A STUDY OF OSTEOCYTE APOPTOSIS BY REGION AND QUADRANT IN MURINE CORTICAL BONE

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ABSTRACT

A Study of Osteocyte Apoptosis by Region and Quadrant in Murine Cortical Bone

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Osteocytes undergo apoptosis to spatially and temporally initiate bone remodeling. This study investigates the distribution of apoptotic osteocytes within different quadrants and regions of cortical bone and compares the frequency of osteocyte apoptosis to regional factors associated with bone remodeling. Specifically, the quantity of apoptosis was compared to levels of the bone morphagenic protein antagonists noggin and gremlin. Samples of unloaded right tibial bone obtained from C57/Bl/6 mice underwent TUNEL staining for apoptotic osteocytes and were counterstained with methyl green to detect osteocyte viability. Cross sectional areas of bone were divided into four quadrants (cranial, caudal, medial, and lateral) and three regions (proximal, midshaft, and distal) for analysis. Densities and percentages of osteocytes were measured within each area. While the results show that there were no differences among quadrants, regional variations were found in osteocyte apoptosis. A significantly higher density of apoptotic osteocytes was found in the midshaft region which also displayed higher levels of BMP antagonists. Using regression analysis, a positive linear relationship between apoptotic osteocytes and gremlin was established while noggin showed a negative linear correlation for the percentage of apoptotic osteocytes. Further studies are needed to observe the distribution of apoptotic osteocytes within loaded bone to confirm the exact relationship between osteocyte apoptosis and bone remodeling.

Keywords: osteocyte apoptosis, bone remodeling

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I. INTRODUCTION

1.1 Bone Biology

Bone Function and Properties

Bone is a complex and dynamic tissue essential in the maintenance of the human body. Its main functions include locomotion, support of organs, protection, attachment sites for muscles, mineral homeostasis, and blood cell formation (Marieb 2005). As the load bearing structure of the body, bone must be stiff enough to resist deformation, flexible enough to absorb energy from loading, and light enough to allow mobility (Bilezikian 2008). These properties are achieved through its unique structure and composition.

Bone Structure

The external surface of bone is covered by the periosteum, a double layered membrane. The outer fibrous layer serves as an insertion point for ligaments and tendons while its inner osteogenic layer is more cellular and vascular. Perforating fibers secure the periosteum to inner bone matrix. Beneath the periosteum a combination of dense cortical bone and porous trabecular bone constitute the main bone matrix. Most internally, a delicate inner membrane, the endosteum, lines the trabecular bone and canals within the cortical bone (Marieb 2005).

Cortical or compact bone has porosities ranging from 5 to 10 percent. It is found surrounding the marrow cavity and forms the shafts of long bones. This stiffer bone responds slowly to changes in loads and shifts accordingly. The main structural unit of cortical bone is the osteon. Concentric rings of lamella or matrix tubules form osteons. They are cylindrical in shape and arranged parallel to the long axis of bone, giving bone the ability to withstand

torsion and bending stresses. The center of each osteon contains a Haversian canal which consists of small blood vessels and nerves. Haversian canals are connected by short transverse Volkmann's canals, containing blood vessels that also connect to the outside surfaces of bone. Compact bone also contains temporary resorption cavities created by osteoclasts during the initial phases of remodeling (Figure 1).

Trabecular, also known as cancellous or spongy bone, is extremely light and porous with porosities between 75 to 95 percent. It consists of trabeculae, plates or struts that are randomly arranged in a sponge-like three dimensional matrix. This provides resilience and shock absorption in areas such as the epiphyseal region of long bones. Due to its large surface area and many voids, trabecular bone partakes in metabolic functions.

Figure 1. Cortical and trabecular bone structure (www.iofbonehealth.org, 2007)

Bone Matrix

Bone is composed of an extracellular and cellular component. The extracellular makeup accounts for about 90% of its volume while the cells and blood vessels make up the remaining 10%.

The extracellular matrix includes an inorganic phase responsible for about two thirds of its weight and an organic phase that comprises the remaining one third. The inorganic matrix or mineral phase is made up of hydroxyapatite crystals, tightly packed mineral salts that are responsible for bones hardness and resistance to compression. The organic phase primarily consists of type I collagen and noncollagenous proteins. This phase is also known as osteoid and plays an important part in bones flexibility and tensile strength, allowing it to stretch and twist (Marieb 2005).

Bone Cells

The cellular component of bone includes osteoclasts, osteoblasts, osteocytes, and bone lining cells. They can be categorized into two main cell types, bone resorbing cells or bone forming cells.

Osteoclasts are large multinuclear cells that resorb bone. They are formed by the fusion of monocytes that originate from hematopoietic stem cells within the bone marrow. Generally located on the surface of bone, osteoclasts are very mobile, resorbing at a rate of 10 microns per day. They are distinguished by their characteristic ruffled border where enzymes are secreted to help break down proteins.

Osteoblasts are mononuclear cuboidal cells that form new bone. They are derived from mesenchymal cells located in the bone marrow. Their formation is believed to be triggered by mechanical stress on bone (Martin, Burr et al. 1998). Osteoblasts produce osteoid, the organic portion of the bone matrix, at a rate of 1 micron per day.

Osteocytes are former osteoblasts buried within the bone matrix. They sit in cavities called lacunae and communicate with each other through tiny tunnels known as canaliculi.

Processes from adjoining cells are connected through gap junctions and maintain the signals for cell to cell communication. These pathways allow for communication between cells as well as within cells to maintain the bone matrix. Making up more than 90% of bone cells, osteocytes are the most abundant cell type yet their function is the least understood. They are thought to play an important role in the signaling mechanism responsible for bone remodeling because of their ideal location and high numbers within the bone matrix.

Once osteoblasts become inactive and old, they form bone lining cells. They are thin elongated cells that appear flattened, covering the surface of bone. Bone lining cells maintain communication through gap junctions and are thought to play a similar role as osteocytes in terms of sensing mechanical strain and monitoring mineral transfer. They are also believed to initiate a bone remodeling response (Martin, Burr et al. 1998). Rodan proposed a theory suggesting bone lining cells as the controller of bone remodeling (Rodan 1992). Their ideal anatomic location allows them to transmit signals that initiate new BMU's. Bone lining cells possess receptors for parathyroid hormone and vitamin D and their response to certain activation signals is consistent with the appearance of osteoclasts. This provides a way for osteoclasts to indirectly respond to hormonal and physical signals interpreted by bone lining cells (Martin, Burr et al. 1998).

Figure 2. The structure of a Basic Multicellular Unit (BMU). The upper half is a longitudinal section through an active BMU while the lower half is an image depicting the positioning of the bone cells. The blue osteoclasts can be seen on the leading edge while the green osteoblasts follow (Robling, Castillo et al. 2006).

1.2 Bone Remodeling

Modeling vs. Remodeling

Forty years ago two processes were discovered responsible for the sculpting and shaping of bone, modeling and remodeling. Modeling occurs during growth and results in changes in bone size and shape. Bone is resorbed in some places and deposited at others, but never at the same location. Once the adult skeleton has matured, modeling is severely reduced. Throughout a lifetime, bone strength is maintained by remodeling which removes damaged bone as a consequence of fatigue or varying load conditions. Remodeling's unique coupling mechanism involves bone first being resorbed and then deposited in the same location, resulting in little change of bone mass. This process is responsible for replacement of the skeleton every ten years (Manolagas 2000).

Process

Bone remodeling involves a group of cells that work together three dimensionally to form a basic multicellular unit (BMU) (Figure 2). They can be found on the inner surface of

bone, specifically intracortical and trabecular bone (Jilka 2003). In cortical bone, BMU's tunnel through with a team of approximately 10 osteoclasts in front followed by hundreds of osteoblasts in the rear. A vascular capillary, nerve supply, and connective tissue are also part of the middle of the BMU (Manolagas 2000). Bone remodeling is activated by a chemical or mechanical signal that causes osteoclasts to form from precursor cells. Osteoclasts remove bone at rate of 40 microns/ day creating a resorption space at a targeted area. Once osteoclasts have finished eroding, they disappear, allowing osteoblasts to take over. The transition from osteoclast activity to osteoblast activity is approximately a 10 day period known as the reversal phase. This phase can be identified by the cement line which marks the beginning of bone formation, separating new osteons from older ones. Osteoblasts assemble on the bottom of the resorption space and deposit layers of new bone known as osteoid or unmineralized bone matrix. The mineralization of osteoid lasts about 3 months where mineral is deposited within the collagen fibers. Once deposition finishes, an empty space is left in the middle for the Haversian canal which helps deliver nutrients to the BMU during formation of the new osteon. The final stage of remodeling is known as quiescence or resting (Figure 3). Osteoblasts either become osteocytes embedded in the bone matrix or flatten out into bone lining cells. This new unit formed is called a secondary osteon (Martin, Burr et al. 1998). Individual BMU's have a total lifespan of about 6-9 months. Bone remodeling in trabecular bone follows the same sequence of events as in cortical bone but travels across bone in a slightly different manner. Rather than tunneling through bone, cells remove and replace trench-like packets of bone along the surface of the trabeculae (Manolagas 2000).

Figure 3. The bone remodeling cycle (Hill 1998).

Purpose

Bone is dynamic and responds to strains placed on it. Frost's mechanostat theory describes the mechanical adaption of the skeleton in relation to strain magnitudes. This theory is based on the idea that bone maintains a physiologic range of strain values. Any deviation from this range generates an adaptive response that aims to return bone to its physiologic value. When strains exceed this range due to loading, bone remodeling is inhibited, resulting in bone formation. Similarly, when strains drop due to a decrease in loading bone remodeling is increased, resulting in bone loss (Martin, Burr et al. 1998).

The concept that bone adapts to its surrounding mechanical environment was made popular by Julius Wolff. Mechanical loading strongly influences bone remodeling. Disuse or lack of loading, most common in bedridden individuals, accelerates bone resorption leading to a loss of bone mass. Fatigue damage typically caused by overuse can also stimulate remodeling. Bone remodeling serves the main purpose of replacing and repairing damaged

bone tissue. Disuse and damage are two mechanical factors responsible for the activation of bone remodeling (Robling, Castillo et al. 2006).

