THE ROLE OF THE OSTEOCYTE IN
ORTHODONTIC TOOTH MOVEMENT IN
THE RAT DENTO-ALVEOLAR COMPLEX

Doctor of Clinical Dentistry (Orthodontics)

Thesis

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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
</tr>
<tr>
<td>AEC</td>
<td>3-Amino-9-ethylcarbazole</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMU</td>
<td>Basic metabolic unit</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony stimulating factor</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
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<tr>
<td>hMSC</td>
<td>Human mesenchymal stem cells</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IMVS</td>
<td>Institute of Medical and Veterinary Science</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
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<tr>
<td>IR</td>
<td>Immunoreactive</td>
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<tr>
<td>IU</td>
<td>International units</td>
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<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>LRP 5/6</td>
<td>Lipoprotein receptor related protein 5/6</td>
</tr>
<tr>
<td>M</td>
<td>Molar (molarity)</td>
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### Abbreviations of length

<table>
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<tbody>
<tr>
<td>mm</td>
<td>Millimetre</td>
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<tr>
<td>μm</td>
<td>Micron</td>
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<tr>
<td>nm</td>
<td>Nanometre</td>
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### Abbreviations of time

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<tr>
<td>d</td>
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<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>s</td>
<td>Second</td>
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<tr>
<td>wk</td>
<td>Week</td>
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<td>y</td>
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### Abbreviations of volume

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<tr>
<td>l</td>
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<tr>
<td>ml</td>
<td>Millilitre</td>
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<tr>
<td>μl</td>
<td>Microlitre</td>
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### Abbreviations of weight

<table>
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<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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Declaration

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Dr Dinesh Sanmuganathan

Dated:
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SECTION 1
Introduction

Since the middle of the 19th century the importance of mechanical stimuli in the maintenance and structure of skeletal tissues has been recognised.[1] It is now accepted that skeletal tissue is dynamic, incorporating cycles of bone resorption and bone formation; a process which helps to restore the skeleton while preserving its structural integrity.[2] This remodelling is organised by cells of the osteoblast and osteoclast lineage and involves a complicated network of cell-cell and cell-matrix interactions, systemic hormones, locally produced cytokines and growth factors.[3]

Orthodontic and orthopaedic practice have much in common, in that both fields involve a fundamental understanding of bone biology, particularly the relationship that mechanical stress has on the various cell types found in bone. Mechanical strain on a tooth, as opposed to bone, is a more complicated phenomenon requiring changes in both the periodontal ligament (PDL) as well as the supporting alveolar bone, which are tissue types that house vastly different cell populations and remodelling characteristics. The signal transduction events that convert the mechanical strain to a molecular biological response, and subsequent remodelling of the supporting tissues, are yet to be fully elucidated.[3] Bone biologists have recently ascribed to the osteocyte a key role in this mechanotransduction, a role that was traditionally given to the osteoblast. A more fundamental understanding of the cellular and molecular elements that regulate and drive these events is not only pivotal in understanding how these processes occur but also needed for development of a means to control them to make orthodontic tooth movement more efficient. [4]
LITERATURE REVIEW
Overview of Alveolar Bone Structure

The skeleton makes up approximately 20% of human body weight. Its major functions incorporate mechanical support and motility, protection of internal organs, storage and metabolism of Ca2+ and phosphate, and regulating haematopoiesis. Bone is dynamic in nature, being controlled by changes in hormones, growth factors, mechanical loading, nutrition and other as yet unidentified factors.[5]

Within mammals the teeth are attached to the bones of the jaws via an attachment gomphosis apparatus consisting of cementum, periodontal ligament and alveolar bone. This apparatus allows enough flexibility to withstand the forces of mastication.[6] Alveolar bone specifically refers to that bone containing the sockets for the teeth. It consists of an outer cortical plate, a central spongiosa and bundle bone (the bone lining the alveolus). The cortical plates are continuations of the compact bone of the principal mass of the maxilla and mandible. Bundle bone provides the attachment for the PDL fibre bundles.[7]

Like all other bone, alveolar bone is in a state of constant remodelling through an orchestrated interplay between the removal of old bone and its replacement with new bone.[6] There is considerable variation, at the histological level, in the morphology of alveolar bone produced by resorption or deposition of bone as it responds to the functional demands placed on it.
Cells of Alveolar Bone

Bone effector cells of the osteoclast and osteoblast lineage are responsible for the size, shape and strength of bones. The joint activity of these two cell types drive the turnover, repair and developmental sculpting of the skeleton including both modelling and modelling drift.[8]

Osteoclast

The multinucleated osteoclast represents the main bone resorption cell.[7] Osteoclasts are derived from bone marrow haematopoietic stem cells sharing the same precursor as macrophages.[5] It is by far the largest of the bone cells (50 to 100 μm) and their precursors. They are usually found in association with bony surfaces, occupying shallow depressions known as Howship's lacunae.[6] Mature osteoclasts have abundant mitochondria, numerous lysosomes, and free ribosomes.[9] However, the most remarkable morphological feature of the osteoclast is their ruffled border which is a complex system of finger-shaped projections of the membrane, the function of which is to mediate the resorption of the calcified bone matrix.[10] This border is completely surrounded by another specialized area, called the clear zone. The cytoplasm within this zone has a uniform appearance, containing bundles of actin-like filaments. Immediately underneath the osteoclast, the clear zone seals off a resorbing compartment on the bone surface. It is the ability of the clear zone to seal off this area of bone surface that allows the formation of a microenvironment suitable for the operation of the resorptive apparatus.[9] Within the ruffled border membrane is an ATP-driven proton pump (the so-called vacuolar H1-ATPase). This pump creates an acidic environment within the resorption site which is responsible for the dissolution of the mineral component (hydroxyapatite) of the bone matrix.[9] Matrix metalloproteinases (MMP's) and cathepsins K, B, and L are secreted by the osteoclast into the area of bone resorption and are responsible for the degradation of the protein
components of the matrix, chiefly collagen.[11] Degraded bone matrix components are endocytosed along the osteoclast’s ruffled border and subsequently transcytosed to the contralateral membrane area, where they are released into the extracellular environment.[12] Osteoclasts feature the presence of high amounts of the phosphohydrolase enzyme, tartrate-resistant acid phosphatase (TRAPase), and this feature is commonly used for the detection of osteoclasts in bone specimens.[9]

![Figure 1: A light micrograph indicating large multinucleated osteoclasts resorbing an old osteon.[6]](image)

**Osteoblast**

Osteoblasts arise from multipotent mesenchymal stem cells that have the capacity to differentiate into osteoblasts, adipocytes, chondrocytes, myoblasts, or fibroblasts.[13] The proteins that constitute the bone matrix are produced and secreted by the fully differentiated osteoblasts.[14] This matrix is subsequently mineralized under the control of the same cells.[9]
Osteoblasts, when most active, form a layer of cuboidal cells with strong basophilic cytoplasm, and a prominent nucleus that lies towards the basal end of the cell. There is a pale juxtanuclear area indicating the site of the Golgi complex. They also contain a prominent rough endoplasmic reticulum (RER) with numerous vesicles and mitochondria. Microtubules and microfilaments are present and particularly prominent beneath the secreting membrane of the cell. As bone deposition proceeds, osteoblasts become incorporated into the matrix as osteocytes.[15]

Osteoblasts do not function alone but are found in groups along the bone surface, where they line the layer of bone matrix that they are producing. Toward the end of the matrix-secreting period, 15% of mature osteoblasts are entrapped in the new bone matrix and differentiate into osteocytes. On the contrary, some cells remain on the bone surface, becoming flat lining cells.

Bone formation occurs in three successive phases: the production and the maturation of osteoid matrix, followed by mineralization of the matrix. In normal adult bone, these processes occur at the same rate so that the balance between matrix production and mineralization is equal. Initially, osteoblasts produce osteoid by rapidly depositing collagen. This is followed by an increase in the mineralization rate to equal that of collagen synthesis. In the final stage the rate of collagen synthesis decreases and mineralization continues until the osteoid becomes fully mineralized.[9]

As stated above, a major product of the bone-forming osteoblast is type I collagen. Initially, this protein is secreted in the form of a precursor, which contains peptide extensions at both the amino-terminal and carboxyl ends of the molecule. Proteolytic removal of these propeptides and additional extracellular processing results in mature three-chained type I collagen molecules that assemble themselves into a collagen fibril. Numerous other proteins are synthesized by the osteoblast. These proteins, such as
osteocalcin and osteonectin are incorporated into the bone matrix and constitute 40% to 50% of the noncollagenous proteins of bone.[9] Glycosaminoglycans are other osteoblast-derived proteins and are attached to one of two small core proteins: PGI (or biglycan) and decorin. Numerous other proteins such as osteopontin, bone sialoprotein, fibronectin, vitronectin, and thrombospondin serve as attachment factors that interact with integrins.

While responsible for producing the osteoid matrix, mature osteoblasts play an essential role in creating the microenvironment that allows the mineralization of this osteoid by deposition of hydroxyapatite.[16] Local concentrations of calcium and phosphate are regulated by osteoblasts in such a way as to promote the formation of hydroxyapatite crystals. Osteoblasts express relatively high amounts of alkaline phosphatase, an enzyme thought to play a role in bone mineralisation.[9] This is highlighted by the fact that a genetic deficiency of alkaline phosphatase leads to hypophosphatasia, a condition characterised by defective bone mineralization.[17] The precise mechanism of mineralisation and the role of alkaline phosphatase in this process still remains unclear. Bone mineralisation lags behind matrix production and, in remodelling sites in the adult bone, occurs at a distance of 8–10 μm from the osteoblast. Matrix synthesis determines the volume of bone but not its density. Mineralisation of the osteoid increases the density of bone by removal of water, but does not alter its volume.[9]

i) Osteoblast differentiation
Marrow mesenchymal stem cells (MSC's) are pluripotent progenitors that can differentiate into bone, cartilage, muscle and fat cells.[5] Many factors have been implicated in regulating osteoblast differentiation (and subsequent bone formation) from these mesenchymal stem cells. Bone morphogenetic protein 2 (BMP-2), BMP-6 and BMP-9 are among the most potent inducers of osteogenic differentiation. The bone
morphogenetic proteins (BMP’s) are a large family of dimeric proteins within the Transforming Growth Factor β superfamily of cytokines.[18] They were originally identified as the active components within the osteo-inductive extracts derived from bone and they are now known to be involved in a wide range of signalling functions that mediate tissue interactions during development. The discovery of Smad-mediated signals revealed the precise functions of BMPs in osteoblast differentiation.[19] Transcription factors, Runx2 and Osterix, are essential molecules for inducing osteoblast differentiation, as indicated by the fact that both Runx2-null mice and Osterix-null mice have neither osteoblasts or bone tissue.[19]

There is increasing evidence suggesting that the canonical Wnt signalling pathway may play as important a role in regulating osteoblastic differentiation.[20] The Wnt family consists of a large number of secreted glycoproteins that are involved in embryonic development, tissue induction and axis polarity.[20] Wnt ligands bind to seven transmembrane-spanning receptors of the frizzled family and coreceptors of the LRP5/6 gene, leading to an accumulation of the intracellular signalling molecule β-catenin which is translocated to the nucleus where it initiates transcription of target genes.[21] Loss-of-function mutations in the LRP5 gene cause the low-bone-mass phenotype of the autosomal recessive disorder osteoporosis-pseudoglioma syndrome and conversely, gain in function mutations of LRP5 result in patients with a high-bone-mass phenotype. These findings suggest that balanced function of LRP5 is critical to osteoblastic proliferation and differentiation.[20]

