Ca\(^{2+}\) to many downstream pathways and is believed to be tethered to the L-VSCC [153]. Each CaM molecule can bind four Ca\(^{2+}\) ions, and once bound it undergoes structural changes that expose hydrophobic residues, allowing it to bind to a large number of downstream pathways that lead to proliferation and differentiation [154, 155]. When CaM is not bound to Ca\(^{2+}\), studies have indicated that it acts to slowly inactivate the L-VSCC [156], however once it is bound Ca\(^{2+}\)-dependent inactivation of the L-VSCC occurs rapidly. Only one Ca\(^{2+}\)/CaM molecule is necessary for this to occur [157].

The concentration of CaM molecules has been found to be much higher around the L-VSCC compared to the rest of the cell [157]. This suggests that CaM either acts activate numerous signaling pathways or amplify a single Ca\(^{2+}\) signal. Two specific pathways studied in osteoblasts are the CaM kinase and calcineurin pathways. Ca\(^{2+}\)/CaM activate a large family of CaM-dependent protein kinases. CaMKII, a member of the CaM kinase family only requires interaction with Ca\(^{2+}\)/CaM for full activation [158, 159]. CaMKII phosphorylates cAMP response element binding protein (CREB) and that has been shown to affect the production of RANKL and OPG [160, 161]. Ca\(^{2+}\)/CaM can also activate the phosphatase calcineurin. When activated, calcineurin dephosphorylates nuclear factors activated T cells (NFAT) which are transcription factors usually found inactive in the cytoplasm [162]. Two nuclear localization sequences are exposed when NFAT is dephosphorylated allowing for
NFAT dependent gene transcription [163]. NFAT has been shown to affect osteoblast proliferation and differentiation [164, 165].

If the L-VSCC and TRPV4 are scaffolded together then TRPV4 could serve to enhance the intracellular Ca\(^{2+}\) concentration once the L-VSCC undergoes Ca\(^{2+}\)-dependent inactivation and further propagate the Ca\(^{2+}\)/CaM signaling pathways. When CaM is activated by Ca\(^{2+}\) entering the L-VSCC, the Ca\(^{2+}\)/CaM complex can bind to the CaM binding domain on the C-terminus of TRPV4. This would activate the channel and allow for additional Ca\(^{2+}\) entry and subsequent activation more CaM molecules (Figure 4.1).
Figure 4.1: **Proposed model of L-VSCC and TRPV4 interaction.** Ca$^{2+}$ enters through the L-VSCC activating CaM which quickly inactivates the channel. Ca$^{2+}$/CaM bind to the TRPV4 CaM binding domain activating TRPV4 and allowing additional Ca$^{2+}$ entry to further activate CaM and downstream signaling.
Ultimately, increased mechanical load leads to bone formation and mineralization. Mineralization is dependent on osteoblasts secreting Ca\(^{2+}\) and PO\(_4\)\(^{-}\) filled vesicles at the end of collagen fibrils to serve as a nucleation core and propagate hydroxyapatite crystallization. The lack of mineralization at secondary ossification sites seen in patients with TRPV4 mutations that cause Metatropic Dysplasia indicate that the channel may play a role in this process. Decreased mineralization could be caused by either decreased chondrocyte apoptosis in the growth plate, preventing osteoblasts from moving in and mineralizing the matrix, or a defect in osteoblast proliferation and/or mineralization. My data looking at the role of TRPV4 in proliferation found that it necessary for proper expansion of osteoblast populations. For my final study I examined the role TRPV4 could potentially have in mineralization by treating osteoblasts with ruthenium red and RN-1734 throughout the time they were allowed to mineralize in culture and then stained with von Kossa. What I found was little difference between the control group and the RN-1734 treated group, however mineralization was almost completely inhibited by the ruthenium red (Figure 3.6). Since ruthenium red is used as a general TRPV blocker, and in Chapter 2 I found only TRPV2 and TRPV4 to be present in MC3T3-E1 cells, this data suggests that TRPV2 is vital for mineralization.

No other studies have been performed to date looking into the role of TRPV2 in mineralization or any other osteoblast function. TRPV2 has only been studied in osteoclast function where it was found to necessary for Ca\(^{2+}\) oscillations induced by RANKL which is required for osteoclastogenesis [141]. Research utilizing other cell lines have shown that TRPV2 is generally stored in intracellular pools and translocate to the plasma membrane when stimulated by insulin-like growth factor
IGF is necessary for bone mineralization, seen when IGF-1 receptor knockout mice exhibited decrease trabecular bone volume, connectivity and a decreased rate of mineralization of osteoid despite hyperactivity of osteoblasts and osteoclasts [143]. This information points to TRPV2 as a mediator of IGF induced mineralization. The Ca\(^{2+}\) influx from TRPV2 could play a role in the transport of the nucleation core vesicles to the plasma membrane or during the fusion of the vesicle to the membrane.