Fatigue Microdamage

Fatigue, creep, and other similar mechanical processes can permanently alter the microstructure of bone when loaded (Martin 2003). Every day activities subject bone to repetitive loading which initiates microcracks, first observed by Frost, within the mineralized bone matrix. The accumulation of microdamage is undesirable because it can lead to stress fractures, increased bone fragility, and overall degeneration of bone health. However, bone is unique in its ability to remove microdamage through bone remodeling.

Since microdamage can only be removed through remodeling, it has been proposed that microcracks initiate bone remodeling. Osteonal remodeling increased when bones were impulsively and repetitively loaded suggesting bone remodeling was initiated by loads (Burr, Martin et al. 1985). Fatigue loading within physiologic strains increased microdamage (Burr, Martin et al. 1985). Experimental studies in canine bones that were cyclically loaded to create damage showed that resorption spaces were more likely to be associated with microcracks than by chance (Mori 1993). However, it still remained unclear whether the cracks accumulated at preexisting resorption spaces or if they actually initiated new remodeling. Burr and Mori further demonstrated in canine bone that cracks caused the resorption spaces, verifying that remodeling followed fatigue damage, removing and replacing it (Mori 1993). A more recent study on adult rat ulna, which unlike canine bone does not characteristically Haversian remodel, showed that fatigue loading activated intracortical remodeling. This was

significant because damage-activated remodeling was introduced in a species that does not typically remodel their bone (Bentolila, Boyce et al. 1998).

While it has been established the remodeling is a mechanism for dealing with fatigue damage, exactly how cells are targeted for remodeling remains poorly understood. Much research has focused on the osteocyte as an important bone cell in the signaling pathway guiding bone turnover.

1.3 The Osteocyte

Mechanosensors

Osteocytes are the most abundant, longest lived, and least understood cell type compared to osteoblasts and osteoclasts. They are regularly spaced throughout the mineralized bone matrix in cave-like lacunae. Lacunae are connected via long slender processes known as canaliculi (Figure 4). These processes connect osteocytes with each other and bone lining cells, allowing for communication throughout the entire bone matrix (Bonewald 2006). Intercellular contacts known as gap junctions allow for communication with other cell types (Doty 1981). However, immediately surrounding the osteocyte the matrix does not calcify, forming a three dimensional network of lacunae and canaliculi, important in the detection of mechanical strain (Burger and Klein-Nulend 1999). Because of their large numbers, distribution through the matrix, and high degree of interconnectivity, osteocytes are thought to be the main cell regulating bone mass and structure biochemically through strain (Lanyon 1993). As a result, they play a pivotal role in bone remodeling.

Figure 4. Osteocyte network. Osteocyte lacunae (A) are connected via canaliculi (B) allowing communication. A Haversian space resides in middle (C) (Martin, Burr et al. 1998).

Bone responds to mechanical loading by mechanotransduction, a process in which a physical force is converted into a signal that elicits a cellular response (Robling, Castillo et al. 2006). Within the bone matrix, osteocytes act as strain receptors and transducers, adopting the role of mechanosensors (Marotti 1996).

Mechanical Simulation

How mechanical loading is sensed by osteocytes remains somewhat of a mystery. Mechanical loading, stress, causes a small deformation of the calcified matrix, strain. The osteocyte network with its lacuna-canalicular porosity is thought to be the site of mechanosensing in bone tissue (Figure 5). When force is applied to bone, it elicits several potential cellular stimuli. Originally, osteocytes were thought to respond directly to the mechanical deformation of the bone matrix. However, it is more likely that fluid flow of

canalicular interstitial fluid is the stress derived factor that informs osteocytes about the level of bone loading (Skerry, Bitensky et al. 1989; Cowin, Moss-Salentijn et al. 1991).

Figure 5. Mechanotransduction in bone (Klein-Nulend, Semeins et al. 1995).

In order to sense mechanical strain, osteocytes must respond to mechanical loading applied to bone. An *in vivo* study intermittently loading turkey ulnae showed an increase in the number of osteocytes expressing glucose-6-phosphate dehydrogenase (G6PD), a marker of cell metabolism with increased strain (Skerry, Bitensky et al. 1989). Strain magnitude varied between .05 and 2% similar to local strains found in bone as a result of physiological loads. Intermittent loading at physiological strain magnitude activated strain related changes of metabolic processes in osteocytes. *In vivo* experiments demonstrated osteocytes produce high levels of intracellular messengers such as NO and prostaglandins in response to pulsating fluid flow and are the most responsive bone cell to fluid flow (Klein-Nulend, Semeins et al. 1995). The generation of these potential signaling molecules indicates that osteocytes may function as mechanosensors of local strain in bone.

In various animals that were physiologically loaded, Rubin found maximal strains of 0.2%-0.3% (Rubin 1984). However, in vitro studies of isolated stretched bone cells showed they were sensitive to strains between 0.7% and 10% (Murray and Rushton 1990). Interestingly, intact bone tissue was sensitive to lower strain levels in comparison to individual bone cells which required higher strains to elicit a cellular response. Local strains at the cellular level were higher than overall strains of the entire whole bone because whole bone is stiff due to the mineralization of the extracellular matrix (Rubin 1984). It is that local force derived from the bulk strain of the whole bone that activates osteocytes. Smaller matrix strains are amplified into larger signals detected by osteocytes. Therefore, it was proposed that the strain derived interstitial fluid flow through the lacunocanalicular network was directly related to the amount of strain of the bone (Cowin, Moss-Salentijn et al. 1991).

The mechanism that detects strain within bone may be the flow of interstitial fluid which stimulates the cell (Cowin, Moss-Salentijn et al. 1991). Streaming potentials and chemotransport were two possible stimuli in the activation of bone cells, but Bakker proved that shear stress was the flow-derived mechanism (Bakker, Soejima et al. 2001). When bone is loaded, interstitial fluid is squeezed through the nonmineralized matrix surrounding osteocytes towards the Haversian canal. Fluid flow causes a shear stress on osteocytes' cell membrane that deforms the cells within their lacunae and the dendrites within their canaliculi (Weinbaum, Cowin et al. 1994).

While osteocytes are sensitive to fluid shear stress, their viability may be dependent on nitric oxide (NO) production that varies with strain. The tip of the cutting cone of a BMU is a predicted area of low canalicular flow resulting in low NO production, causing osteocytes to

enter apoptosis (Burger, Klein-Nulend et al. 2003). Basal levels of NO under adequate fluid flow prevent apoptosis and promote the survival of osteocytes. Other auto paracrine signaling molecules such as prostaglandins may be involved in osteocyte viability. Mechanically stressed chicken osteocytes generated an increase in prostaglandin E2 production which is known to also inhibit apoptosis (Ajubi, Klein-Nulend et al. 1996). The factors responsible for triggering decreased osteocyte viability or apoptosis are affected by mechanical loading in bone.

1.4 Osteocyte Apoptosis

Osteocytes can undergo apoptotic cell death in tissue undergoing rapid remodeling (Noble 2003). Recently, there has been a growing interest in apoptotic osteocytes as part of the signaling mechanism responsible for spatially initiating bone remodeling.

Osteoclasts and osteoblasts undergo apoptosis, or programmed cell death (Steller 1995). Apoptosis refers to a form of cell suicide or programmed cell death characterized by specific morphological changes (Figure 6). These include cytoplasmic shrinkage, nuclear chromatin condensation, DNA degradation, and cytoplasmic blebbing. Cells lose contact with neighboring cells and fragment into apoptotic bodies that are phagocytosed by surrounding cells without generating an inflammatory response. This is not to be confused with another type of cell death, necrosis. This is characterized by cell swelling, chromatin flocculation, loss of membrane integrity, and a general local inflammatory response. This controlled deletion of cells plays an opposing role to mitosis in regulating cell populations (Kerr, Wyllie et al. 1972). Apoptosis plays a key role in development and homeostasis of the skeleton. Recent studies have focused on the role of apoptotic osteocytes as a possible therapeutic target in the prevention of bone loss.

Figure 6. The difference between apoptotic and necrotic cells (Gewies 2003).

Osteocyte apoptosis is regulated by mechanical strain. It has been shown that physiological levels of strain by stretching or pulsatile fluid flow prevent apoptosis of cultured osteocytes (Bakker, Klein-Nulend et al. 2004; Plotkin, Mathov et al. 2005). There appears to be a U shaped relationship between mechanical strain and osteocyte survival. In regions of physiological levels of strain in rat ulna, the number of apoptotic osteocytes remained low suggesting that nondamaging strain promotes osteocyte viability. Low levels of strain and damagingly high levels of strain induced apoptosis and subsequent remodeling (Noble, Peet et al. 2003). Similarly, reduced mechanical forces induced by unloading increased the number of apoptotic osteocytes in vivo (Aguirre, Plotkin et al. 2006). In rat hindlimbs, unloading increased the percentage of apoptotic osteocytes which were associated with osteoclastic bone resorption (Basso and Heersche 2006). Mechanical strain stimulates osteocytes to evoke some sort of response in remodeling. Specifically the apoptosis of osteocytes may be important in the targeting mechanism that drives bone adaptation.

Apoptosis and Bone Remodeling

Apoptosis disrupts the osteocyte network that leads to bone remodeling. Apoptotic osteocytes have been associated with high rates of bone turnover (Noble, Stevens et al. 1997). In areas of growing bone, apoptotic osteocytes were present in a nonuniform distribution suggesting a relationship between osteoclastic resorption and programmed cell death. Osteocyte apoptosis in developing bone was observed in places where intense bone resorption took place (Bronckers, Goei et al. 1996). Estrogen withdrawal in rats and glucocorticoid treatment induced osteocyte apoptosis which is associated with high rates of bone turnover (Tomkinson, Gevers et al. 1998; Weinstein, Nicholas et al. 2000). Both lead to deleterious effects on bone which may be caused by the disruption of the osteocyte canalicular network. Microdamage can alter the osteocytes normal environment in a way that induces apoptosis. A model of the lacunocanalicular network was developed to simulate the effects of interstitial fluid flow after fatigue loading. This model demonstrated that microcracks caused by overloading altered fluid flow by decreasing the transport of signaling molecules to osteocytes and thereby disrupted the network (Tami, Nasser et al. 2002).