**Osteocyte**

The osteocyte represents a terminally differentiated non-proliferative cell of the osteoblast lineage. As osteoblasts secrete bone matrix (osteoid), some of them become embedded in lacunae and are from that point referred to as osteocytes. As such, the osteocyte is considered non-migratory because it is entrenched within the
bone matrix. Each osteocyte extends numerous (perhaps as many as 60) small cytoplasmic processes within bone canaliculi.[8,22] They form a network permeating the entire bone matrix. These long cell processes are rich in microfilaments that are organized during the formation of the matrix and before its calcification. It has been shown that these cytoplasmic processes are approximately half the diameter of the canaliculi in which they reside, leaving a sufficient gap for two processes to lie side by side.[23] Each osteocyte communicates with its neighbours and with the surface lining cells of bone by means of gap junctions.[24] A gap junction allows ions and compounds of low molecular weight to pass between the two neighbouring cells without having to pass into extracellular space.[25] Theoretically, this allows the cells to communicate with each other, forming an extensive syncitial network through bone.[22] Osteocytes are, therefore, in close communication with bone-lining cells at quiescent bone surfaces, osteoblasts at sites of new bone formation and the pericytes of capillaries and sinusoids supplying nutrients to osteocytes and other bone cells. They are also in indirect gap-junction communication with the osteoblast precursor cells of the marrow stroma.[24]

Figure 2: A schematic diagram of a functional syncytium comprising osteocytes, osteoblasts, bone marrow stromal cells and endothelial cells. [9]
i) **Size of the osteocytes**

Osteocyte size varies among species. The murine osteocyte lacuna dimension is of the order of 5 μm by 20 μm [23] with a gap present between the cell and the lacuna wall. The canaliculi range between 50 and 100 nm in diameter, where the cytoplasmic processes, as discussed, are approximately half the diameter of the canaliculi.[23]

ii) **Distribution**

The distribution of osteocytes and their dendritic processes in lamellar bone, and to a lesser extent woven bone, is not entirely random. In theory, this distribution is set at the time of new bone formation.[8] Polarity is displayed by osteocytes in terms of the distribution of their cell processes. The cell membrane facing the bone surface has the highest number of these processes termed vascular dendrites.[26] Pallumbo et al. hypothesised that the maximum functional length of these cell processes might be the determining factor in incorporating the associated osteoblast into bone matrix.[26]

Osteocytes represent the most common cell type in bone.[24] While their lacuna/canalicular system only represents 1% of bone fluid volume, it has substantial surface area for molecular exchange, estimated to be some 400 times that of the entire Haversian and Volkmann system combined and more than 100 fold that of the trabecular bone surface.[27] This vast internal surface area potentially provides a mechanism to quickly alter bone mineral homeostasis, growth factor content and degree of mineralisation of bone. Osteocytes must have the capability to inhibit mineralization on this large bone surface as, in their absence, micropetrosis (mineralisation of the canaliculi and to a lesser extent the osteocyte lacunae) occurs.[28] As the osteoblast becomes encased in bone matrix, the metabolic activity of the osteoblast/osteocyte decreases, despite the fact that the osteocyte has been historically thought to be a metabolically inert cell due to its number of cellular organelles.[8] In fact, osteocytes are capable of significant molecular synthesis and
modification. The nervous system is a similar type of communication system, comprising large numbers of low activity cells in a syncitial network, which has been claimed to represent the most efficient design for the transmission of metabolically expensive signals over long distances.[29]

iii) Formation of the cells—lineage transitions

The early stages of any lineage are poorly defined and this is particularly so for cells derived from the mesenchymal stem cells within the bone marrow environment. These stem cells, of which the osteocyte is one derivative, are particularly problematic due to the increasing levels of cell plasticity being reported.[30]

Osteocyte formation is the result of an infrequent lineage transition from bone surface resident osteoblasts to this non-proliferative terminally differentiated cell (it is estimated only 10-20% of osteoblasts differentiate into non-proliferative osteocytes).[24] The second end-form of the osteoblastic lineage is the bone lining cell.[31] During the process of bone formation, an osteoblast, will be entombed in matrix as an “osteoid osteocyte” as outwardly advancing newly formed osteoid is laid down. As the cell is carried away, the future osteocyte maintains contact with the advancing osteoblasts at the surface via extending cellular processes.[24] The surrounding osteoid matrix then undergoes mineralisation under the control of enzymes, including osteocyte-derived cacein kinase II.[32] However, the exact cellular/molecular mechanisms that regulate this process are not fully understood. It has been hypothesised that osteocytes produce signals that decrease the bone apposition rate of osteoblasts during refilling of BMUs (basic multicellular units) hence enabling the recruitment of osteoblasts to become osteocytes.[24] The evolution of an osteoblast to osteocyte involves a number of significant changes including reduced susceptibility to apoptotic death, permanent removal from the cell cycle and production of dendritic processes. As such it would be expected that changes in gene expression at this time would be prominent.[24]
iv) **Osteocyte Receptors**

Mature osteocytes express most of the receptors known to play important roles in bone metabolism. These include ER \(\alpha\) and \(\beta\) (oestrogen receptor \(\alpha\) and \(\beta\)), PTH, vitamin D3, corticosteroids, and TGF-\(\beta\). Certain osteocytic receptors, ligands and molecular transporters are commonly associated with components of nerve tissue, suggesting a role for some components of the nervous system in bone function. Osteocytic production of nerve growth factor after fracture and the presence of glutamate transporters are intriguing given the proposed function of the osteocytes as a syncitial sensing and information transfer system.[8]

v) **Functional Role of Osteocytes in Bone**

The definition of an osteocyte is descriptive of its location as opposed to its function. Bone biologists are still unclear about the precise role of the osteocyte in bone but several theories have been postulated concerning their function and their contribution to the process of bone remodelling.[8] These theories include; osteolysis, maintaining systemic mineral homeostasis, sensing the strains produced in response to mechanical loading of bones, production of signals that affect osteoblast/osteoclast function as well as the expression of molecules that directly affect the turnover process.[24,33] If the majority of these theories are correct then the osteocyte is a multi-functional “smart cell” that acts as the conductor of the orchestra that is local bone turnover.[8]
Osteoblastic and Osteocytic Responses to Mechanical Stimuli

The architecture of bone is sensitive and shaped by mechanical forces.[34] Unloaded bone, such as in the case of long-term bed rest or space travel, is resorbed whereas increased exercise or gravity is associated with increased bone mineral density.[35] Bone cells can respond to various mechanical stimuli including stretch, fluid flow and hydrostatic pressure. However, in the literature these responses are widely varied.[34] Osteoblast proliferation on mechanical stimulation has been found in some studies, whereas others have found decreased cell numbers. Burger and Veldhuijzen hypothesised that differential responses to mechanical stimulation are based on the level of strain applied. They reason that osteoblasts respond to high stress levels with increased proliferation but physiologic levels of stress decrease cell numbers.[36] The level of differentiation of human osteoblasts has been shown to affect the response of the osteoblast to mechanical strain.

Weyts et al. showed that physiologic stretch levels induce apoptosis in young osteoblast cultures (7-day).[34] In more mature cultures, apoptosis was not triggered by the same level of stretching and in day 14 cultures stretching increased cellular proliferation. Protection of older cultures against stretch-induced apoptosis was not related to accumulation of a mineralised matrix, or the absence of a matrix to adhere to in young cultures, because collagen I matrix was provided to all cells from day 1 of culture. The authors conceptualised that the differentiation-dependent changes in stretch responses are due to intrinsic changes in mechanosensitivity of human osteoblasts during differentiation and may reflect differential expression or maturation of a mechanosensor (i.e., integrins, G protein coupled receptors, ion-channels, or the cytoskeleton). As such, the absence of a response on the level of apoptosis or proliferation in late stages of osteoblast differentiation may not necessarily reflect insensitivity of cells in this phase to mechanotransduction; but the tight regulation of
this response by differentiation does imply its significance and is congruent with the role of the osteoblastic lineage as a mechanotransductor in bone. [34]

Given that osteocytes are terminally differentiated osteoblasts, it is a logical extrapolation that they, too, possess a mechanosensory apparatus. However, because osteocytes, unlike osteoblasts, are housed in a mineralised matrix they are ideally positioned to detect changes in environmental perturbations. It has, therefore, been postulated that the osteocyte plays a central role in orchestrating local bone remodelling where physical loading triggers them to modulate local bone homeostasis.[8] Osteocytes most likely detect these changes and fatigue-induced bone microdamage through their dendritic processes extending throughout bones within the canalicular system [37] and their apoptosis might provide site-specific repair signals.[38] In support of this notion, induction of microcracks in rat ulnae by fatigue loading induced apoptosis of osteocytes adjacent to microcracks, but not in distant osteocytes.[39] More importantly, resorption of the affected sites followed. Very high strains also increased osteocyte apoptosis in rat ulnae, possibly by signalling to surface lining osteoblasts.[39]

It has also been theorised that control of osteoclast and osteoblast apoptosis is important in the overall control of bone remodelling.[40] The control mechanisms are not fully understood but the fluid flow hypothesis proposes that locally evoked strain derived from the displacement of fluid in the canaliculi plays an important role.[41] When loading occurs, interstitial fluid is squeezed through the thin layer of non-mineralized matrix surrounding the cell bodies and cell processes, resulting in local strain at the cell membrane and activation of the affected osteocytes.[41]

Osteocyte lacunal density has been found to be variable, not only between individuals [42] and with age [43], but also under altered mechanical stimuli.[43] The exact location
of the mechanosensor in the osteocyte is still to be determined but the presence of a primary (non-motile) cilium on osteocytes has been reported and is known to sense mechanical stimuli in other cell types.[44] It has also been found that a mesh of extracellular material, primarily proteoglycans, is present in at least some canaliculi and might assist in the amplification of fluid flow derived from mechanical signals.[45] These cells respond both in vivo and in vitro to increased load-induced strains by modifying a number of important molecules.[8] Anabolic signals, such as nitric oxide, prostaglandins, and ATP, are released within seconds of osteocyte loading.[46] A few minutes after the onset of mechanical loading, glucose 6-phosphate dehydrogenase, a marker of cell metabolism, is elevated in osteocytes [47], and an increase in c-FOS mRNA is observed within 2 hrs.[48] Within 4 hours, transforming growth factor β and insulin-like growth factor mRNA expression are increased.[49]

Increases in osteocyte-specific markers have also been published, including E11/gp3.8, dentin matrix protein 1 (found in a tooth movement model), matrix extracellular phosphoglycoprotein (MEPE), and sclerostin.[50] The targets for all these signals, through the network of cell-cell communications, are bone-surface cells and osteoblasts. It was reported recently that SOST (the gene for sclerostin) and sclerostin protein levels were dramatically reduced by mechanical loading of bone.[51] Mechanical strain may also trigger osteocytes to send signals for activation of the bone resorption cascade through expression of activator for NF-κB ligand (RANKL), secretion of macrophage colony-stimulating factors (M-CSF), and through their own apoptosis at the sites of micro-damage or micro-cracks.[50] Gene expression analysis suggests that osteocytes indirectly control osteoclast differentiation through modulation of RANKL expression in osteoblasts. In addition, humoral factors produced and released through canaliculi into the bone marrow may regulate the differentiation and activity of osteoclasts.[52] With these types of control mechanisms, it is plausible to
conclude that the osteocyte acts as the chief mechanosensor in bone, which has also been recently confirmed by targeted ablation of osteocytes in a mouse model.[52]