Overall, I can draw several conclusions from my data. The first is that TRPV4 channels are not the “mechanosensors” in MC3T3-E1 osteoblasts that respond to fluid shear stress. I have shown that inhibition of TRPV4 has no affect on ATP release or actin stress fiber formation, two responses generally seen following mechanical stimulation. In addition, to my data, the conductance of TRPV4 channels does not match that of the MSCC. The MSCC has a conductance of 18pS [67] and is an inwardly rectifying channel, meaning ions only flow into the cell. TRPV4 on the other hand is a nonrectifying channel so ions can flow both in and out of the cell at a conductance of 61.4 pS inward and 98.9 pS outward [118]. Although TRPV4 is not the “mechanosensor” it does seem to play a role in the mechanotransduction process by sustaining the Ca\(^{2+}\) response seen following mechanical load. This Ca\(^{2+}\) response could amplify the Ca\(^{2+}\)/CaM signaling seen following activation of the L-VSCC and play an important role in osteoblast proliferation.

4.2 Future Directions

A number of studies can be done to further examine the role of TRPV4 function in osteoblasts. siRNA studies knocking down TRPV4 in osteoblast and
osteocytes cell lines will provide more specific data regarding the channels role in normal cellular functions and with regards to mechanotransduction.

It would also be possible to examine scaffolding proteins found beneath the channel. Ca\(^{2+}\) is a ubiquitous signaling molecule which controls a large variety of cellular actions. In order for one molecule to perform sure an abundant amount of tasks it must have specific proteins to bind to. Determining what is scaffolding the TRPV4 channel would give a more detailed explanation of its function.

The TRPV4 knockout mouse created by Liedtke et al., and would be an ideal way to perform in vitro studies. The cre-lox-mediated excision was used for gene targeting and generation of the null allele. Exon 12 of the TRPV4 gene was targeted which codes for the pore-loop and adjacent transmembrane domain [167]. The skeleton of these mice has already been characterized and they show normal bone development. The differences have been seen in extreme cases of mechanical stimulation. Tibial loading on these mice could be used to extend on the data presented by Mizoguchi et. al., stating that TRPV4\(^{-/-}\) prevented unloading induced bone loss [96]. The rate at which bone formation occurs and if these mice become desensitized to load could be examined to see if TRPV4 plays a role in desensitization of osteoblasts.

The results from the mineralization data pose questions about the role of TRPV2 in osteoblast behavior that can be further investigated. It would be interesting to determine how TRPV2 mediates mineralization. One possibility would be in the trafficking of the nucleation core vesicles to the membrane. If these vesicles remain sequestered within the cell then this would explain the decreased mineralization.
Another option would be to look at the docking proteins necessary for vesicle fusion with the membrane.

Mutations in TRPV4 have been linked to a rare but severe skeletal phenotype known as metatropic dysplasia. The phenotype is characterized by a small chest cavity, severe kyphoscoliosis, shortened limbs with dumbbell shaped bones, small flat vertebrae and increased cartilage in the joints due to defective ossification. Several mutations have been found in various sites of the TRPV4 gene which affect different parts of the channel [86]. The proliferation data from this thesis may offer some insight into a therapeutic target for patients with this genetic disorder. During the bone formation process, chondrocytes lay down a cartilage matrix and undergo apoptosis when osteoblasts move in to mineralize the matrix. If the osteoblasts are not proliferating then there will be a decreased number migrating into the cartilage area and less mineralization can occur.

The data presented in this thesis begins to elucidate the role of TRPV4 in normal osteoblast behavior and its role in mechanotransduction but not as the hypothesized “mechanosensor.” Future work utilizing specific knockdown of TRPV4 will further clarify the role of this channel in osteoblasts and bone physiology.
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APPENDIX A

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Kristen Howell

Dr. Genever,

I am a master's student in the Department of Biology at the University of Delaware. I am currently finishing up my master's thesis which focuses on aspects of bone biology. For the introduction I would like to include an image of the long bone structure and came across a nice image in the Biomedical Tissue Research Group image library under "bone structure." I was hoping, with your permission, to use it in my thesis. Here is a link to the picture I am referring to
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I will of course reference your webpage and copyright it to the University of York.

Thank you,

Kristen Howell

Paul Genever
paul.genever@york.ac.uk

Jan 10

90
Dear Kristen
Of course, that’s fine, thanks for asking. I hope it goes well.
Best wishes

Paul

-----Original Message-----
From: Kristen Howell [mailto:khowell@udel.edu]
Sent: 09 January 2012 18:33
To: paul.genever@york.ac.uk
Subject: Permission to use one of your images

Dr. Genever,

I am a master’s student in the Department of Biology at the University of Delaware. I am currently finishing up my master’s thesis which focuses on aspects of bone biology. For the introduction I would like to include an image of the long bone structure and came across a nice image in the Biomedical Tissue Research Group image library under "bone structure." I was hoping, with your permission, to use it in my thesis. Here is a link to the picture I am referring to http://www.york.ac.uk/res/btr/Image%20Library/Bone%20structure.jpg

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Thank you,

Kristen Howell
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<td>Principal Investigator:</td>
<td>Randall L. Duncan, Ph.D.</td>
</tr>
<tr>
<td>Common Name:</td>
<td>Mice and Rats</td>
</tr>
<tr>
<td>Genus Species:</td>
<td>Mus musculus and Rattus norvegicus</td>
</tr>
<tr>
<td>Pain Category: (please mark one)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Breeding or holding where NO research is conducted</td>
</tr>
<tr>
<td>C</td>
<td>Procedure involving momentary or no pain or distress</td>
</tr>
<tr>
<td>D</td>
<td>Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)</td>
</tr>
<tr>
<td>E</td>
<td>Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation</td>
</tr>
</tbody>
</table>

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IACUC Approval Signature: [Signature]

Date of Approval: 15 July 2010