Osteocyte apoptosis follows load induced microdamage. Verborgt showed that dying osteocytes appeared after fatigue induced bone microdamage in rats (Verborgt, Gibson et al. 2000). Apoptotic osteocytes were not widespread but rather localized to areas containing microcracks. These areas were later found to be the site of osteoclastic resorption. Furthermore in additional fatigue loaded rats evidence of the proapoptotic gene product Bax were found adjacent to sites of microdamage where bone resorption occurs. In contrast, the anti apoptotic gene product Bcl-2 was expressed at some distance from the microcracks

(Verborgt, Tatton et al. 2002). By expressing these gene products, osteocytes demonstrate the capability to protect themselves from apoptosis. They appear to exercise some level of control in the regulation and directing of resorption associated with microdamage and apoptosis.

Temporal and Spatial Relationship

Apoptotic osteocytes are not randomly distributed within the bone matrix but are generally found in the vicinity of resorption areas. In chicken radii, osteocyte apoptosis increased following osteotomy and was followed by the appearance of osteoclasts (Clark, Smith et al. 2005). The temporal relationship between osteocyte apoptosis and intracortical osteoclast appearance supports the theory that osteocyte apoptosis plays an important role in the bone remodeling process. Also, Aguirre showed that osteocyte apoptosis preceded recruitment of osteoclasts to areas of unloading and subsequent bone loss. After three days of tail suspension, apoptotic osteocytes prevailed around endosteal regions of cortical bone where resorption occurred by day 18. In contrast, the control ambulatory mice exhibited a random distribution of apoptotic osteocytes in cortical bone (Aguirre, Plotkin et al. 2006). In rabbit tibial midshaft, Hedgecock demonstrated the first quantitative evidence of a regional and linear correlation between osteocyte apoptosis and intracortical remodeling in animals that normally remodel their bone (Hedgecock, Hadi et al. 2007).

Osteocyte ablated mice were resistant to unloading induced bone loss and osteoclastic resorption. Instead they displayed characteristics of fragile bone indicating that osteocytes play a crucial role in the sensing of mechanical changes caused by unloading (Tatsumi, Ishii et al. 2007). Osteocytes may serve as beacons for osteoclast recruitment and the targeted initiation of bone remodeling (Manolagas 2000).

Detection of Apoptosis

Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) is a method used to detect apoptotic cell death by end labeling fragmented DNA. Morphologically, DNA degradation of apoptotic cells generates DNA fragments within the nucleus through endonucleases (Wyllie, Kerr et al. 1980). Using Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT) enzyme, the assay incorporates biotinylated nucleotide at the 3'-OH DNA ends. Horseradish peroxidase-labeled streptavidin binds to the biotinylated nucleotides. A DAB solution composed of peroxidase substrate, hydrogen peroxide, and diaminobenzidine detects the biotinylated nucleotides causing the apoptotic nuclei to stain dark brown.

A methyl green counterstain is used to detect all viable osteocytes. Viable nuclei are stained blue-green, clearly distinguishing them from the dark brown TUNEL stained nuclei.

Other signaling mechanisms (BMPs)

Although there is much evidence suggesting osteocytes as the mechanosensors responsible for initiating bone remodeling, another theory suggests variations in bone morphogenetic proteins (BMPs) could play a role in initiating bone remodeling. BMPs are local regulators of osteoblast differentiation from mesenchymal cells that promote osteoblastic maturation and function. BMP's can be regulated by extracellular antagonists produced by osteocytes, which bind to BMPs, preventing signaling and thereby affecting osteoblast and BMU activity. Examples include noggin, gremlin, follistatin, and sclerostin. Osteocyte apoptosis may affect levels of BMP antagonists, thereby providing a possible signaling pathway in bone remodeling.

1.5 Study Goals

The objective of this study is to calculate the density of apoptotic and viable osteocytes by region and quadrant in unloaded murine cortical bone and attempt to correlate their regional variability with possible factors that signal bone remodeling. A previous study has quantified and correlated the regional variability of osteocyte apoptosis and bone remodeling in rabbit tibial midshafts (Hedgecock, Hadi et al. 2007). This study will focus on the right hind tibia of murine specimens. Densities of osteocytes will be compared between four anatomic quadrants (cranial, caudal, lateral, and medial) and three regions (proximal, midshaft, and distal) of the limb. A spatial correlation between the distribution of apoptotic osteocytes and levels of chemoattractants measured in a previous study will be examined. If a correlation exists, we can hypothesize that osteocytes undergoing apoptosis regulate the signal that directs bone remodeling. To further pursue this hypothesis, a future study will investigate the regional variability of apoptotic osteocytes in loaded murine cortical bone. The subjects in the current study will be used as control animals.

As the search for a better understanding of bone remodeling continues, more attention is being directed towards osteocyte viability and its role in guiding bone turnover. Bone diseases such as osteoporosis are commonly characterized by an imbalance during this process in which the rate of osteoclasts resorbing bone differs from the rate of osteoblasts forming bone. This results in an overall loss in bone mass and strength. Apoptotic osteocytes disrupt the osteocyte network responsible for maintenance of the skeleton making them a therapeutic target in the treatment of bone disorders and prevention of bone loss.

II. MATERIALS AND METHODS

Four C57 Black 6 Taconic mice (C57Bl/6) were procured at 7 to 9 weeks of age (Taconic, Oxnard, CA), housed in microisolation chambers at the University's vivarium, and sacrificed at 8 to 12 weeks of age. A previous study on male and female C57Bl/6 measured the changes in cortical bone and length of tibiae, bone weight, and composition to determine skeletal maturity which occurred between 3 and 6 months (Somerville, Aspden et al. 2004). Since the mice used in this study were sacrificed at around 3 months, they appear to have reached young adulthood and possibly full skeletal maturity.

2.1 Sacrifice

Perfusion fixation was performed on each animal by being anesthetized with 25% Avertin (0.15ml/10g) through an intraperitoneal injection and placed on a heating pad. Shaved limbs were taped to the heating pad and the skin was separated from the muscle between the abdomen and thoracic cavity. A thoracotomy was performed and a small incision was made in the left ventricular apex to inject a heparinized vasodilator cocktail followed by a perfusion of Histochoice (Amresco) to fix the animal. A small incision was made in the hindlimb to observe clearance of blood. Once fixed, limbs were dissected and placed in microcentrifuge tubes with Histochoice to post fix at 4 degrees Celsius.

Hindlimb samples were transferred into a decalcification solution to soften the bone prior to embedding for 3 to 4 days. The decalcification solution was composed of 150 grams disodium EDTA dehydrate and 15 grams NaOH. Water was added until the pH of the solution reached 7.4 (between 700-800ml), yielding an approximate 15% EDTA solution.

2.2 Specimen Preparation and Processing

Following sacrifice, tissue fixation, and decalcification, processing followed. Once the bones were softened and decalcification was completed, the limbs were placed in a tissue processor. After dehydration, tissue samples were embedded in paraffin using the Shandon Excelsior ES system (Thermo Fisher Scientific, Waltham, MA). Sections 7 μm thick were cut from each block using the Leica RM2255 rotary microtome (Leica Microsystems, Bannockburn, IL). Sections were floated on a warm distilled water bath (Boeckel Scientific, Feasterville, PA) and adhered to the slides (Thermo Fisher Scientific). Two wrinkle free sections were placed on each slide and allowed to air dry for 24 hours. To prepare for staining, slides were incubated in a 40°C oven for twenty minutes or until the paraffin lost its opacity. Sections were stained using the DeadEnd Colorimetric TUNEL (Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end labeling) System (Promega Corp., Madison, WI) to identify cellular DNA fragmentation characteristic of apoptosis.

2.3 TUNEL staining and Osteocyte Apoptosis

Sections were deparaffinized in xylene for 5 minutes, rehydrated by a graded alcohol series (8 minutes in 100%, 3 minutes in 95%, 3 minutes in 85%, 3 minutes in 70%, and 3 minutes in 50%), and then set out to air dry for 20 minutes. After a 5 minute phosphate buffered saline (PBS) rinse, sections were refixed in Histochoice for 15 minutes. A 20 $\mu\text{g}/\text{mL}$ proteinase K solution was used to digest the sections for 10 minutes at room temperature. After PBS rinses and another refixing in Histochoice, equilibration buffer was placed on the slides for 10 minutes. After equilibration, 100 μL of the TUNEL reaction mixture (1 part biotinylated nucleotides, 1 part rTdT enzyme, and 98 parts buffer) was added to each section and then

covered with plastic coverslips to evenly distribute the reagent. Sections were allowed to incubate (1 hour in a humidifying chamber at 37°C) which allowed the nick end-labeling reaction to occur. Sections were placed in a sodium-chloride sodium-citrate (SSC) wash to terminate the reaction, followed by PBS rinses to remove unincorporated biotinylated nucleotides. Then they were treated with 0.3% H₂O₂ for 5 minutes to block endogenous peroxidases, rinsed in PBS, and treated with streptavidin horseradish peroxidase for 30 minutes. After PBS rinses, slides were developed for approximately 10 minutes with diaminobenzidine (DAB) substrate for visualization of the peroxidase. Slides were rinsed briefly in distilled water and then mounted with Permount (Thermo Fischer Scientific) and coverslipped. Slides that served as positive controls were treated with DNase I (Promega Corp., Madison, WI) to artificially create DNA fragmentation while slides with no rTdT enzyme in the reaction mixture served as the negative control. Positive and negative controls were stained separately to prevent contamination.

To reveal non apoptotic nuclei separate sections were deparaffinized in xylene for 5 minutes, rehydrated by a graded alcohol series (8 minutes in 100%, 3 minutes in 95%, 3 minutes in 85%, 3 minutes in 70%, and 3 minutes in 50%), and then set out to air dry for 20 minutes. Sections were then rinsed briefly in deionized water for 5 minutes, stained with 2% methyl green (Aldrich Chemical Co., St. Louis, MO) for 30 seconds, and then rinsed briefly in distilled water (until clear). All slides were mounted with Permount (Thermo Fischer Scientific) and coverslipped.