**Orthodontic Tooth Movement**

The skeleton is subject to intermittent loads during locomotion and other forms of physical activity. Similarly, alveolar bone is loaded intermittently during mastication, but is subject to a more continuous deformation or strain during orthodontic tooth movement.[53] In non-alveolar bone, the application of mechanically induced strain appears to trigger primarily an osteogenic response [53], without any apparent resorptive effect. Furthermore, this osteogenic response of bone to external loading appears to be due to a reactivation of quiescent bone-lining cells [54], and is not dependent on a preliminary phase of bone resorption [55]. Alveolar bone, however, fundamentally differs from the rest of the skeleton in this response as when it is subject to a continuous physical strain, it undergoes both resorption (osteoclastogenesis) and deposition (osteogenesis), the extent of which is dependent upon the magnitude, direction, and duration of the applied force.[3] The balance favours bone deposition on the side of the alveolar wall undergoing tension and bone resorption on the side of the wall undergoing compression, allowing the tooth to “move” along the force vector being applied to it.(Figure 3) Osteoclastogenesis and osteogenesis are not only pivotal for orthodontic tooth movement but also for tooth eruption.[4]
Mechanosensing is the phenomenon by which cells are capable of sensing structural changes in the extra-cellular matrix (ECM), caused by external loading. Mechanical forces exerted on teeth by orthodontic appliances strain both the extracellular matrix (ECM) and cells of the periodontium (alveolar bone, PDL, gingiva, and associated blood vessels and neural elements). The effect is initially physical in nature and is followed closely by a biological response.[50] This interaction generates significant changes in both the structural and functional components of the ECM, cell membrane, cytoskeletal elements, nucleus, and several other cytoplasmic organelles that subsequently synthesise and mobilise a variety of molecules inside and outside the cells.[3,56]

Cell adhesion molecules, like the integrins, allow direct transmission of the tensile, compressive, and shear forces from the ECM into the cell and vice versa, and are considered essential for cellular survival, growth, and mobility.[50] This transmission helps to maintain the cells in their active form, termed tensegrity, capable of responding
rapidly to various mechanical, physical, and other challenges. In addition to the energy provided by the mechanical load, this sensing triggers a biochemical reaction that provides the additional energy required for the biological system to respond to the elevated environmental demands.[57] Each cellular group within paradental tissue is equipped with mechanosensors and as such a mechanical stimulus may activate multiple sets of these sensors, leading to a series of separate downstream cellular events in various tissue types.[50] Subsequent changes in cytoskeletal protein structure and function propagate the signalling process into the nucleus and, ultimately the genome, via signalling proteins (such as hedgehog, transforming growth factors) and calcium ions. The eventual outcome of these chains of events is enhanced or suppressed gene expression, with transference of the signal back to the cytoplasm through mRNAs, reaching the ribosomes, generating protein synthesis and secretion and an associated cellular response that could include mitosis, cell motility or programmed cell death.[58] (Figure 4) This process is termed mechanotransduction which simply is the conversion of a mechanical input into a biochemical one.[50]

The unique feature of mechanotransduction in orthodontics is the interaction that occurs between various tissues, both mineralized and non-mineralized, and their associated neurovascular elements. For tooth movement to be perpetuated these tissues need to be remodelled, a task performed by cells of the alveolar bone (osteoblasts, osteoclasts, and osteocytes), PDL (gingival fibroblasts), blood vessels (endothelial cells), and neural tissues (dendritic and neural cells). Other cell types essential for this process are derivatives of the immune system (inflammatory cells and osteoclast progenitors). There is an individual response pattern for each of these tissues as far as the mechanosensing, transduction, and response mechanisms are concerned.[3,58]
Figure 4: The sequence followed by cells in mechanosensing, transduction and response.[50]
i) Mineralised tissue response to applied mechanical load

When an orthodontic force is applied to a tooth it bends alveolar bone and both compresses and stretches the PDL. This leads to a sequence of events within the dento-alveolar complex where the alveolar bone adjacent to the compressed PDL becomes the site of intense bone resorption, while the stretched PDL interfaces with sites of active osteogenesis. As the alveolus bends there is flow of bone interstitial fluid, evoking shear stress in the mineralized extracellular matrix (ECM) and deformation of the alveolar bone osteocytes in the lacunae and of the dendrites in the canaliculi. Osteoblasts, which maintain direct contact with osteocytes will, in turn, respond to the osteocytic signal one of two ways depending on the direction of the applied force. The first response, on the “tension” side is a direct one where the osteoblasts initiate and are responsible for appositional bony deposition. The second response, on the “compression” side results in the activated osteoblasts conveying signals to approaching osteoclasts, enticing these cells to start resorbing the alveolar bone, and informing them on the proper time to cease their resorptive activities. These osteoclasts for bone resorption arise from conversion within the local population of monocytes/macrophages or migration of progenitor cells from alveolar bone marrow cavities to the strained PDL.[59] Within the compression side osteocyte signalling mechanisms, such as sclerostin, also inhibit osteoblast differentiation, function and mineralisation.
Figure 5: The sequence of bone remodelling stages during orthodontic treatment. The roles played by osteocytes osteoblasts and osteoclasts are illustrated.[50]

ii) Non-mineralised tissue response to applied mechanical load

Gingival and periodontal fibroblasts differ in their roles in paradental tissue remodeling. While PDL fibroblasts are predominantly involved in the synthesis and degradation of their own ECM, gingival fibroblasts are involved in bone remodelling events.[50] A reduction in collagen production (type I and IV) has been reported in the compressed PDL, as well as increased type IV collagen production at PDL tension sites after 72 h of force application.[60]
The role of blood vessels in orthodontic tooth movement has been the subject of recent scrutiny, where angiogenesis and remodeling of existing blood vessels help functional adaptation to the new environment created. Reports show an initial reduction and a later increase in the number of PDL blood vessels following orthodontic force application.[60,61] These blood vessels also play a major role in the mechanical force-induced sterile necrosis that occurs in the compressed periodontium by acting as a source of cytokines and chemokines, where demonstrable increases in expression of IL-1β, IL-1 receptor, IL-6, IL-6 receptor, IL-8 receptor, IL-11, and TNF-α have been shown.[50]

Neural elements also play a role where released neurotransmitters, such as Substance P and calcitonin gene related peptide (CGRP), interact with endothelial cells of the paradental capillary network, enticing them to bind circulating leukocytes, promoting their migration into the paradental ECM.[62] These migratory leukocytes in turn produce ample amounts of chemokines and cytokines upon entering these tissues. It is this combination of cytokines, along with those produced by the native paradental cells, that evoke and maintain the remodeling events of the PDL and alveolar bone.[63]

In summary, orthodontic tooth movement involves the use of a mechanical strain that is subsequently converted to a desirable, and somewhat predictable, biological response within the dento-alveolar complex. The effectors of this force-induced remodeling are the cells of the paradental tissue that use mechanosensing, transduction, and response phenomena to respond to applied mechanical forces.[50]
Sclerostin

Sclerostin is the protein product encoded by the SOST gene, located on chromosome 17q12-q21.[64] Sclerostin is a known negative regulator of osteoblast differentiation and function and acts as one inhibitor to bone formation.[8,65] In its role as a key inhibitor, sclerostin helps determine the normal extent of bone formation and, consequently, protects against the deleterious effects of uncontrolled bone growth.[66] Its effect is highlighted by two rare high bone mass diseases, Sclerosteosis and Van Buchem disease, both of which have been linked to inactivating mutations in the SOST gene.[65,67] The administration of an anti-sclerostin antibody has been shown to increase bone formation and restore the bone loss upon ovariectomy in rodents.[68] It has been suggested that the inhibition of sclerostin (through sclerostin neutralizing antibodies) may, therefore, be an attractive strategy for conditions in which bone loss is a significant component, such as postmenopausal and senile osteoporosis.[66] Conversely, transgenic mice overexpressing Sost exhibit low bone mass.[69]

On the basis of its amino acid sequence, sclerostin belongs to the DAN family of glycoproteins.[21] This group of proteins has been shown to antagonize both bone morphogenetic proteins (BMP’s) and the Wnt signalling pathway, growth factors of pivotal importance in bone formation.[8,70] BMPs and Wnts are critical for osteoblastogenesis because they provide the initial and essential stimulus for commitment of multipotential mesenchymal progenitors to the osteoblast lineage.[71] Similarly, sclerostin has been demonstrated to potently antagonise and negatively regulate several members of the bone morphogenetic protein (BMP) family.[72] Sclerostin is also believed to work by binding to lipoprotein receptor-related protein 5/6 (LRP5/6), inhibiting canonical Wnt-signalling and thus impairing osteoblast differentiation and function.[65] Although this binding to LRP5/6 antagonises Wnt signalling, sclerostin and Wnt do not appear to compete for binding of this co-
receptor.[21] The precise mechanism through which sclerostin expression inhibits Wnt-mediated bone formation is still unclear, where it may be transported to the bone surface by the osteocytic canaliculi or it may induce another signalling molecule in osteocytes which is then transported to the osteoblasts to inhibit bone formation.[21] While most of the literature points to sclerostin operating through inhibition of BMP and Wnt pathways, it cannot be ruled out that it may have its own, as yet unknown, receptors to which they bind.[70]

It is believed that sclerostin inhibits bone formation via a number of ways. These include:

1. negatively regulating osteoblast differentiation;
2. negatively regulating osteoblast proliferation;
3. suppressing mineralisation by osteoblastic cells and
4. stimulating osteoblast apoptosis.[21,70]

**Figure 6:** A schematic drawing demonstrating the possible inhibitory effects of sclerostin on the proliferation and differentiation of osteoblasts and its agonistic effects on osteoblast apoptosis.[70]
Sclerostin is a factor that was thought to be exclusively expressed by the osteocyte, where high levels have been detected in their lacunae and canalicular system.[70,72] It has been demonstrated that osteoclasts, osteoblasts, bone lining cells (retired osteoblasts that have ceased forming bone and cover most of the bone surface) and periosteal osteoblasts exhibited no sclerostin staining.[66] While postnatally it is clear that osteocytes and no other cells in bone express sclerostin, recent evidence has shown distinct immunolocalisation of SOST in the cementocytes and mineralising periodontal ligament cultures.[21,72] This is hardly surprising given that cementum and bone are rather similar hard tissue substances, where osteocytes and cementocytes, together with their canalicular network, share many morphological and cell biological characteristics. Unlike osteocytes in bone there is no clear evidence that cementocytes have a functional role in the tissue homeostasis of cementum.[72] While SOST protein expression seems to be limited to terminally differentiated cells within an embedded mineralised matrix, SOST mRNA is expressed by many non-mineralised tissues, especially during embryogenesis, including the heart, aorta, kidney and liver.[21]

The distribution of sclerostin-positive osteocytes is not homogenous throughout bone where section mapping has shown a differential spatial relationship, with sclerostin-negative osteocytes located significantly closer to bone surfaces.[66] In vivo and in vitro murine and human studies have shown that recently embedded osteocytes, including those within unmineralised osteoid, were almost all negative for sclerostin.[21] As such, sclerostin protein expression by new osteocytes is delayed until the cells have matured and are surrounded by a mineralised matrix.[66] It has been postulated by Poole et al. that sclerostin secretion by newly embedded osteocytes is delayed such that the cells must mature or receive a later signal that triggers sclerostin expression. They conceptualise that newly embedded osteocytes secrete sclerostin only after the local onset of mineralization to inhibit cortical bone formation and osteon infilling by cells of the osteoblast lineage.(Figure 7) This allows a sufficient, but not
excessive, amount of cortical bone to be formed to fill in osteonal and other canals without compressing their contents. As such, trabecular thickness is maintained at an appropriate thickness.[66] In contrast, active osteoblasts within forming osteons are protected from inhibition by sclerostin by a layer of sclerostin-negative osteocytes, permitting bone formation to continue at specific sites (Figure 7) including the periosteum. As such, the osteocyte has a regulatory role in maintaining bone structure and strength through differential expression of sclerostin. The observations by Poole et al. shift the emphasis away from sclerostin as a possible regulator of preosteoblast proliferation (through interaction with bone morphogenetic proteins) toward a role in determining bone’s microarchitectural development through a very precise geographical expression and a selective local modulator of mature osteoblast function.[66] In a similar way, sclerostin expression in cementum may be a method for development of cemental microarchitecture.