2.4 Image Capture, Processing, and Analysis

To quantify osteocyte apoptosis, slides were observed under full-spectrum white light using a BX41 polarizing light microscope (Olympus Optical Co., Ltd., Center Valley, PA) at 40x magnification. Images were captured using a Retiga EXi color camera (QImaging, Surrey, BC, Canada) and the Q Capture Pro imaging program (QImaging). Using Adobe Photoshop, individual images were combined to create a single image of the entire cross section. A Ronchi ruler with known lines at a size of 150 lines per mm was used to calibrate the images at 40x magnification. Image analysis was performed using Image J (Wayne Rashband(NIH)) where images were calibrated with the Ronchi ruler, yielding total field dimensions of 0.23 mm by 0.17 mm. Bone area was measured in square millimeters using the freehand selection tool.

Figure 7. Cross sectional views of murine right tibia (A) TUNEL stained sections. (B) Methyl green stained sections. Cr=cranial, Ca=caudal, M=medial, L=lateral. To the right is an image of a right tibia and fibula divided regionally.

For analysis, each cross section of bone was divided into four anatomic quadrants (cranial, lateral, caudal, and medial) and three regions (proximal, medial, and distal) along the entire bone (Figure 7). Osteocyte lacunae were divided into three categories based on the staining of their nucleus: brown (Br), blue (Bl), and empty. Lacunae with brown nuclei represented TUNEL- positive cells, apoptotic osteocytes (Figure 8), while lacunae with blue nuclei represented TUNEL-negative cells, viable osteocytes (Figure 9). Empty lacunae were characterized by a distinct lacunar wall (Figure 8). Similarly, stained cells were only counted that resided within lacunae, eliminating cells residing in Haversian spaces, cells that accidentally migrated over bone tissue during the staining procedure, or cells located in a different plane. The frequency of osteocyte apoptosis and viable osteocytes were expressed as a percentage of total cells and a density (number per unit tissue area). Total lacunar density was found as well. Osteocyte percentage and density were measured within each of the four quadrants and across the three regions as well as total osteocyte density per subject.

Figure 8. TUNEL stained image.

Figure 9. Methyl green stained image.

2.5 Statistical Analysis

Data was tested using general linear models (GLM, ANOVA) in Minitab 16 (Minitab) to measure the differences in apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes. General linear models were performed to test the differences of the interaction using factors of quadrant and region across all subjects along with Tukey's comparison. P values less than 0.05 were considered significant.

Regression analyses were performed using BMP antagonist expression levels found in a previous study. BMP expression level was compared to apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes at each of the twelve region/quadrant combinations. A regression test on

slope determined whether there was a significant relation or not. If the slope equaled zero, no relation could be determined.

2.6 BMU Identification

BMU's are three dimensional units used to quantify bone remodeling that typically travel through cortical bone in a longitudinal direction. When examining cross sectional views of bone, BMU's can be identified two dimensionally by their unique morphological features. The most distinct is the cement line that defines the boundary of the secondary osteon. Within the cement wall lies a space for the Haversian canal surrounded by concentric lamellae. The radius of the lamellae depends on the remodeling stage of the osteon. An example of a BMU can be seen in Figure 10. For analysis, BMUs were identified across all subjects by region and quadrant.

Figure 10. BMU within murine cortical bone. The arrow marks the outline of cement wall.

III. RESULTS

3.1 Osteocyte Densities

A summary of apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes from all individual subjects are listed in Table 1. The total lacunar density was compared to previous studies measuring osteocyte densities in mice to validate this study. A similar study looking at BMP antagonist expression levels and using some of the same mice as the current study produced an overall osteocyte density of 676 per mm^2 in mice hindlimbs (Mosher 2010). When the effect of osteoprotegerin, a protein affecting the formation of osteoclasts was studied, densities of 750-800 per mm^2 were reported in mice tibial diaphysis using H and E staining (Bucay, Sarosi et al. 1998). Our measurements yielded a total lacunar density of 599 per mm^2 with a standard deviation of 121 per mm^2 . While this number was slightly lower than the densities found in the Bucay study, it was similar to the density calculated by Mosher.

Table 1. Apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes by individual subjects. Standard deviations in parentheses.

SUBJECT	APOPTOTIC OSTEOCYTE DENSITY($\#/\text{mm}^2$)	PERCENTAGE APOPTOTIC OSTEOCYTES(%)	TOTAL LACUNAR DENSITY($\#/\text{mm}^2$)	VIABLE OSTEOCYTE DENSITY($\#/\text{mm}^2$)	PERCENTAGE VIABLE OSTEOCYTES(%)
45	217.91	41.53	556.33	396.33	67.36
90	248.58	37.96	738.62	697.07	75.67
93	210.30	33.38	645.56	476.71	71.69
94	72.14	17.48	455.70	353.61	70.88
Overall	187.2(78.5)	32.59(10.61)	599.1(121.1)	480.9(152.9)	71.40(3.41)

Mean values and standard deviations of apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable

osteocytes across all subjects by quadrant are summarized in Table 2. Results from the general linear models are shown in appendix A. Quadrantal mean values and standard deviations across all subjects from individual regions were calculated in Tables 2a-2c. P-values comparing the differences between each quadrant are also listed.

As displayed in Table 2, no significant differences appear in apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, or percentage of viable osteocytes between quadrant comparisons. Similarly, no differences appeared when all subjects were analyzed by region (Table 2a-2c).

Table 2. Quadrantal differences of apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects. Standard deviations in parentheses.

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	205.1(83.2)	33.99(11.20)	641.9(131.0)	518.8(161.7)	71.43(3.21)
Caudal(Cd)	159.8(67.2)	30.06(10.36)	554.5(105.4)	445.5(158.4)	70.56(4.70)
Medial(M)	200.3(93.5)	30.96(11.00)	668.1(144.7)	510.3(121.1)	70.84(1.75)
Lateral(L)	189.8(85.5)	35.02(10.84)	544.8(127.2)	440.1(144.4)	73.00(4.25)
Cr-Cd	.7035	.8543	.5681	.6586	.9833
Cr-M	.9688	.7968	.9921	.8579	.9993
Cr-L	.9937	.9935	.9200	.8579	.9983
Cd-M	.9210	.9994	.4045	.7108	.9952
Cd-L	.8434	.9493	.9066	.9828	.9972
M-L	.9975	.9135	.7971	.9998	1.000

Table 2a. Mean quadrantal data gathered from the *proximal region* for apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects. Standard deviations in parentheses.

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	168.1(69.7)	29.67(9.60)	592.7(111.6)	437.8(93.5)	68.96(4.38)
Caudal(Cd)	157.7(86.4)	29.59(12.92)	519.6(83.9)	368.0(44.6)	69.15(3.15)
Medial(M)	164.2(93.6)	28.86(12.28)	600.4(112.4)	453.6(64.4)	68.99(1.32)
Lateral(L)	156.5(86.0)	32.07(11.85)	478.2(96.8)	357.5(57.6)	72.08(5.49)
Cr-Cd	.9987	1.000	.8153	.6065	.9999
Cr-M	.9999	.9998	.9997	.9912	.9878
Cr-L	.9981	.9940	.5452	.5016	.7658
Cd-M	.9997	.9998	.7683	.4531	.9932
Cd-L	1.000	.9934	.9574	.9973	.7959
M-L	.9994	.9861	.4957	.3630	.9102

Table 2b. Mean quadrantal data gathered from the *midshaft region* for apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects. Standard deviations in parentheses.

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	290.67(11.74)	46.16(7.76)	710.6(106.6)	613(176)	73.24(3.42)
Caudal(Cd)	220.1(41.7)	38.53(8.37)	628.2(69.5)	535(200)	71.04(7.45)
Medial(M)	294.40(.402)	38.44(3.15)	791.8(78.5)	602.3(105.1)	72.00(1.95)
Lateral(L)	279.2(50.8)	44.67(4.92)	688.9(34.7)	591.8(68.2)	73.31(4.07)
Cr-Cd	.2889	.6626	.7214	.9486	.9620
Cr-M	.9994	.6555	.7297	.9999	.9926
Cr-L	.9842	.9949	.9908	.9988	1.000
Cd-M	.2593	1.000	.2833	.9651	.9964
Cd-L	.4011	.7790	.8557	.9784	.9585
M-L	.9652	.7722	.5877	.9998	.9912

Table 2c. Mean quadrantal data gathered from the *distal region* for apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects. Standard deviations in parentheses.

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	214.7(23.0)	39.44(9.82)	602.2(93.6)	436.0(40.4)	65.00(8.48)
Caudal(Cd)	148.59(5.18)	31.91(3.36)	511.4(74.9)	360.4(64.3)	62.21(1.89)
Medial(M)	153.4(42.8)	30.9(15.4)	570(183)	403.1(108.7)	63.10(15.4)
Lateral(L)	207.1(58.9)	32.31(11.12)	658.4(63.8)	401.33(12.62)	58.10(9.17)
Cr-Cd	.4169	.8934	.8537	.6904	.9912
Cr-M	.4679	.8544	.9913	.9564	.9970
Cr-L	.9967	.9069	.9567	.9497	.8951
Cd-M	.9992	.9996	.9509	.9130	.9997
Cd-L	.5007	1.000	.6140	.9221	.9735
M-L	.5584	.9990	.8637	1.000	.9551

Mean values and standard deviations of apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects by region are summarized in Table 3. Results from the general linear models are listed in appendix A. Table 3 demonstrates significant differences between the proximal and midshaft regions in the apoptotic osteocyte density, percentage of apoptotic osteocytes, total lacunar density, and viable osteocyte density. The percentage of viable osteocytes showed a significant difference between the proximal and distal regions, while the apoptotic osteocyte density, viable osteocyte density, and percentage of viable osteocytes showed a significant difference between the midshaft and distal regions. Regional mean values and standard deviations across all subjects from individual quadrants were listed in Table 3a-3d. A significant difference was only seen in the viable osteocyte density of the lateral quadrant between the proximal and midshaft regions. Furthermore, the viable osteocyte density in the

lateral quadrant showed a marginally significant difference between the midshaft and distal regions.

Table 3. Regional differences of apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects. Standard deviations in parentheses.

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Proximal(P)	160.5(77.4)	29.84(10.90)	547.4(95.4)	403.1(66.9)	69.79(3.64)
Midshaft(MS)	266.0(24.7)	42.54(6.48)	689.7(69.2)	584(160)	72.46(4.54)
Distal(D)	180.3(22.22)	34.29(8.94)	574.0(93.9)	399.2(54.6)	62.64(7.10)
P-MS	.0009	.0176	.0047	.0002	.5930
P-D	.7431	.6510	.6783	.9940	.0073
MS-D	.0113	.1627	.0563	.0005	.0016

Table 3a. Mean regional data gathered from the *cranial quadrant* for apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects. Standard deviations in parentheses.

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Proximal(P)	168.1(69.7)	29.67(9.60)	592.7(111.6)	437.8(93.5)	68.96(4.38)
Midshaft(MS)	290.67(11.74)	46.16(7.76)	710.6(106.6)	613(176)	73.24(3.42)
Distal(D)	214.7(23.0)	39.44(9.82)	602.2(93.6)	436.0(40.4)	65.00(8.48)
P-MS	.1187	.2378	.5051	.3078	.6976
P-D	.6136	.5329	.9948	.9998	.7304
MS-D	.3855	.7613	.6035	.3532	.3858

Table 3b. Mean regional data gathered from the *caudal quadrant* for apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects. Standard deviations in parentheses.

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Proximal(P)	157.7(86.4)	29.59(12.92)	519.6(83.9)	368.0(44.6)	69.15(3.15)
Midshaft(MS)	220.1(41.7)	38.53(8.37)	628.2(69.5)	535(200)	71.04(7.45)
Distal(D)	148.59(5.18)	31.91(3.36)	511.4(74.9)	360.4(64.3)	62.21(1.89)
P-MS	.5847	.6363	.3745	.3219	.8905
P-D	.9869	.9667	.9929	.9968	.3073
MS-D	.5594	.8027	.3855	.3479	.2305

Table 3c. Mean regional data gathered from the *medial quadrant* for apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects. Standard deviations in parentheses.

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Proximal(P)	164.2(93.6)	28.86(12.28)	600.4(112.4)	453.6(64.4)	69.99(1.32)
Midshaft(MS)	294.40(40.2)	38.44(3.15)	791.8(78.5)	602.3(105.1)	72.00(1.95)
Distal(D)	153.4(42.8)	30.90(15.4)	570(183)	403.1(108.7)	63.1(15.4)
P-MS	.2160	.6705	.3298	.2672	.9576
P-D	.9842	.9809	.9641	.8147	.6322
MS-D	.2212	.8037	.2995	.1743	.5424

Table 3d. Mean regional data gathered from the *lateral quadrant* for apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes across all subjects. Standard deviations in parentheses.

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Proximal(P)	156.5(86.0)	32.07(11.85)	478.2(96.8)	357.5(57.6)	72.08(5.49)
Midshaft(MS)	279.2(50.8)	44.67(4.92)	688.9(34.7)	591.8(68.2)	73.31(4.07)
Distal(D)	207.1(58.9)	32.31(11.12)	658.4(63.8)	401.33(12.62)	58.10(9.17)
P-MS	.2628	.4513	.0852	.0188	.9757
P-D	.7405	.9997	.1297	.6695	.1485
MS-D	.6155	.5159	.9200	.0502	.1510

3.2 Regression Analysis

Linear regression analysis of BMP antagonist levels versus apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes was performed (Figures 11-15). Levels of BMP antagonists, noggin and gremlin, were used from a previous study (Mosher 2010) and shown in Table 4. Noggin expression displayed an overall negative correlation except in the percentage of viable osteocytes but showed a significant relationship in the percentage of apoptotic osteocytes and marginally significant relationships with viable osteocyte density and percentage of viable osteocyte. Conversely, gremlin expression demonstrated all positive correlations and significant relationships between expression and apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes (Table 5).

Table 4. BMP antagonist levels (Mosher 2010).

Region	Quadrant	Noggin	Gremlin
Proximal	Cranial	2.33	1.59
Midshaft	Cranial	1.75	2.02
Distal	Cranial	2.35	1
Proximal	Caudal	2.63	1.47
Midshaft	Caudal	2	1.98
Distal	Caudal	1.96	1
Proximal	Medial	2.49	1.47
Midshaft	Medial	2.06	1.87
Distal	Medial	1.76	1
Proximal	Lateral	2.55	1.38
Midshaft	Lateral	1.8	1.97
Distal	Lateral	2.14	1.5

Table 5. Linear relationships between BMP antagonist expression and apoptotic osteocyte density, viable osteocyte density, total lacunar density, percentage of apoptotic osteocytes, and percentage of viable osteocytes.

Noggin	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Slope	-0.0029	-0.0311	-0.0018	-0.0018	0.0029
R ²	0.2696	0.3572	0.2563	0.3101	0.0020
P-value	0.0836	0.0401	0.0931	0.0600	0.8890

Gremlin	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Total Lacunar Density	Viable Osteocyte Density	%Viable Osteocytes
Slope	0.005	0.0372	0.0029	0.0032	0.054
R ²	0.5382	0.3397	0.4480	0.6595	0.4796
P-value	0.0066	0.0467	0.0173	0.0013	0.0125

Figure 11. Regression analysis of BMP expression versus apoptotic osteocyte density. Data from proximal region is represented with the diamond (◆) symbol, midshaft region is represented with the circle (●) symbol, and distal region is represented with the triangle (▲) symbol. Cranial, caudal, medial, and lateral points are colored blue, red, green, and yellow respectively. All points indicating noggin levels are solid shapes, while all points indicating gremlin levels are shape outlines.

Figure 12. Regression analysis of BMP expression versus percentage of apoptotic osteocytes. Data from proximal region is represented with the diamond (◆) symbol, midshaft region is represented with the circle (●) symbol, and distal region is represented with the triangle (▲) symbol. Cranial, caudal, medial, and lateral points are colored blue, red, green, and yellow respectively. All points indicating noggin levels are solid shapes, while all points indicating gremlin levels are shape outlines.

Figure 13. Regression analysis of BMP expression versus total lacunar density. Data from proximal region is represented with the diamond (◆) symbol, midshaft region is represented with the circle (●) symbol, and distal region is represented with the triangle (▲) symbol. Cranial, caudal, medial, and lateral points are colored blue, red, green, and yellow respectively. All points indicating noggin levels are solid shapes, while all points indicating gremlin levels are shape outlines.

Figure 14. Regression analysis of BMP expression versus viable osteocyte density. Data from proximal region is represented with the diamond (◆) symbol, midshaft region is represented with the circle (●) symbol, and distal region is represented with the triangle (▲) symbol. Cranial, caudal, medial, and lateral points are colored blue, red, green, and yellow respectively. All points indicating noggin levels are solid shapes, while all points indicating gremlin levels are shape outlines. BMP Expression level

Figure 15. Regression analysis of BMP expression versus percentage of viable osteocytes.
 Data from proximal region is represented with the diamond (◆) symbol, midshaft region is represented with the circle (●) symbol, and distal region is represented with the triangle (▲) symbol. Cranial, caudal, medial, and lateral points are colored blue, red, green, and yellow respectively. All points indicating noggin levels are solid shapes, while all points indicating gremlin levels are shape outlines.

3.3 BMU Analysis

Our samples showed low levels of BMU activity which was to be expected due to their unloaded condition. The summary of the number of BMUs positively identified by quadrant and region is shown in Table 6.

Table 6. Number of BMUs identified across all subjects

Region	Quadrant	BMUs
Proximal	Cranial	3
Proximal	Caudal	2
Proximal	Medial	0
Proximal	Lateral	1
Midshaft	Cranial	2
Midshaft	Caudal	1
Midshaft	Medial	0
Midshaft	Lateral	0
Distal	Cranial	0
Distal	Caudal	0
Distal	Medial	0
Distal	Lateral	0

IV. DISCUSSION

The objective of this study was to investigate apoptotic osteocytes as a possible regulator of signaling factors in the bone remodeling process. The frequency of osteocyte apoptosis was measured by region (proximal, midshaft, and distal) and quadrant (cranial, caudal, lateral, and medial) in unloaded murine cortical bone. To determine variability, their spatial distribution was correlated with the location of levels of the BMP antagonists, noggin and gremlin, that were measured in a previous study. The distribution of total lacunar density and viable osteocyte frequencies were quantified as well and correlated with the same BMP antagonist levels. We hypothesized that a variation in the location of apoptotic osteocytes would coincide with varying levels of either one or both of the BMP antagonists. BMP antagonist expression is thought to play a role in the initiation of bone remodeling. If a correlation does exist, we believe that osteocytes undergoing apoptosis are part of the regulatory mechanism that stimulates and directs bone remodeling in a spatial and temporal manner. If no correlation can be made, then apoptotic osteocytes may not affect the signaling pathway responsible for bone remodeling. Using mice as models were advantageous because they are small, affordable, easily obtained, and may be used for gene expression studies in the future. Analysis was performed on the tibial hindlimbs as opposed to forelimbs because of their larger cross sectional area and easy subsection to mechanical loading that will be performed in future studies.