As discussed earlier, osteocytes are thought to sense loading stimuli and regulate remodelling and bone turnover processes. The findings by Poole suggest that osteocytes, which have access to surface osteoblasts and bone lining cells through their dendritic connections, may provide the key inhibitory signal (sclerostin) that inactivates bone surfaces. Mechanical loading on non-alveolar bone was found to reduce sclerostin expression, thereby providing a mechanism by which bone formation upon mechanical strain is increased.[70]
Figure 7: Schematic diagram of the proposed regulation of a remodelling cortical osteon by osteocytic sclerostin expression.[66]

Sclerostin has been reported to selectively initiate the apoptosis of osteoblastic cells. Human mesenchymal cells treated with sclerostin displayed a marked increase in caspase activity and elevated levels of fragmented histone-associated DNA in these cells were detected.[73] This phenomenon could serve as one of the mechanisms by which sclerostin modulates the survival of osteoblasts and ultimately osteoblastic function. Sclerostin was also found to significantly decrease alkaline phosphatase (ALP) activity and the proliferation of differentiating human mesenchymal cells (hMSC).
Sclerostin-induced changes in ALP activity and the survival of hMSC cells were partially restored by BMP-6, suggesting the involvement of additional growth factors.[73]

Intermittent parathyroid hormone application has strong anabolic effects on bone. SOST and sclerostin expression was found to be reduced in vitro and in vivo in response to parathyroid hormone treatment, and SOST is a direct target gene of parathyroid hormone.[74,75] This suggests that downregulation of SOST expression may play a role in mediating parathyroid hormone action in bone.

Thus, one way for osteocytes to regulate bone remodelling would be by altering the secretion of sclerostin. Accordingly, it was shown that mechanical stimulation in vivo reduced the expression of sclerostin by osteocytes.[76] Also, it is possible that osteocyte death is a signal for bone formation because the level of sclerostin would decrease. Sevetson et al. demonstrated that the osteoblast differentiation factor Cbfa1/Runx2 increases SOST expression.[77] Up to now, there is some disagreement on the effect of parathyroid hormone (PTH) on SOST expression. One study showed that intermittent PTH treatment reduced SOST expression.[74] In contrast, Bellido et al. found that PTH given intermittently to mice did not alter the levels of SOST, but PTH given continuously did decrease SOST expression.[75]

Somewhat interestingly, Poole et al. found no reduction in the recruitment of osteocytes (as inferred from osteonal lacunar density) in the osteons of bone specimens removed at operation from 3 cases of sclerosteosis and 3 controls. This finding suggests that sclerostin primarily influences the later stage of bone formation rather than earlier events such as osteocyte recruitment.[66]
**Apoptosis**

Programmed cell death, termed apoptosis, is an essential cellular mechanism used by organisms to reduce cell number in order to attain precise control of organ development and function. In its simplicity it is a form of individual cell suicide. When cells become apoptotic they contract, lose attachment to their neighbours, undergo chromatin condensation and margination and cleavage of the DNA into inter-nucleosomal size fragments which retain their organelles. This is followed by packaging of the cell contents into membrane-bound vesicles (apoptotic bodies) that have specific membrane surface molecule signatures that aid their rapid removal from the system by phagocytic cells.[40,78] This rapid deletion allows apoptosis to occur with minimal or no inflammatory response in direct contrast to necrosis.[80] Apoptosis is not initiated simply to delete cells. The death process gives rise to a large number of signalling molecules that can dramatically modulate the behaviour of cells in the locality of the deletion and far beyond.[78]

i) *Pathways of apoptosis*

Two major signalling pathways control apoptosis.[79] One is initiated by death receptors, members of the TNF receptor family with an intracellular death domain, and the other is the mitochondrial pathway that involves mitochondrial release of cytochrome c.[40] Regardless of the source of activation, the final mechanistic pathway in apoptosis is the activation of a family of proteolytic enzymes called caspases.[80] These enzymes induce the morphological features of apoptosis by cleaving specific substrates.
Figure 8: Model of death ligand/death receptor and mitochondria-mediated classical apoptosis signalling, including Bcl-2 members. [40]

**ii) Osteoblast and osteocyte apoptosis**

Osteoblast apoptosis plays a critical role during embryonic limb development, skeletal maturation, bone turnover in the adult skeleton, and in bone repair and regeneration processes.[80] This balance of proliferation, cell differentiation, apoptosis and cell progression to the osteocyte phenotype affects the pool of osteoblasts available to maintain bone homeostasis. It is clearly evident from the literature that there are many factors which affect osteoblast survival but the mechanistic pathways by which cell survival is affected or compromised are not well understood.[73]

Osteoblast apoptosis explains the fact that 50–70% of the osteoblasts initially present at a remodelling site of human bone cannot be accounted for after counting the lining cells and osteocytes at that site.[9] Osteocyte apoptosis could be of importance to the origination and/or progression of the bone multicellular unit (BMU), the unit responsible for a localised area of bone remodelling. Osteocytes are the only cells in bone with the infrastructure to sense the need for remodelling at a specific time and place and, as such, it makes sense that they could drive this turnover. Furthermore, osteocytes are in direct physical contact with lining cells on the bone surface and targeting of osteoclast
precursors to a specific location on bone depends on a “homing” signal given by lining cells.[37] What seems evident now is that the life span of the cells that form the BMU (that is the osteoclasts, osteoblasts, and osteocytes) is an important regulator of bone mass, strength and turnover.[40]

iii) Regulation of osteoblast and osteocyte apoptosis

It is now appreciated that all major regulators of bone metabolism including bone morphogenetic proteins (BMPs), Wnts, other growth factors and cytokines, integrins, oestrogens, androgens, glucocorticoids, PTH and PTH-related protein (PTHrP), immobilization, and the oxidative stress associated with aging contribute to the regulation of osteoblast and osteocyte life span by modulating apoptosis. Moreover, osteocyte apoptosis has emerged as an important regulator of remodelling on the bone surface and a critical determinant of bone strength, independent of bone mass.[83] Osteoblast apoptosis has been shown in vitro to be stimulated by the activation of death receptors with TNF or CD95 ligand.[82,83] While osteoblasts and osteocytes express CD95 and its ligand, their in vivo role in the apoptosis of these cells is yet to be elucidated.[40]

iv) Osteocyte Apoptosis and its Role in Targeted Bone Removal

One of the more characteristic osteocyte behaviours is their apoptotic death.[8] The impact of a loss of cells from a functional syncitium has an impact not only on neighbouring cell types but also those in communication via the canalicular system. Frost found that with increasing age there was an increasing incidence of empty osteocyte lacunae in human bone.[28] Reduced osteocyte lacunal density has also been noted in osteoporotic bone [84], and individuals with fewer osteocyte lacunae in their bone have a reduced ability to repair accumulating microdamage.[85] It was initially thought that necrosis was responsible for osteocytic death under these conditions; however, it is now known that osteocyte apoptosis is a relatively common
event in both pathological and healthy human bone.[39] Noble and coworkers have hypothesised that apoptosis of osteocytes helps target the resorption process to specific areas following microdamage.[86] To support this hypothesis, large numbers of apoptotic osteocytes have been observed to surround resorption pits induced in response to fatigue microdamage.[38] Osteocytic apoptosis associated with regions of damage was shown to precede osteoclastic activity raising the possibility that it may be the apoptotic process that generates the targeting signal for osteoclastic bone destruction.[86] When conditional ablation of osteocytes has occurred, the bones demonstrated a dramatic increase in bone resorption, a decrease in bone formation, trabecular bone loss and loss of response to unloading.[52] The results of these data can be interpreted two ways. It may indicate osteocytes play a role in the inhibition of osteoclastic resorption or alternatively the increased resorption could be due to the death-induced signals from osteocytes.[8] Parfitt postulated that there are two types of bone remodelling in adults: targeted and background or untargeted remodelling. He has suggested that no more than 10–20% of remodelling in adult humans normally falls into the first category. [87] This has not been tested in alveolar bone, and as such it would be interesting to test the model with orthodontic tooth movement, which one would assume would be a form of targeted bone remodelling.
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SECTION 2
Statement of Purpose

Within bone biology there has been a paradigm shift away from the osteoblast, and toward the osteocyte being the chief cell responsible for governing local bone turnover. Sclerostin may play a key role in this regulation as it has been shown to be exclusively and differentially expressed by osteocytes in response to mechanical strain in non-alveolar bone. Interestingly its effect within alveolar bone, as far as we know, has yet to be investigated. Previous studies have looked at sclerostin expression in bone subject to either mechanical loading (bony apposition) or unloading (bony resorption) but due to the unique remodelling characteristics of alveolar bone, our experimental design will allow for both phenomena to be looked at within the one model.

1. Aims

Paper 1 – To examine changes that occur in the distribution of osteocytes positively stained for sclerostin in the alveolar bone of orthodontically stimulated rat teeth.

Paper 2 – To examine whether there is a linear association between the rate of orthodontic movement experienced by a tooth and the differential sclerostin expression across it.

2. Hypotheses

Paper 1 - The null hypothesis to be investigated is there is no statistically significant difference in the expression of sclerostin by osteocytes in the alveolar bone of a rat molar when subject to orthodontic tooth movement.

Paper 2 - The null hypothesis to be investigated is there is no statistically significant association between differential sclerostin expression around a tooth and the amount of orthodontic movement it achieves.
ARTICLE ONE

DIFFERENTIAL SCLEROSTIN EXPRESSION IN RAT ALVEOLAR BONE
EXPOSED TO MECHANICALLY INDUCED TOOTH MOVEMENT

FOR SUBMISSION TO ARCHIVES OF ORAL BIOLOGY
Abstract

Objective: Sclerostin, a negative regulator of osteoblast differentiation and function, has been shown to be exclusively and differentially expressed by osteocytes in response to mechanical strain. The aim of the study was to examine changes in this distribution within the alveolar bone of orthodontically stimulated rat teeth.

Design: Orthodontic 100cN NiTi pullcoil springs were lightly ligated to the right maxillary first molar and maxillary incisors of 12 Sprague-Dawley rats. The rats were divided into two groups with sacrifice at either 7 or 14 days. The left first maxillary molars from each animal served as internal controls. Six animals from a third, matched group did not have an orthodontic spring attached and were sacrificed to provide an additional untreated external control group. Serial horizontal frozen sections were obtained to allow direct comparison between untreated and experimental sides of the mesial first maxillary molar root. Immunohistochemical tissue labelling was performed, involving the avidin-biotin peroxidase technique and goat polyclonal anti-mouse sclerostin (SOST) as the primary antibody. Histomorphometric analysis of digital photomicrographs allowed comparison and quantification of immunolabelled osteocytes within alveolar bone.