Previous correlations of apoptotic osteocytes with high rates of bone turnover sparked the possibility of a tight spatial and temporal link between dying osteocytes and fatigue induced

microdamage followed by subsequent remodeling. After it was confirmed apoptosis was not a result of remodeling but rather a part of the pathway leading to it, a causal pathway linking apoptotic osteocytes and the regulation of bone remodeling was established. Rat bones subjected to hyperphysiological strains produced apoptotic osteocytes in areas surrounding microdamage that were preceded by the tunneling of osteoclasts, a marker of the beginning of bone resorption (Noble, Peet et al. 2003). As the mechanosensors within bone, osteocytes and their viability greatly influence bone remodeling. Osteocyte ablated mice showed altered activities of osteoclasts and osteoblasts during unloading induced bone loss (Tatsumi, Ishii et al. 2007). Similarly, without the apoptosis of osteocytes, remodeling cannot occur. An in vivo rat ulnar model used a pan caspase inhibitor to suppress osteocyte apoptosis that normally resulted from fatigue induced microdamage. As a result, the activation of osteoclastic resorption within the cortex was completely prevented. This proved that not only was apoptosis necessary to stimulate activation of intracortical remodeling but also that osteoclastic resorption was directly related to the amount of apoptosis. A possible dose-response relationship may exist further emphasizing the role apoptotic events play in determining the course of remodeling (Cardoso, Herman et al. 2009). In addition to a temporal relationship, a spatial relationship between apoptotic osteocytes and bone remodeling exists. The location of apoptotic osteocytes within bone may guide osteoclasts to a targeted location for bone resorption to begin. A study on mice performed ovariectomies to induce bone loss by creating an estrogen deficiency. Osteocyte apoptosis occurred in the femoral cortical bone in a certain area of the cortex that coincided with the region of subsequent bone resorption (Emerton, Hu

et al. 2009). It is clear that the death of osteocytes, not other bone cell types, triggers the activation of localized remodeling of bone.

Since the crucial role of apoptosis has become apparent, current studies have focused on looking at its triggers in an attempt to gain a more complete understanding of how it affects bone turnover. The trigger for apoptosis could come from a variety of sources including molecular mechanisms, chemoattractants, or a cellular source of signals. Apoptotic osteocytes themselves may release a signal that initiates a bone remodeling response. An *in vivo* study showed that osteocyte apoptotic bodies are capable of initiating osteoclastic bone resorption without the addition of pro osteoclastogenic factors. Osteocyte apoptotic debris was shown to stimulate osteoclasts (Kogianni, Mann et al. 2008). Alternatively, apoptosis may be controlled by various gene products. Finding its exact selective mechanism remains difficult because it is regulated by the interaction of many molecules through several different pathways. Bax and Bcl-2 are two gene products that control apoptosis in a complimentary pathway. Surrounding areas of microcracks in fatigue loaded rats, Bax expression promoted osteocyte apoptosis while Bcl-2 expression seen at a distance from microdamage protected cells from undergoing apoptosis. Surviving cells could be a signal source or promote an osteoclast response by releasing an anti apoptotic response in areas surrounding apoptotic osteocytes (Verborgt, Tatton et al. 2002). In addition, sclerostin, a protein product of the SOST gene, has been shown to initiate apoptosis through the activation of caspase 3. Sclerostin is a negative regulator of bone formation through the modulation of osteoblast function and survival (Sutherland, Geoghegan et al. 2004). Expression of this gene was detected strongly in osteocytes suggesting its role in bone homeostasis (Winkler, Sutherland et al. 2003). Apoptosis is regulated by the

interaction of many molecules through different pathways. While its selective mechanism remains a mystery, its importance in the skeletal development and regulation of bone regeneration makes it a key topic for current research.

Quantifying the number and location of apoptotic osteocytes within bone is helpful in determining where and how bone remodeling is targeted. In a previous study, Hedgecock measured the regional variability in bone turnover parameters, osteocyte apoptosis, and osteocyte density in rabbit tibial midshafts. Cross sectional areas of bone were divided into cranial, caudal, lateral, and medial quadrants. A significant correlation was found in areas between bone remodeling parameters and osteocyte apoptosis suggesting they release a signal that attracts or directs bone remodeling (Hedgecock, Hadi et al. 2007). Further investigation into the precise location of apoptotic osteocytes within bone and their relation to other possible bone remodeling stimuli will offer important information. Taking a look at regional factors associated with bone remodeling, Mosher quantified regional and quadrantal differences of BMP antagonist levels and osteocyte densities in unloaded murine cortical bone. He compared BMP antagonist expression levels with varying osteocyte densities. While no significant differences were shown between quadrants, significant regional differences were found between the BMP antagonist levels of gremlin and osteocyte densities indicating some sort of relationship and influence on bone remodeling (Mosher 2010).

The results of this study focused on quantifying the variation of apoptotic osteocytes in the cross sectional area of unloaded mice hindlimbs in an attempt to provide more insight into possible factors of initiation for bone remodeling. By comparing the results to Mosher's previous study measuring BMP antagonist levels, we were able to provide interesting results

and affirmation of our initial hypothesis. Similar regions and quadrants were analyzed in both studies to make drawing correlations and comparisons easier.

Quadrantal Analysis

While the cranial region had the highest density of apoptotic osteocytes and the lateral region had the highest percentage of apoptotic osteocytes, no statistical difference was found in the distribution of osteocytes within quadrants. No quadrantal differences were seen in the percentage of apoptotic osteocytes, total lacunar density, viable osteocyte density, and percentage of viable osteocytes as well. Mosher's study showed similar results with no differences between quadrants with respect to viable osteocyte density, total lacunar density, percentage of viable osteocyte, and gremlin expression (Mosher 2010). This overall lack of variation among quadrants is not uncharacteristic of unloaded bone conditions.

Regional Analysis

While there was a lack of quadrantal differences, regional variations were found between subjects supporting our hypothesis of regional variation in osteocyte apoptosis. A significantly higher density of apoptotic osteocytes in the midshaft region was seen in comparison to both proximal and distal regions. Also, a greater percentage of apoptotic osteocytes were observed in the midshaft region compared to the proximal region. Coincidentally, in Mosher's previous study, the midshaft region demonstrated higher gremlin expression than both the proximal and distal regions as well (Mosher 2010). This indicates some relationship between BMP antagonists and apoptotic osteocytes that is confirmed by the regression analysis. Regression analysis of gremlin showed significant moderate positive linear correlations between gremlin expression and apoptotic osteocyte density ($R^2=.54$, $p\text{-value}=.01$)

and the percentage of apoptotic osteocytes ($R^2=.34$, $p\text{-value}=.05$). While regression analysis for noggin showed a negative linear correlation for the percentage of apoptotic osteocytes ($R^2=.36$, $p\text{-value}=.04$), no other significant correlations were established.

Regional differences were also noted in the total lacunar density, where midshaft densities were significantly higher than the proximal densities. Regression analysis of gremlin showed a significant positive linear correlation between its expression and total lacunar density ($R^2=.45$, $p\text{-value}=.02$) while no correlation was seen in noggin expression.

Viable osteocyte densities in the midshaft were higher than in the proximal and distal regions while the percentage of viable osteocytes was higher in the proximal and midshaft when compared to the distal region. This is comparable to Mosher's regional analysis in which the midshaft had greater viable osteocyte densities than the proximal and distal region. A significant positive correlation between gremlin expression and viable osteocyte density ($R^2=.66$, $p\text{-value}=.00$) and the percentage of viable osteocytes ($R^2=.48$, $p\text{-value}=.01$) was established through regression analysis.

Comparing our results to BMP antagonists gives new insight into possible factors controlling bone remodeling. Regional differences found in previous and the current study suggest the midshaft as a possible target location for osteoclast recruitment in remodeling since an increase in all osteocyte densities occurred in that specific region. Furthermore a positive correlation was made between gremlin and apoptotic osteocytes, viable osteocytes, and total osteocytes in both studies suggesting gremlin could be an important part of the signaling pathway responsible for bone resorption. These possibilities are helpful in honing in on the exact mechanism responsible for bone remodeling.

This study confirmed the hypothesis that a variation in the location of apoptotic osteocytes coincided with a variation in the BMP antagonist levels of gremlin within unloaded bone. While a direct link between viable or apoptotic osteocytes and gremlin has not been established, the close association between the gene product sclerostin, apoptosis, and bone morphagenic protein antagonists suggests the need for a further investigation of these possible initiation factors. Since bone morphagenic proteins are responsible for skeletal homeostasis and bone growth, the binding of their antagonists to them prevents them from binding to signaling factors, causing BMPs to lose control of bone formation. The two extracellular antagonists, noggin and gremlin, used for comparison in this study, have been previously examined separately to explore their individual role in bone formation. In vivo overexpression of these BMP antagonists was conducted in mice using an osteocalcin promoter. Noggin is known to decrease the effects of BMPs on osteoblast function and impair osteoblastogenesis and osteoclastogenesis. Overexpression caused a decrease of trabecular bone volume due to a decrease in osteoblastic activity but not osteoblast number (Devlin, Du et al. 2003). Similarly, over expression of gremlin has been shown to lead to bone loss. Gremlin is expressed in osteoblasts and increases after BMP exposure. It regulates BMP activity by a local feedback mechanism. Skeletal markers showed a decrease in bone mineral density and overall weakening of the bone. The number of osteoclasts and osteoblasts was shown to decrease indicating decreased bone remodeling. Mechanisms of inhibition included a reduction in the replication of the osteoblasts or differentiation of mesenchymal stem cells toward mature osteoblasts (Gazzerro, Pereira et al. 2005). Overexpression of both noggin and gremlin in mice led to an overall inhibition of bone formation. Both bind to BMP-2,-4, and -7 which play a key

role in the regulation of bone function and fracture repair, but noggin binds with a higher affinity than gremlin (Zhao, Harris et al. 2002). Overall BMP antagonists have been shown to play a role in the formation of bone especially in controlling osteoblast function. However, their specific activities in terms of interacting with BMPs, each other, bone remodeling, and apoptosis is still under investigation.

The exact role of osteocyte death in the signaling of bone remodeling remains unclear. Whether apoptotic osteocytes release a stimulatory or inhibitory signal, or are the actual signal itself, they somehow activate the initiation of targeted bone remodeling along with other various factors in the signaling cascade. A better understanding of this remains a topic of much current research.

Limitations

Several limitations have been made in the current study. Subjects were housed in the University's vivarium and looked after by other students. Since no external loads were applied, we assumed each subject had similar daily activity levels as we were unable to monitor them. Differences in activity levels could have affected the levels of mechanically induced bone remodeling and therefore osteocyte levels. The mice used in the current study and Mosher's previous study were assumed to experience the same daily activity levels so comparisons could be easily made between the two.

Using histology presented some limitations as well. The majority of the tissue gathering including sample sacrifice, fixation, decalcification, tissue processing, and embedding was handled by other lab students using similar samples but performing different studies. As a result, bone samples were processed and sectioned with the surrounding muscle tissue

attached. While the muscle tissue acted as a guide in helping determine the orientation of the bone, it was excess that may have contributed to problems arising while sectioning using the microtome. Uncontrollable tissue damage resulted in the form of torn, ripped, and folded sections. Distal and midshaft regions proved to be the hardest regions to gather decent tissue sections from, most likely due to their smaller cross sectional areas. This prevented analysis of a complete data set across all three regions from individual subjects.

There are several limitations in the apoptosis detection technique. Much concern lies in clearly distinguishing between apoptosis and necrosis since they are both forms of cell death. Detection of apoptosis was previously limited to morphology, but as more information about the cell death pathway is constantly being uncovered, other aspects of cell death can be analyzed. TUNEL staining using the DNA fragmentation technique is used for detection in this current study. While DNA fragmentation in necrosis is characterized by random, late, and usually single stranded breaks, opposite of apoptosis, DNA fragmentation may not necessarily signify apoptosis. Fixation, embedding, and sectioning the tissue before staining may lead to fragmentation that results in false positive staining. Ideally, the best approach for apoptosis identification should include a combination of techniques such as in situ end labeling and morphological criteria or ISEL and immunohistochemistry to ensure the most accurate results (Stadelmann and Lassmann 2000).

In addition, the combination of the TUNEL stain and the methyl green counterstain resulted in problems identifying viable osteocytes during co-staining. Our study performed separate TUNEL and methyl green stains on slides to more clearly distinguish viable osteocytes. However, overstaining may have occurred due to extra clumping and false positive staining.

Figuring out a way to effectively stain using both TUNEL and methyl green on one section would eliminate the need for double the amount of tissue sections as well as reduce the amount of overstaining. Developing a consistent counting technique used during analysis proved challenging as well. Rules were established to identify apoptotic osteocytes, viable osteocytes, and empty lacunae to ensure valid comparisons between data sets of each study. Only osteocytes with a clearly defined lacunar wall were counted and osteocytes outside of the plane were not included.

Applications and Future Work

Osteocyte apoptosis is fundamental to the life cycle of the skeleton. During growth it is responsible for the expansion of the skeleton and during old age it plays a role in osteoporosis.

The current study solely looked at unloaded bone samples to locate areas of apoptotic and viable osteocytes within hindlimbs. The results of this study can be used as a control for future studies comparing other possible initiation factors of bone remodeling. Since mechanical loading is known to induce apoptosis and subsequent bone remodeling, a future study on the regional variability of apoptotic osteocytes in loaded murine cortical bone would be a simple next step. Similar considerations for future studies on bone remodeling stimuli could explore the role of apoptosis as a spatial and temporal marker in response to stimuli such as estrogen withdrawal or disuse.

Studies of osteocyte apoptosis have been performed using mainly animal models. Hedgecock performed a quantitative analysis of bone remodeling factors and osteocyte apoptosis in rabbit hindlimbs (Hedgecock, Hadi et al. 2007). Other research has mainly used

mice and rats to study apoptosis in vivo (Tomkinson, Gevers et al. 1998; Weinstein, Jilka et al. 1998; Verborgt, Gibson et al. 2000; Noble, Peet et al. 2003). Using different possibly larger animal models could potentially drive the research in a direction where it could be relatable to the human skeleton, which is the ultimate goal. Until then, the observations from these studies can only be speculated in regards to the human skeleton.

While TUNEL remains the standard for immunohistochemical detection techniques, other techniques could be explored to identify apoptosis. The phenomenon itself is difficult enough to detect because it represents such a tiny fraction of the cell's life span (Jilka, Weinstein et al. 2007). Previously mentioned as a limitation, in situ nick end labeling has been attacked for its lack of specificity but is not the only apoptotic technique available to identify apoptotic cells. Aside from being mistaken for necrotic cells, apoptotic cells may also be mistaken for oncotic cells which also undergo DNA fragmentation during cell death. Newer, more specific methods that have been developed to detect single stranded DNA or caspase-3 which is known to play a central role in the apoptotic process and has so far not been detected in oncosis (Van Cruchten and Van den Broek 2002).

Apoptosis of bone cells has been the topic of much current research due to its role in the signaling pathway guiding bone turnover. Since impairment or dysregulation of bone remodeling is the main cause of most skeletal diseases, apoptotic osteocytes may act as a therapeutic target for their treatment. These skeletal disorders include post menopausal osteoporosis, osteoarthritis, and Paget's disease. Induction of apoptosis may be a potential therapeutic tool for treating these diseases (Hughes and Boyce 1997). Drugs currently used today for the management of osteoporosis alter the frequency of apoptosis in bone cells such

as increasing osteoclast apoptosis or increasing osteoblast apoptosis in an attempt to correct the imbalance that leads to diminished bone mass (Weinstein and Manolagas 2000). Also, identifying the selective pathways of apoptosis is important in finding potential targets for new therapeutic strategies (Hock, Krishnan et al. 2001). Cell death by apoptosis plays a significant but complex role in bone physiology and pathology.

Conclusion

Identifying the location of apoptotic osteocytes within unloaded murine hindlimbs provides insight into a better understanding of the spatial and temporal relationship between dying osteocytes and bone remodeling. This study examines the frequency of apoptotic osteocytes by quadrant and region within cross sections of bone and correlates their regional variability with possible factors that signal bone remodeling. Quantitative evidence of a strong linear correlation between osteocyte apoptosis and gremlin expression specifically in the midshaft region offers a potential area for bone remodeling to be directed. Further examination of the distribution of apoptotic osteocytes in bone undergoing super-physiologic loading conditions to induce bone turnover would provide more information on the relationship between apoptotic osteocytes and bone remodeling. Understanding the role of apoptosis is essential in maintaining overall skeletal tissue homeostasis and developing treatments in the preventative care of skeletal pathologies.

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Appendix A

General linear models across all subjects by quadrant

General Linear Model: TUNEL OD versus Quadrant

Factor	Type	Levels	Values
Quadrant	fixed	4	caudal, cranial, lateral, medial

Analysis of Variance for TUNEL OD, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Quadrant	3	7219	7219	2406	0.43	0.736
Error	24	135728	135728	5655		
Total	27	142948				

S = 75.2020 R-Sq = 5.05% R-Sq(adj) = 0.00%

Unusual Observations for TUNEL OD

Obs	TUNEL OD	Fit	SE Fit	Residual	St Resid
15	62.073	205.996	28.424	-143.922	-2.07 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Quadrant	N	Mean	Grouping
cranial	7	216.4	A
lateral	7	206.0	A
medial	7	198.3	A
caudal	7	172.9	A

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals

Response Variable TUNEL OD

All Pairwise Comparisons among Levels of Quadrant

Quadrant = caudal subtracted from:

Quadrant	Lower	Center	Upper	
cranial	-67.35	43.50	154.4	(-----+-----+-----+-----+)
lateral	-77.80	33.05	143.9	(-----+-----+-----+-----+)
medial	-85.48	25.37	136.2	(-----+-----+-----+-----+)

-80 0 80 160

Quadrant = cranial subtracted from:

Quadrant	Lower	Center	Upper	
lateral	-121.3	-10.45	100.40	(-----+-----+-----+-----+)
medial	-129.0	-18.13	92.72	(-----+-----+-----+-----+)

-80 0 80 160

-80 0 80 160

Quadrant = lateral subtracted from:

Tukey Simultaneous Tests

Response Variable TUNEL OD

All Pairwise Comparisons among Levels of Quadrant

Quadrant = caudal subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
cranial	43.50	40.20	1.0822	0.7035
lateral	33.05	40.20	0.8223	0.8434
medial	25.37	40.20	0.6311	0.9210

Quadrant = cranial subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
lateral	-10.45	40.20	-0.2599	0.9937
medial	-18.13	40.20	-0.4511	0.9688

Quadrant = lateral subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
medial	-7.685	40.20	-0.1912	0.9975

General Linear Model: Viable OD versus Quadrant

Factor Type Levels Values
Quadrant fixed 4 caudal, cranial, lateral, medial

Analysis of Variance for Viable OD, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Quadrant	3	26519	26519	8840	0.62	0.606
Error	24	339840	339840	14160		
Total	27	366360				

S = 118.996 R-Sq = 7.24% R-Sq(adj) = 0.00%

Unusual Observations for Viable OD

Obs	Viable OD	Fit	SE Fit	Residual	St Resid
1	737.237	487.241	44.976	249.996	2.27 R
2	676.991	413.630	44.976	263.361	2.39 R

R denotes an observation with a large standardized residual.