Results: A comparison of the immunohistochemical SOST staining showed a statistically significant (p<0.05) increase in the difference in positively stained osteocytes between the mesio-palatal (compression zone) and disto-buccal (tension zone) alveolar bone surfaces in both the day 7 and day 14 treated groups when compared to external and internal controls.

Conclusion: Data suggest that sclerostin expression is altered in both the compression and tension zone of alveolar bone in rat molars subject to orthodontic tooth movement.
**Introduction**

Bone is subject to intermittent loads during locomotion and other forms of physical activity. Similarly, alveolar bone is also loaded intermittently during mastication, but is subject to a more continuous deformation or strain during orthodontic tooth movement.\[1\] In non-alveolar bone, the application of mechanically induced strain appears to trigger primarily an osteogenic response \[2\], without any apparent resorptive effect. Alveolar bone, however, fundamentally differs from the rest of the skeleton when subject to a continuous physical strain. It undergoes both resorption (osteoclastogenesis) and deposition (osteogenesis), the extent of which is dependent upon the magnitude, direction, and duration of the applied force. This balance favours bone deposition on the side of the alveolar wall undergoing tension and bone resorption on the side of the wall undergoing compression, allowing the tooth to “move” along the force vector being applied to it. \[3\]

This mechanotransduction, the conversion of a physical strain into a biological response, involves a complex series of interactions between disparate tissue types, both mineralized and non-mineralized, and their associated neurovascular elements. For tooth movement to be perpetuated these tissues need to be remodelled, a task performed by cells of the alveolar bone (osteoblasts, osteoclasts, and osteocytes), periodontal ligament (gingival fibroblasts), blood vessels (endothelial cells), and neural tissues (dendritic and neural cells). Each of these tissues has an individual response pattern as far as the mechanosensing, transduction, and response mechanisms are concerned.\[1,\]4\]

The osteocyte represents a terminally differentiated non-proliferative cell of the osteoblast lineage. As osteoblasts secrete bone matrix (osteoid), some become embedded in lacunae and are, from that point, referred to as osteocytes. As such, the osteocyte is considered non-migratory because it is entrenched within this bone matrix.
Osteocytes extend numerous small cytoplasmic processes within canaliculi, forming a network that permeates the entire bone matrix.[5,6] Each osteocyte communicates with its neighbours and with the surface lining cells of bone by means of gap junctions, theoretically allowing for an extensive syncitial network through bone.[6] It is the density, rigid housing, extensive distribution and interconnectivity of this cell population that has led bone biologists to believe that these cells are ideally positioned to serve as the main mechanosensors within bone and to be the chief orchestrators of the response to this mechanical strain through the effector cells responsible for skeletal resorption and formation.[7] The discovery of sclerostin, a mechanically modulated osteocyte-specific factor which has shown a predilection for reaching effector cell populations (osteoblasts), has provided a long sought-after molecular basis for osteocytic reception, initiation, and spatial control of mechanotransduction.

Among bone cells, sclerostin is exclusively expressed by the osteocyte, in which high levels have been detected in both their lacunae and canalicular system.[8,9] Sclerostin, the protein product of the Sost gene [10], is a potent inhibitor of bone formation.[5,11] It is believed that sclerostin inhibits bone formation through numerous pathways, including negative regulation of osteoblast differentiation and proliferation, as well as suppression of mineralisation by osteoblastic cells and the stimulation of osteoblast apoptosis.[8] In its role as a key inhibitor, sclerostin helps determine the normal extent of bone formation and, consequently, protects against the deleterious effects of uncontrolled bone growth.[12] Its effect is highlighted by two rare high bone mass diseases, Sclerosteosis and Van Buchem disease, both of which have been linked to inactivating mutations in the SOST gene.[11,13] Previous studies have investigated the regulation of Sost/Sclerostin in non-alveolar bone by the mechanical environment using a model for either enhanced loading or reduced loading, but not simultaneously.[10] By looking at alveolar bone, a tissue that undergoes separate and distinct areas of bony apposition and resorption in response to mechanical loading, a model is provided in
which the relationship these phenomena have with Sost/Sclerostin expression can be studied concurrently. The null hypothesis to be investigated is that there is no differential expression of sclerostin in the alveolar bone of the rat molar when subject to orthodontic tooth movement. We hypothesize that the side of the alveolar bone undergoing resorption (compression side) will have an increase in sclerostin expression and conversely the side undergoing deposition (tension side) will have a reduction in Sost expression in response to a continuous strain.

**Materials and Methods**

Approval for the study was obtained through the University of Adelaide Animal Ethics Committee number M/023/2006 and the experimental procedures and care of animals was in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

**i) Animals**

Eighteen, eight week old Sprague-Dawley rats were used for this study and were contained at the Animal House facility of the Medical School of the University of Adelaide. The rats weighed between 250 - 350g and were fed commercially manufactured standard pellets (Parastoc Feed, Ridley AgriProducts, Murray Bridge, Australia) and water, ad libitum.

**ii) Appliances and Animal Manipulations**

All rats were anaesthetised via a subcutaneous injection containing a combination of Ketamine (100mg/ml) (Ketamil Injection, Troy Laboratories, Smithfield, Australia) and Xylazine (Xylazil, 20mg/ml) at a dosage of 2ml/kg. The two drugs were mixed at the ratio 2:1 (Ketamine:Xylazine) and diluted 1:1 with sterile water for injection. In twelve animals, a 100cN superelastic nickel titanium closing spring (Sentalloy, GAC, New
York, USA) was bonded to the right maxillary first molar and to the maxillary incisors using 0.010” stainless steel ligatures and light cured bonding material (Z100 composite resin, 3M ESPE, St. Paul, USA) (Figure 1). This was to impart a mesially directed heavy force to the right maxillary first molar. Another six animals, which were not subjected to orthodontic movement, were used to establish age-matched, untreated external controls.

Figure 1: The closed coil spring was placed between the maxillary rat incisor and first molar imparting a mesially directed force on the molar, and an equal and opposite distally directed force on the incisor (diagram modified from Ren et al. 2004).[14]

Animals were provided with identification numbers and sacrificed at day 0, 7 or 14 as shown in Table 1. A power study was undertaken where, for a sample of 18, there was an 80% power to detect a moderate effect (effect size ≥ 0.75) when comparing the two sides, assuming a 2-tailed p-value of 0.05 to indicate statistical significance.

<table>
<thead>
<tr>
<th>DAY</th>
<th>0</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 1: Sample sizes. Apart from Day 0, an orthodontic spring was attached to the maxillary right first molar of all animals.
iii) Harvesting of tissue

The animals were sacrificed via cardiac left ventricular perfusion with 4% paraformaldehyde, after an intraperitoneal administration of chloral hydrate 5g/100ml H₂O (5mL). Of the twelve animals that had spring placement performed at day 0, six animals were perfused at 7 days and six animals were perfused at 14 days. The six untreated animals were also sacrificed, using the same perfusion protocol involving 200ml 0.1% sodium nitrite, and a 400ml solution of 50:50 para-formaldehyde and p-benzoquinone under positive pressure. Each animal had the maxilla removed and stored in the 50:50 fixative solution for two hours, followed by 4% EDTA decalcifying solution pH 7.4 for 8 weeks. The decalcified tissue was impregnated with an embedding medium (OCT, Sakura Finetek, Torance, CA, USA) for cryosectioning.

iv) Section preparation

Horizontal sections 25μm thick were obtained using a Leitz 1720 cryostat at -20°C. The maxillae were orientated so that the crowns of teeth were uppermost. Sections were collected sequentially from the furcation in an apical direction to the root apex (Figure 2). Such sections allowed for direct comparison between untreated and experimental sides of the alveolar bone circumference.

![Figure 2: Schematic representation of the level at which the sections were cut through maxillary first molar (M₁) and maxillary second molar (M₂) roots below the furcation.](image)
Serial sections were placed consecutively over 12 slides, so that each slide contained sections representing different levels of the alveolar bone for comparison (Figure 3). Positive controls were provided via separate slides of rat tibia included in the staining runs.

Figure 3: Arrangement of specimen sections on slides.

v) Immunohistochemistry

The even-numbered slides from each animal were used for immunohistochemical labelling of sclerostin. Slides from the different groups were labeled in the same solutions simultaneously with positive control tissue (rat tibia) known to have SOST-immunoreactive tissue. The avidin-biotin peroxidase complex technique was used involving goat anti-mouse-SOST polyclonal antibody (R & D Systems, MN, USA) as the primary antibody. The anti-mouse-SOST was diluted from lyophilised form (200μg/ml) to 1:75 in PBS. The optimal primary antibody concentration used was found through a pilot study using similar tissue to the area studied. Secondary antibody labelling and detection were accomplished using the Vectastain Universal Quick-Kit (Vector Lab. Inc.) with 3-amino-9-ethylcarbazole (AEC) as the chromogen. For a negative control, primary antibody was omitted (R & D Systems, MN, USA).

vi) Histoquantification

The immunolabelled sections were photographed at 20x magnification (Leica Q550 IW and Leica QWin image analysis software) and stored as JPEG files of 300 – 350
kilobytes. For image quantification, a protocol developed by Robling et al. was used. The number of sclerostin positive osteocytes, defined as those osteocyte cell bodies exhibiting red (AEC) staining (Figure 4), and the number of sclerostin-negative (Sclr-) osteocytes, defined as those osteocyte cell bodies exhibiting blue (haematoxylin) staining, were counted on each section. To measure a depth effect, two areas of interest within each side of interest were measured. The percentage of sclerostin-positive cells (Sclr+) was calculated as the number of Sclr+ cells divided by the total number of cells (Sclr+ plus Sclr-) in four areas of interest.

**Figure 4:** The *middle panel* shows a transverse section of the mesial root immunolabelled for sclerostin at a magnification of 5x. At this power individual osteocytes cannot be identified. The orange arrow represents the direction of orthodontic tooth movement, where the mesio-palatal surface in this case represents the area of “compression” and the disto-palatal surface the area of “tension”. At this magnification a representative section of alveolar bone from the areas of tension and compression are chosen. The *upper* and *lower panels* are photomicrographs taken at a magnification of 20x. The *upper panel* represents the mesio-palatal region of interest (area of “compression”). Here sclerostin positive osteocytes can be visualized by the red staining of their cell bodies and/or cell processes within the canaliculae. The *lower panel* represents the
disto-buccal surface (area of “tension”). The upper and lower panels have been divided into two region of interest, R1 and R2. R1 was compared to R2 to measure a potential depth effect, with R1 being the region of alveolar bone closest to the molar. A percentage difference between positively stained osteocytes on mesio-palatal (MP) and disto-buccal (DB) surfaces was calculated and compared between the control groups (internal and external) and treated groups (7 days and 14 days).

vii) Statistics and Error Study

All statistical analysis was performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). An error study involved re-counting the total number of osteocytes in 40 sections that were picked at random (10 from each region), and re-measured. Systematic error was assessed with the paired $t$ test, and random error was assessed with the coefficient of reliability. Definitive data analysis was carried out with a linear mixed effects model and a Type 3 test of fixed effects.
Results

i) Error Study

Recounting of the total number of osteocytes in each of the four regions showed no statistically significant difference between the two sets of replicates. From the data in Table 2, it can be seen that the confidence interval includes zero in all cases and the $p$ values are all acceptable. On examination of the values for the coefficient of reliability in Table 2, there was no statistically significant random error in the study. This was also confirmed by the fact that the means and standard deviations of the differences were small.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean Difference 1-2</th>
<th>SD</th>
<th>95% CI</th>
<th>$P$</th>
<th>$\pi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP count (R1)</td>
<td>1.3</td>
<td>3.1</td>
<td>0.13</td>
<td>0.39</td>
<td>1.0</td>
</tr>
<tr>
<td>MP count (R2)</td>
<td>1.1</td>
<td>2.3</td>
<td>0.04</td>
<td>0.10</td>
<td>1.0</td>
</tr>
<tr>
<td>DB count (R1)</td>
<td>2.2</td>
<td>4.2</td>
<td>0.32</td>
<td>0.84</td>
<td>1.0</td>
</tr>
<tr>
<td>DB count (R2)</td>
<td>1.8</td>
<td>3.7</td>
<td>0.11</td>
<td>0.24</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 2: Error study for total osteocyte count ($n = 40$).

ii) Comparison of region 1 and region 2 within treated group

To test for a difference in the proportion of positive osteocytes between region 1 and region 2, a linear mixed effects model was fitted to the data. In the model, region (1 vs. 2) was entered as a fixed effect (predictor variable), while rat ID was entered as a random effect to account for the dependence between repeated measurements from the same rat. Table 3 shows the mean proportion of positive osteocytes for each region, as estimated by the linear mixed effects model.
### Table 3: Adjusted means of proportion of positively stained osteocytes.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Region</th>
<th>Mean</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region</td>
<td>Region 1</td>
<td>0.43</td>
<td>0.015</td>
</tr>
<tr>
<td>Region</td>
<td>Region 2</td>
<td>0.43</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Table 4 shows the significance of predictor variables in the linear mixed effects model. In this case there was no evidence for a difference between the two regions (p = 0.94).

### Table 4: Type 3 Tests of Fixed Effects.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>region</td>
<td>1</td>
<td>83</td>
<td>0.01</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Overall conclusion

Within the groups there was no evidence for a difference in the mean proportion of positive osteocytes between region 1 and region 2 (mean 0.43 vs. 0.43, p = 0.94). Subsequently, for all further analyses, region was ignored.

**iii) Comparison of day 7 and day 14 right side of treated group**

For each RHS tooth within the treated group, the difference in the proportion of positive osteocytes between the MP (pressure zone) and DB (tension zone) locations was calculated. To test whether this outcome (the difference) was shared between day 7 and day 14 treated rats, a linear mixed effects model was fitted to the data. Day (7 vs 14) was entered as a fixed effect in the model, while rat ID was entered as a random effect (Table 5).
### Adjusted Means

<table>
<thead>
<tr>
<th>Effect</th>
<th>day</th>
<th>Mean</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>day</td>
<td>7</td>
<td>0.23</td>
<td>0.027</td>
</tr>
<tr>
<td>day</td>
<td>14</td>
<td>0.16</td>
<td>0.027</td>
</tr>
</tbody>
</table>

*Table 5*: Adjusted means of the treated group (right side) at day 7 and day 14.

On day 7 the mean difference in the proportion of positive osteocytes between the pressure and tension locations was 0.2253, whereas on day 14 the mean difference was 0.1601.

There was no significant difference in the outcome between the two groups (p = 0.11) (Table 6).

### Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>day</td>
<td>1</td>
<td>12</td>
<td>2.92</td>
<td>0.1133</td>
</tr>
</tbody>
</table>

*Table 6*: Type 3 tests of fixed effects of the treated group (right side) at day 7 and day 14.

**Overall conclusion**

In the right side of the treated group, there was no evidence for a difference in the outcome (MP-DB proportion of positive osteocytes) between the day 7 and day 14 treated rats (mean 0.2253 vs. 0.1601, p = 0.11). Subsequently for all further analyses the day 7 and day 14 treated rats were pooled together into a single 'treated' group.
iv) Comparison of right side MP and DB differences between treated and control groups

For each right side tooth, the difference in the proportion of positive osteocytes between the pressure (MP) and tension (DB) locations was calculated. To test whether this outcome differed between control and treated rats, a linear mixed effects model was fitted to the data. Group (control vs treated) was entered as a fixed effect in the model, while rat ID was entered as a random effect. There was a small difference between the MP and DB locations in the control rats (mean = 0.0008), whereas for the treated rats the difference was much larger (mean = 0.1927) (Tables 7 & 8).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Group</th>
<th>Mean</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>group</td>
<td>Control</td>
<td>0.00</td>
<td>0.03</td>
</tr>
<tr>
<td>group</td>
<td>Treatment</td>
<td>0.19</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Table 7:** Adjusted means of difference in positively stained osteocytes between the MP and DB surfaces of the treated and control groups

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>group</td>
<td>1</td>
<td>18</td>
<td>33.88</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

**Table 8:** Type 3 test of fixed effects in positively stained osteocytes between the MP and DB surfaces of the treated versus control groups
Overall conclusion

There was a significant difference between control and treated rats in the outcome (MP-DB proportion of positively stained osteocytes) (mean 0.0008 vs. 0.1927, p < 0.0001).

v) Comparison of MP and DB differences between left and right sides in treated group

For each tooth, the difference in the proportion of positive osteocytes between the pressure (MP) and tension (DB) locations was calculated. To test whether this outcome differed between left and right sides within the treated group, a linear mixed effects model was fitted to the data. Side (left vs. right) was entered as a fixed effect in the model, while rat ID was entered as a random effect (Tables 9 & 10).

<table>
<thead>
<tr>
<th>Adjusted Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>Side</td>
</tr>
<tr>
<td>Side</td>
</tr>
</tbody>
</table>

Table 9: Adjusted means of difference in positively stained osteocytes between in MP and DB surfaces between left and right sides in the treated group.

<table>
<thead>
<tr>
<th>Type 3 Tests of Fixed Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>side</td>
</tr>
</tbody>
</table>

Table 10: Type 3 test of fixed effect in positively stained osteocytes between in MP and DB surfaces between left and right sides in the treated group.
**Overall conclusion**

There was a significant difference between left and right sides in treated rats in the outcome (MP-DB proportion positive osteoctyes) (mean -0.0316 vs. 0.1927, p<0.0001).

**Discussion**

The main objective in this study was to determine if mechanical stimulation altered sclerostin regulation within the alveolar bone of rats. Our results indicate that sclerostin levels are tightly regulated by mechanical strain evidenced by a differential SOST expression across the “pressure” (mesio-palatal) and “tension” (disto-buccal) sides of alveolar bone of an orthodontically treated sample when compared to both a non-orthodontically strained and an internal control group.

No statistically significant regional differences were found within the disto-buccal or mesio-palatal surfaces of either the control or tested groups. This was somewhat unexpected and could have resulted from the force levels in the closed coil spring being too high. Heavy forces were used to guarantee tooth movement, but the amplitude could have exceeded the discriminatory mechanosensory threshold of the osteocytes measured. Also, if the area we measured was too small, the force may have been sensed over a larger distribution of osteocytes. This area of measurement was somewhat curtailed by local anatomy, where often the mesial and mesio-buccal root of the first molar lie in close proximity to each other. No statistical differences were found between corresponding sites in the day 7 and day 14 test group. This was expected given the fact that we were using a closed coil spring that, due to its composition (Nickel-Titanium), exerted a continuous force even though the length between which it was activated was reducing as the teeth came towards each other. A difference may have been detected had the spring been made from a material that had
a steeper force-deflection curve (eg stainless steel) or had the spring been removed after a 7 day period.

While the mechanistic pathway of sclerostin has yet to be fully elucidated, several pathways have been suggested in the literature. Wnt signalling has been shown to be crucial in the mechanotransduction cascade, as indicated by the observation that the Lrp5-deficient mouse skeleton is unable to respond anabolically to mechanical stimulation.[15] Several experiments have demonstrated enhanced Wnt signalling in bone cells as a result of mechanical stimulation. For example, stretched osteoblasts harvested from transgenic mice exhibited activation of canonical Wnt signalling after stretching.[16] Robling and co-workers have shown the inhibitory effects of sclerostin on Lrp function and bone formation, implicating it as a mechanically suppressed signal, providing an attractive regulatory mechanism that permits enhanced Wnt signalling upon mechanical stimulation.[10]

The mineralisation of bone, that is the formation of hydroxyapatite, is a highly regulated process. The details of this regulation are also not fully understood but sclerostin has recently been proposed as a potential source of control of mineralisation within mature bone.[17] The TNF-family member, TWEAK, inhibits mineralisation by human osteoblasts. This action was accompanied by TWEAK inducing the formation of both SOST/sclerostin mRNA and protein.[18] When human osteoblasts had recombinant exogenous sclerostin applied to them, it was found that mineral deposition was highly sensitive to sclerostin, with concentrations as low as 10 ng/ml inhibiting cell layer-associated Ca2+. The inhibition of mineralisation by sclerostin was associated with reduced expression by the osteoblasts of mRNA encoding the bone formation markers osteocalcin and collagen I, and increased expression of the pre-osteocyte marker E11. This implies that sclerostin acts in part by inhibiting the progression of cells through to the mature osteocyte stage.[17] Consistently, the same study also showed that DMP1
expression was also reduced in response to sclerostin treatment.[17] These results indicate that sclerostin inhibits the differentiation of human osteoblasts and/or their ability to regulate mineral deposition.

Our study showed that osteocytes on either side of alveolar bone under mechanical strain had a different response to alteration in sclerostin expression, facilitating tooth movement in the direction of the force vector applied to it. It would be interesting to know how this discriminatory mechanotransduction takes place, and whether it lies within the osteocyte, or is downstream to signals from the periodontal ligament. Another issue that remains unclear is the identity of the upstream factors that control sclerostin expression and whether sclerostin can be modulated by known classical early mediators of mechanotransduction (e.g. ATP, Ca, PGE2, and NO) or if sclerostin is controlled by other novel mechanisms.

The osteocyte-specific expression profile of sclerostin provides a long sought-after molecular mechanism by which osteocytes per se can relay mechanical information biochemically to the effector cell populations (chiefly osteoblasts and osteoclasts). From the results of this study, we hypothesise that when significant local strains are sensed by the osteocyte cell population on the side of tension, sclerostin levels are decreased, and the local osteoblast cell populations are released from their suppressed Wnt signaling state, ultimately resulting in new local bone formation in the region. Conversely, when significant local strains are sensed by the osteocyte cell population on the side of compression, sclerostin levels are increased with continued Wnt signalling suppression, leading to a prevention of local bone formation.
Conclusion

Sclerostin levels were significantly altered in the alveolar bone around rat molars subject to orthodontic tooth movement. These data highlight the role of the osteocyte in mechanically induced bone formation and suppression and provide the first known evidence for osteocyte-specific control of mechanotransduction in alveolar bone.

Furthermore, sclerostin presents a novel mechanism for modulating alveolar bone turnover and mineralisation in response to mechanical strain. Pharmacologic modulation of sclerostin signalling emerges as an obvious potential therapy for improved treatment efficiency in orthodontic treatment.
Acknowledgments

Funding: The Australian Society of Orthodontists Foundation for Research and Education provided partial funding for the project.

Conflict of interest: There is no foreseeable conflict of interest.

Ethical approval: The ethics approval was obtained from the University of Adelaide Animal Ethics Committee (M/023/2006).

Statistician: Mr Thomas Sullivan, Statistician, Discipline of Public Health, University of Adelaide.

Bone biologist: Dr Brendon Noble, Scottish Mechanotransduction Consortium, University of Edinburgh Medical School, Edinburgh, UK.
References


ARTICLE TWO

RATE OF ORTHODONTIC TOOTH MOVEMENT AND DIFFERENTIAL SCLEROSTIN EXPRESSION IN THE RAT

FOR SUBMISSION TO THE ARCHIVES OF ORAL BIOLOGY
Abstract

Objective: Osteocytic changes involving the release of sclerostin have been shown to occur in the alveolar bone of teeth subjected to orthodontic tooth movement. The aim of this study was to examine whether differential sclerostin expression around a tooth has a linear association with measured orthodontic tooth movement in the rat.