Quadrant	N	Mean	Grouping
cranial	7	487.2	A
medial	7	481.7	A
lateral	7	436.9	A
caudal	7	413.6	A

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Viable OD
All Pairwise Comparisons among Levels of Quadrant
Quadrant = caudal subtracted from:

Quadrant = lateral subtracted from:

	Difference	SE of		Adjusted
Quadrant	of Means	Difference	T-Value	P-Value
medial	44.73	63.61	0.7033	0.8948

General Linear Model: Total Lacunar D versus Quadrant

Factor	Type	Levels	Values
Quadrant	fixed	4	caudal, cranial, lateral, medial

Analysis of Variance for Total Lacunar D, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Quadrant	3	40203	40203	13401	1.00	0.410
Error	24	321905	321905	13413		
Total	27	362108				

S = 115.813 R-Sq = 11.10% R-Sq(adj) = 0.00%

Unusual Observations for Total Lacunar D

Obs	Total Lacunar D	Fit	SE Fit	Residual	St Resid
15	366.866	589.877	43.773	-223.011	-2.08 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Quadrant	N	Mean	Grouping
medial	7	646.5	A
cranial	7	629.1	A
lateral	7	589.9	A
caudal	7	548.3	A

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals

Response Variable Total Lacunar D

All Pairwise Comparisons among Levels of Quadrant

Quadrant = caudal subtracted from:

Quadrant	Lower	Center	Upper	
cranial	-89.9	80.85	251.6	(-----*-----)
lateral	-129.1	41.60	212.3	(-----*-----)
medial	-72.5	98.25	269.0	(-----*-----)
	-150	0	150	300

Quadrant = cranial subtracted from:

Quadrant	Lower	Center	Upper	
lateral	-210.0	-39.25	131.5	(-----*-----)
medial	-153.3	17.40	188.1	(-----*-----)
	-150	0	150	300

Quadrant = lateral subtracted from:

Tukey Simultaneous Tests

Response Variable Total Lacunar D

All Pairwise Comparisons among Levels of Quadrant

Quadrant = caudal subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
cranial	80.85	61.90	1.3061	0.5681
lateral	41.60	61.90	0.6721	0.9066
medial	98.25	61.90	1.5871	0.4045

Quadrant = cranial subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
lateral	-39.25	61.90	-0.6340	0.9200
medial	17.40	61.90	0.2810	0.9921

Quadrant = lateral subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
medial	56.65	61.90	0.9151	0.7971

General Linear Model: TUNEL ratio versus Quadrant

Factor	Type	Levels	Values
Quadrant	fixed	4	caudal, cranial, lateral, medial

Analysis of Variance for TUNEL ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Quadrant	3	0.01188	0.01188	0.00396	0.38	0.769
Error	24	0.25093	0.25093	0.01046		
Total	27	0.26281				

S = 0.102251 R-Sq = 4.52% R-Sq(adj) = 0.00%

Grouping Information Using Tukey Method and 95.0% Confidence

Quadrant	N	Mean	Grouping
cranial	7	0.4	A
lateral	7	0.4	A
caudal	7	0.3	A
medial	7	0.3	A

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals
 Response Variable TUNEL ratio
 All Pairwise Comparisons among Levels of Quadrant
 Quadrant = caudal subtracted from:

Quadrant	Lower	Center	Upper	-----+-----+-----+-----
cranial	-0.1071	0.043658	0.1944	(-----*-----)
lateral	-0.1214	0.029306	0.1800	(-----*-----)
medial	-0.1571	-0.006384	0.1443	(-----*-----)
				-----+-----+-----+-----
				-0.12 0.00 0.12

Quadrant = cranial subtracted from:

Quadrant	Lower	Center	Upper	-----+-----+-----+-----
lateral	-0.1651	-0.01435	0.1364	(-----*-----)
medial	-0.2008	-0.05004	0.1007	(-----*-----)
				-----+-----+-----+-----
				-0.12 0.00 0.12

Quadrant = lateral subtracted from:

Quadrant	Lower	Center	Upper	-----+-----+-----+-----
medial	-0.1864	-0.03569	0.1150	(-----*-----)
				-----+-----+-----+-----
				-0.12 0.00 0.12

Tukey Simultaneous Tests
 Response Variable TUNEL ratio
 All Pairwise Comparisons among Levels of Quadrant
 Quadrant = caudal subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
cranial	0.043658	0.05466	0.7988	0.8543
lateral	0.029306	0.05466	0.5362	0.9493
medial	-0.006384	0.05466	-0.1168	0.9994

Quadrant = cranial subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
lateral	-0.01435	0.05466	-0.2626	0.9935
medial	-0.05004	0.05466	-0.9156	0.7968

Quadrant = lateral subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
medial	-0.03569	0.05466	-0.6530	0.9135

General Linear Model: Viable ratio versus Quadrant

Factor	Type	Levels	Values
Quadrant	fixed	4	caudal, cranial, lateral, medial

All Pairwise Comparisons among Levels of Quadrant
 Quadrant = caudal subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
cranial	0.013456	0.03708	0.3629	0.9833
lateral	0.007313	0.03708	0.1972	0.9972
medial	0.008808	0.03708	0.2375	0.9952

Quadrant = cranial subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
lateral	-0.006143	0.03708	-0.1657	0.9983
medial	-0.004648	0.03708	-0.1254	0.9993

Quadrant = lateral subtracted from:

Quadrant	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
medial	0.001495	0.03708	0.04031	1.000

General linear models across all subjects by region

General Linear Model: TUNEL OD versus Region

Factor Type Levels Values
 Region fixed 3 distal, midshaft, proximal

Analysis of Variance for TUNEL OD, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Region	2	60954	60954	30477	9.29	0.001
Error	25	81993	81993	3280		
Total	27	142948				

S = 57.2690 R-Sq = 42.64% R-Sq(adj) = 38.05%

Grouping Information Using Tukey Method and 95.0% Confidence

Region	N	Mean	Grouping
midshaft	8	271.1	A
distal	8	180.9	B
proximal	12	161.6	B

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals

Response Variable TUNEL OD

All Pairwise Comparisons among Levels of Region

Region = distal subtracted from:

Region	Lower	Center	Upper	-----+-----+-----+-----
midshaft	18.90	90.17	161.44	(-----*-----)
proximal	-84.37	-19.31	45.75	(-----*-----)

Tukey 95.0% Simultaneous Confidence Intervals
 Response Variable Viable OD
 All Pairwise Comparisons among Levels of Region
 Region = distal subtracted from:

Region = midshaft subtracted from:

Tukey Simultaneous Tests
 Response Variable Viable OD
 All Pairwise Comparisons among Levels of Region
 Region = distal subtracted from:

Region	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
midshaft	185.294	41.85	4.4275	0.0005
proximal	4.007	38.20	0.1049	0.9940

Region = midshaft subtracted from:

Region	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
proximal	-181.3	38.20	-4.745	0.0002

General Linear Model: Total Lacunar D versus Region

Factor Type Levels Values
 Region fixed 3 distal, midshaft, proximal

Analysis of Variance for Total Lacunar D, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Region	2	122099	122099	61049	6.36	0.006
Error	25	240009	240009	9600		
Total	27	362108				

S = 97.9815 R-Sq = 33.72% R-Sq(adj) = 28.42%

Grouping Information Using Tukey Method and 95.0% Confidence

Region	N	Mean	Grouping
midshaft	8	704.9	A
distal	8	585.6	A B
proximal	12	547.7	B

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals
 Response Variable Total Lacunar D
 All Pairwise Comparisons among Levels of Region
 Region = distal subtracted from:

Region = midshaft subtracted from:

Tukey Simultaneous Tests
 Response Variable Total Lacunar D
 All Pairwise Comparisons among Levels of Region
 Region = distal subtracted from:

Region	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
midshaft	119.29	48.99	2.4350	0.0563
proximal	-37.85	44.72	-0.8463	0.6783

Region = midshaft subtracted from:

Region	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
proximal	-157.1	44.72	-3.514	0.0047

General Linear Model: TUNEL ratio versus Region

Factor Type Levels Values
 Region fixed 3 distal, midshaft, proximal

Analysis of Variance for TUNEL ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Region	2	0.068793	0.068793	0.034396	4.43	0.023
Error	25	0.194015	0.194015	0.007761		
Total	27	0.262808				

S = 0.0880943 R-Sq = 26.18% R-Sq(adj) = 20.27%

Grouping Information Using Tukey Method and 95.0% Confidence

Region	N	Mean	Grouping
midshaft	8	0.4	A

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distal      8    0.3  A B
proximal   12    0.3   B

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Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable TUNEL ratio
All Pairwise Comparisons among Levels of Region
Region = distal subtracted from:

Region	Lower	Center	Upper
midshaft	-0.0264	0.08321	0.19284
proximal	-0.1359	-0.03581	0.06427

-----+-----+-----+-----
(-----*-----)
(-----*-----)
-----+-----+-----+-----
-0.12 0.00 0.12

Region = midshaft subtracted from:

Region	Lower	Center	Upper
proximal	-0.2191	-0.1190	-0.01894

-----+-----+-----+-----
(-----*-----)
-----+-----+-----+-----
-0.12 0.00 0.12

Tukey Simultaneous Tests
Response Variable TUNEL ratio
All Pairwise Comparisons among Levels of Region
Region = distal subtracted from:

Region	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
midshaft	0.08321	0.04405	1.8891	0.1627
proximal	-0.03581	0.04021	-0.8907	0.6510

Region = midshaft subtracted from:

Region	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
proximal	-0.1190	0.04021	-2.960	0.0176

General Linear Model: Viable ratio versus Region

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Factor Type Levels Values
Region fixed      3 distal, midshaft, proximal

```

Analysis of Variance for Viable ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Region	2	0.047830	0.047830	0.023915	8.75	0.001
Error	25	0.068323	0.068323	0.002733		
Total	27	0.116153				

S = 0.0522774 R-Sq = 41.18% R-Sq(adj) = 36.47%

Unusual Observations for Viable ratio

Obs	Viable ratio	Fit	SE Fit	Residual	St Resid
11	0.516129	0.620943	0.018483	-0.104814	-2.14 R
12	0.521739	0.620943	0.018483	-0.099204	-2.03 R
28	0.739726	0.620943	0.018483	0.118783	2.43 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Region	N	Mean	Grouping
midshaft	8	0.7	A
proximal	12	0.7	A
distal	8	0.6	B

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals

Response Variable Viable ratio

All Pairwise Comparisons among Levels of Region

Region = distal subtracted from:

Region	Lower	Center	Upper	
midshaft	0.03796	0.10302	0.1681	(-----*-----)
proximal	0.02013	0.07952	0.1389	(-----*-----)

-0.070 0.000 0.070 0.140

Region = midshaft subtracted from:

Region	Lower	Center	Upper	
proximal	-0.08289	-0.02350	0.03589	(-----*-----)

-0.070 0.000 0.070 0.140

Tukey Simultaneous Tests

Response Variable Viable ratio

All Pairwise Comparisons among Levels of Region

Region = distal subtracted from:

Region	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
midshaft	0.10302	0.02614	3.941	0.0016
proximal	0.07952	0.02386	3.333	0.0073

Region = midshaft subtracted from:

Region	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
proximal	-0.02350	0.02386	-0.9848	0.5930