Design: Orthodontic NiTi pullcoil springs(100cN) were ligated to the right maxillary first molar and maxillary incisors of 12 eight week old Sprague-Dawley rats. The rats were divided into two groups; one sacrificed at 7 and the other at 14 days. Serial frozen sections were obtained and immunohistochemical tissue labelling was performed, involving the avidin-biotin peroxidase technique and goat polyclonal anti-mouse sclerostin as the primary antibody. Histomorphometric analysis of digital photomicrographs allowed quantification and comparison of immunolabelled osteocytes resident in the mesio-palatal and disto-buccal alveolar bone of the maxillary first molar mesial root. This differential was expressed as a percentage. The amount of tooth movement between the incisors and first molars was measured using an indirect method and compared with the differential sclerostin staining percentage of each animal. A linear regression analysis was performed using the paired sets of data with GraphPad Prism 5 software.

Results: The analysis showed there was no significant association between differential sclerostin staining and the amount of tooth movement measured at day 7 (p = 0.16) and a significant association at day 14 (p = 0.03).

Conclusion: Tooth movement and sclerostin expression are affected by multiple variables; however, a linear association between osteocyte-driven sclerostin expression and the rate of tooth movement may be present once hyalinised tissue has been eliminated.
Introduction

Alveolar bone is loaded intermittently during mastication, but is subject to a more continuous deformation or strain during orthodontic tooth movement.[1] Alveolar bone significantly differs from the rest of the skeleton in its remodelling response when placed under a mechanical load. The balance favours bone deposition on the side of the alveolar wall undergoing tension and bone resorption on the side of the wall undergoing compression, allowing the tooth to “move” along the force vector being applied to it. This complex cascade of events involves a mechanical signal being converted into a biological response. Recent literature has focused on the osteocyte as the chief mechaonosensor within bone.

Sclerostin, a protein product encoded by the SOST gene, is a known negative regulator of osteoblast differentiation and function, and acts as an inhibitor to bone formation.[2-4] It has also been hypothesised that sclerostin released by the mature osteocyte may have a role in regulating bone mineralisation surrounding the “osteoid osteocyte”. In its role as a key inhibitor, sclerostin helps determine the normal extent of bone formation and, consequently, protects against the deleterious effects of uncontrolled bone growth.[5] Its effect is highlighted by two rare high bone mass diseases, Sclerosteosis and Van Buchem disease, both of which have been linked to inactivating mutations in the SOST gene.[3,6] The administration of an anti-sclerostin antibody has been shown to increase bone formation and restore the bone loss upon ovariectomy in rodents.[7] It has been suggested that the inhibition of sclerostin (through sclerostin neutralizing antibodies) may, therefore, be an attractive strategy for conditions in which bone loss is a significant component, such as postmenopausal and senile osteoporosis.[5] Conversely, transgenic mice overexpressing sclerostin have been shown to exhibit low bone mass.[8]
The results of a previous study (Article 1) indicated that teeth subjected to orthodontic tooth movement showed an increase in the difference between positively stained osteocytes between mesio-palatal and disto-buccal surfaces of alveolar bone around the rat first molar. The present study aims to evaluate a potential association between the amount of this differential and the amount of orthodontic tooth movement achieved over a given period of time. The null hypothesis to be investigated is there is no association between differential sclerostin expression and the amount of orthodontic tooth movement.

Materials and methods

The ethics approval for this study was provided by the University of Adelaide Animal Ethics Committee number M/023/2006 and the experimental procedures and care of animals was in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

i) Animals, Appliances and Animal Manipulations

This study used twelve, eight week old Sprague-Dawley rats which were contained at the Animal House facility of the Medical School of the University of Adelaide.

Anaesthesia for the rats was administered via a subcutaneous injection containing a combination of Ketamine (100mg/ml) (Ketamil Injection, Troy Laboratories, Smithfield, Australia) and Xylazine (Xylazil, 20mg/ml) at a dosage of 2ml/kg. Twelve animals had a 100cN superelastic nickel titanium closing spring (Sentalloy, GAC, New York, USA) ligated to the right maxillary first molar and to the maxillary incisors using 0.010” stainless steel ligatures and a light cured bonding material (Z100 composite resin, 3M ESPE, St. Paul, USA). This imparted a mesially directed force to the right maxillary first molar, using a protocol developed by Noxon et al. (9).
ii) Tooth movement measurements

A protocol developed by Ho [10] for molar-to-incisor distance measurement was used on the anaesthetised animal via direct and indirect means (see Appendix 2). For the direct method, a digital calliper was used to take three sets of readings from the labial surface of the incisor to the mesial surface of the first molar and a mean value was calculated from the three readings. Readings were performed by the same operator, and recorded at day 0, 7 and 14. In the present study, the measurements at day 0 to day 7 and day 0 to 14 were used to form paired data, along with the differential sclerostin staining percentages for each animal (animals were sacrificed at either day 7 or day 14 for immunological staining). The indirect method involved a polyvinyl siloxane impression (Imprint 3, 3M ESPE) of the maxillary arch using a special tray for the anaesthetised animal. Images of the impressions were taken with a MZ16FA stereo microscope and were calibrated using Image analySIS software. Computer software was used to measure the distance between three constructed centroid points representing the incisors and the first molar on the digitised images (Appendix 2).

iii) Harvesting of tissue

The animals were sacrificed via cardiac left ventricular perfusion with fixative solutions, after an intraperitoneal administration of chloral hydrate 5g/100ml H₂O (5ml). The perfusion protocol involving 200ml 0.1% sodium nitrite and a 400ml solution of 50:50 4% para-formaldehyde and p-benzoquinone injected under positive pressure. Of the twelve animals that had spring placement and injection performed at day 0, six animals were perfused at 7 days and six animals were perfused at 14 days. Each animal had the maxilla removed and stored in the 50:50 fixative solution for two hours, followed by 4% EDTA decalcifying solution pH 7.4 for 8 weeks. The decalcified tissue was placed within an embedding medium (OCT, Sakura Finetek, Torance, CA, USA) for cryosectioning.
iv) Section preparation

Horizontal sections 25μm thick were obtained using a Leitz 1720 cryostat at -20°C. The maxillae were orientated so that the crowns of teeth were uppermost. Sections were collected sequentially from the furcation in an apical direction to the root apex (Figure 1).

![Figure 1: Schematic representation of the level at which the section was cut through maxillary first molar (M1) and maxillary second molar (M2) roots below the furcation.](image)

Serial sections were placed consecutively over 12 slides, so that each slide contained sections representing different levels of the alveolar bone for comparison (Figure 2). Positive controls were provided via separate slides of rat tibia included in the staining runs.

![Figure 2: Arrangement of specimen sections on slides](image)
v) Immunohistochemistry

For immunohistochemical labelling of sclerostin, the avidin-biotin peroxidase complex technique was used involving goat anti-mouse-SOST polyclonal antibody (R & D Systems, MN, USA) as the primary antibody. The anti-mouse-SOST was diluted from lyophilised form (200μg/ml) to 1:75 in PBS. The optimal primary antibody concentration was calculated through a pilot study using similar tissue to the area studied. Secondary antibody labeling and detection was accomplished using the Vectastain Universal Quick-Kit (Vector Lab. Inc.) with 3-amino-9-ethylcarbazole (AEC) as the chromogen. Slides from the different groups were labeled in the same solutions along with both positive and negative control tissue (rat tibia) known to have SOST-immunoreactive tissue. For a negative control, primary antibody was omitted (R & D Systems, MN, USA).

vi) Histoquantification

A photomicroscope was used to capture immunolabeled sections. For image quantification, the number of sclerostin positive osteocytes, defined as those osteocyte cell bodies exhibiting red (AEC) staining (Figure 3), and the number of sclerostin-negative (Sclr-) osteocytes, defined as those osteocyte cell bodies exhibiting blue (haematoxylin) staining, were counted on the mesio-palatal (MP) and the disto-buccal (DB) section of alveolar bone surrounding the mesial root of the first maxillary molar (Figure 4). These were areas of interest because the force vector was directed mesio-palatally and, as such, the MP surface was expected to be a compression site (undergo bone resorption) and the DB surface a tension site (undergo bony apposition). The percentage of sclerostin-positive cells (Sclr+) was calculated as the number of Sclr+ cells divided by the total number of cells (Sclr+ plus Sclr−).
Figure 3: Histomicrograph of sclerostin positive osteocytes (Sclr+) exhibiting red (AEC) staining and sclerostin-negative (Sclr-) osteocytes exhibited blue (haematoxylin) staining. Note the diffuse red staining surrounding the cell bodies of sclerostin positive osteocytes possibly indicating the release of sclerostin within the dendritic processes of the osteocyte in their bony canaliculae.

The load-induced change in the samples was calculated for each animal as the percentage of Sclr+ cells in the MP (compression) section minus the percentage of Sclr+ cells in the DB (tension) section. Mean values were calculated for each animal and, along with the corresponding tooth movement measurements, the values were used to form the paired data for a linear regression analysis using Graphpad Prism version 5.00 software (Graphpad, Ca, USA).
Figure 4: The middle panel shows a transverse section of the mesial root immunolabelled for sclerostin at a magnification of 5x. At this power, individual osteocytes can not be identified. The orange arrow represents the direction of orthodontic tooth movement, where the mesio-palatal surface in this case represents the area of “compression” and the disto-palatal surface the area of “tension”. At this power, representative sections of alveolar bone from the areas of tension and compression were chosen. The upper panel represents the mesio-palatal region of interest. The lower panel represents the disto-buccal surface. A percentage difference between positively stained osteocytes in mesio-palatal (MP) and disto-buccal (DB) surfaces was calculated and compared between treated groups (7 days and 14 days).
Results

The tooth movement results are shown in Appendix 3. Measurement calculations were performed using SAS Version 9.1 software (SAS Institute Inc., Cary, NC, USA), and a linear regression analysis of scatter plot diagrams using the paired data was performed (Tables 1 and 2, and Figures 5-8).

<table>
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<tr>
<th>Animal</th>
<th>% of Scl +ve osteocytes (MP)</th>
<th>% of Scl +ve osteocytes (DB)</th>
<th>Differential % of Scl +ve osteocytes (MP-DB)</th>
<th>Distance day 0 to day 7 Indirect (mm)</th>
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Table 1: Tooth movement measured between the right maxillary first molars and incisors over day 0 – day 7. Percentage of osteocytes positively stained for sclerostin was recorded for the mesial root of the treated maxillary first molars.

Figure 5: Scatter plot graph representing the paired data between the differential +ve Sclr percentage and the indirectly measured distance (incisor to 1st molar) from day 0 to day 7. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.4284. Sy.x = 0. 4688. The slope is not significantly non-zero (P = 0.1584).
Table 2: Tooth movement measured between the right maxillary first molars and incisors over day 0 – day 14. Percentage of osteocytes positively stained for sclerostin was recorded for the mesial root of the treated maxillary first molars.

<table>
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<tr>
<th>Animal</th>
<th>% of Scl +ve osteocytes (MP)</th>
<th>% of Scl +ve osteocytes (DB)</th>
<th>Differential % of Scl +ve osteocytes (MP-DB)</th>
<th>Distance day 0 to day 14 Indirect (mm)</th>
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</table>

Figure 6: Scatter plot graph representing the paired data between the differential +ve Sclr percentage and the indirectly measured distance (incisor to 1st molar) from day 0 to day 14. The solid line shows the line of best fit. Dotted lines show 95% confidence levels. R square value = 0.7064. Sy.x = 0. 2508. The slope is considered significant (P = 0.0361).
Discussion

Adult Sprague-Dawley rats were used in this study because of their availability, ease of handling, cost and their biological and structural similarities to human periodontium and alveolar bone. Male rats were used to negate any hormonal-related variables.[11] The rat model also allows for better control of genetic variability, easier monitoring of animal number and conditions (diet and environment), increased sample size and more manageable size of the region to be studied (the maxillary molar). Sprague Dawley rats are an outbred strain of rat and ideally an inbred strain of animal would have allowed for isogenicity, but not necessarily phenotypic uniformity due to individual variations in the environment of each animal. This heterogeneticity may account for an increased individual variability in the response to the treatment effect.[12]

The present study incorporated tissue obtained from a previous study [10], after which the maxillary tissue of twelve treated animals were stored in EDTA medium for up to 18 months in a dark store room at room temperature. Although every effort was made to preserve this tissue in its optimal state, the lengthy storage period may have enabled some protein denaturation.

i) Application of orthodontic force to rat molars

Various methods have been described for application of an orthodontic force to a rat molar, each with varying degrees of success. Waldo and Rothblatt used an orthodontic elastic between rat molars to stimulate orthodontic movement.[13] Orthodontic modules (alastics) have also been used, and are often made from polyurethane, which does not show ideal elastic characteristics. Time, temperature, salivary pH and water absorption have all been found to result in force loss, permanent deformation and strength loss of elastomerics modules.[14] Using this tooth movement model, O’Hara et al. noted that only 66% (26 out of 42) of the animals were found to have the elastic modules retained at the time of sacrifice, where the longer the module was left between the teeth, the
greater the risk of its loss.[15] The study also showed there was no consistency in the
direction of tooth movement and level of response. O’Hara’s tooth movement model
also involved a force being delivered to the tooth via decompression (as opposed to
contraction) of an elastomeric, causing tooth movement to be translatory, tipping or
rotational depending upon the depth of elastomeric module insertion, tightness of
interproximal contact, and duration of application. Unfortunately, there has been
inadequate evidence in the literature regarding the force decay rate, the amount of
force decay and the dimensional changes of elastomeric modules during
compression.[14]

The method used in this experimental sample was based on a modified version of the
Brudvig and Rygh protocol, which advocated the use of a closed coil spring ligated
between an eyelet on an incisor band and the upper first molar.[16-21] The present
study used a continuous force level of 100 cN generated by a NiTi coil spring, although
forces as low as 10cN (which if equated by surface area would be equivalent to a
170cN force exerted on a human molar) have been advocated by Ren et al.[14] While it
could be considered that the force levels delivered in our study were too high, we
wanted to maximise the likelihood of orthodontic tooth movement. A steel ligature was
used to tie in the rat molar, which could have caused localised trauma and a
subsequent inflammatory response. Tooth movement is also based on a modified
inflammatory response and thus this overlap could have confounded our results.
Bonding the spring to the rat molar could alleviate this issue, but had the added risk of
spring loss during mastication. A mesially directed force on the rat maxillary molar was
used in order to oppose the normal physiological conditions these teeth naturally
encounter as they have been shown to distally drift at a rate of 7.7μm/day in adult male
Sprague Dawley rats.[12]
ii) Sclerostin-immunoreactivity

The linear regression analysis showed a statistically significant association between the difference in osteocytes positively stained for sclerostin on either side of the MB root of the maxillary first molar and the amount of tooth movement measured (p < 0.05) from day 0 to day 14. However, there was no significant association between differential sclerostin staining and the amount of tooth movement measured (p > 0.05) from day 0 to day 7. This may be a true representation of the underlying biological phenomena or may result from small sample sizes, variability in measurements or possible method errors.

Studies over the past 100 years have shown that a phenomena termed hyalinization is often observed during the initial phases of tooth movement in both animal and human models. On the pressure side of tooth movement, the first biological event that takes place following force application is a disturbance of blood flow in the periodontal ligament (PDL). Cessation of blood flow results in a sterile cell death in the compressed area of the PDL, a process termed hyalinization. An influx of phagocytotic cells such as macrophages, foreign body giant cells and osteoclasts invade from the adjacent undamaged areas of the PDL and eliminate this necrotic tissue, a process termed undermining resorption. It has been purported that until this hyalinised tissue has been removed, tooth movement will not take place. Hyalinisation is considered to be an undesirable, and somewhat unavoidable sequela of orthodontic tooth movement as the need to remove the necrotised tissue prolongs the initiation of tooth movement. Previous studies have shown that, in a rat model, this hyalinised layer appears as quickly as 24 hours following orthodontic force application; varies vastly and is thought to be related to a multitude of factors including the magnitude and direction of the orthodontic force, and type of tooth movement initiated. The commonly accepted theory within the field of orthodontics was that complete hyalinisation tissue removal was needed before tooth movement could start but it has been recently shown that
even in the later stages of tooth movement small hyalinised patches can still be found.[23] The results of our study tie in to the undermining resorption phenomena as our day 7 readings could be distorted by the fact that not enough hyalinised tissue was removed, and thus the rate of tooth movement was inhibited. An additional confounder could be the fact that bone formation in the deeper region of the pressure side has been previously demonstrated, which would serve to result in fewer sclerostin positive osteocytes.[24] At day 14, it is feasible that enough undermining resorption would have occurred in order for tooth movement to progress, thus allowing for a linearity between sclerostin expression and orthodontic tooth movement.

**Conclusion**

The results of the present study indicate there is a statistically significant association between the differential sclerostin staining intensity and the amount of tooth movement measured (p>0.05) after a 14 day period. This linear association between sclerostin staining intensity and amount of tooth movement achieved suggests that while rate of tooth movement and sclerostin expression are affected by multiple variables, a linear association between the two may be present once hyalinised tissue has been eliminated.
Acknowledgments

Funding: The Australian Society of Orthodontists Foundation for Research and Education provided partial funding for the project.

Conflict of interest: There is no foreseeable conflict of interest.

Ethical approval: The ethics approval was obtained from the University of Adelaide Animal Ethics Committee (M/023/2006).

Statistician: Mr Thomas Sullivan, Statistician, Discipline of Public Health, University of Adelaide.

Bone biologist: Dr Brendon Noble, Scottish Mechanotransduction Consortium, University of Edinburgh Medical School, Edinburgh, UK.
References


SUMMARY
The literature review described the structure and function of alveolar bone, showing it to be a dynamic, complex and highly structured tissue. A particular emphasis was placed on both mechanotransduction, the conversion of a mechanical force into a biologic response and sclerostin, an osteogenic inhibitor uniquely expressed by osteocytes. Alveolar bone was shown to have unique remodelling characteristics when a mechanical strain was placed through it. This response differentiates it from bone found elsewhere in the body and much of the molecular biology that controls this local bone turnover has yet to be elucidated.

In this study, the mineralised response to orthodontic tooth movement was investigated in the dento-alveolar region of Sprague-Dawley rat molars subject to an orthodontic force. The main aim of this study was to examine changes that occur in the distribution of osteocytes positively stained for sclerostin in the alveolar bone of orthodontically stimulated teeth. Furthermore, the study aimed to find out if there was a linear association between the rate of orthodontic movement experienced by a tooth and the differential sclerostin expression across it.

The findings in paper one showed a statistically significant change in the sclerostin expression profile of alveolar bone, when subject to an orthodontic force. This difference was attributable to an increase in sclerostin positive osteocytes on the compression side and a decrease in sclerostin positive osteocytes on the tension side of the rat molar. This is a remarkable finding in that it ascribes a key role to the osteocyte in the processes of orthodontic tooth movement and local alveolar bone turnover.

The second study involved measuring tooth movement, indirectly, within the treated sample and correlating this data with the differential sclerostin staining intensity obtained from the previous study. No significant association was found in the
comparison with the 7 day group; however, a significant association between the amount of differential sclerostin expression and tooth movement was found for the day 14 group. This difference may be attributable and correlate to the length of time taken to remove the hyalinised layer.

There were some limitations to the present study. These were as follow:

- Limited sample size.
- Heavy orthodontic force used to establish movement in the rat molar.
- Length of storage time of decalcified maxillary tissue prior to cryosectioning.
- Thickness of the sections.
- Measurements were only conducted over a 14 day period.
- Only one operator was used to measure tooth movement and count positively stained osteocytes.

Some of the strength of the current study were as follows:

- A protocol was established that routinely delivered tooth movement.
- Power study used to determine sample size.
- Internal and external controls were used.
- Quantitative approach to measure cellular response.
- Error study to determine measurement accuracy.
- Blinding of the operator when measuring data.

Despite the limitations of the study, all the proposed aims have been achieved. The results refuted all the initial null hypotheses and the overall conclusions drawn from the data provided suggest that sclerostin plays a significant role in the conversion of a mechanical signal into a biological response within alveolar bone. Given there are no other studies that have investigated the role of sclerostin within alveolar bone it makes extrapolation and corroboration of our results difficult. It emphasises the need for
supplementary studies to be conducted in this area, particularly given the results shown. Future research should focus on gaining a further insight into the series of events that precede and follow sclerostin expression, and how far downstream in the mechanotransductive sequence the osteocytic response lies. Can the osteocyte be considered to be the chief orchestrator of mechanically driven local alveolar bone turnover or is it merely part of a response mechanism secondary to other local events? Further exploration into the cellular events that are coupled with sclerostin expression, particularly osteoblast apoptosis, control of osteoid mineralisation, and osteoclastic activity could help determine this.
APENDICIES
Appendix 1.

*The Application of Orthodontic Force on the Rat Molars*

Twelve animals were prepared in a previous study. The animals were weighed and anaesthetized to allow for placement of the closed-coil spring, and also prior to perfusion fixation at the end of the study.

1. Placement of Closed-Coil Spring: The rats were anaesthetized with Ketamine (100mg/ml) (Ketamil Injection, Troy Laboratories, Smithfield Australia) and Xylazine (Xylazil, 20mg/ml) via intraperitoneal injection at a dosage of 2ml/kg of body weight. The two drugs were mixed at the ratio of 2:1 (Ketamine: Xylazine) and then diluted 1:1 with sterile water for injection. This allowed a short period of anaesthesia allow for the placement of closed coil springs. Anaesthesia was tested via observation of a plantar reflex and chest movements.

2. Application of Orthodontic Force: The anaesthetised animal was placed onto a holding rack (figure 1). The mouth was gently propped open by two rings attached with elastic bands to the rack. A split-mouth design was used with the experimental side on the right, and the contralateral as the control. A 100 cN super-elastic NiTi closing coil spring (Sentalloy, GAC, New York, USA Catalogue number 10-000-06) was used to impart a mesially-directed force to the right maxillary first molar tooth. The springs were ligated with 0.010” stainless steel ligature wire anteriorly to the maxillary incisors and posteriorly to the cervical region of the right maxillary first molar. Springs were previously calibrated such that the force levels studied were within its superelastic range. Light curing bonding material (3M ESPE Z100 Restorative, St. Paul, USA) was used for bonding the 0.010” stainless steel ligature end of the closed-coil spring to the specific tooth. Care was taken not to injure the surrounding soft tissue (Figure 2).
Figure 1: Animal was placed onto a purpose-built holding rack

Figure 2: Photograph of experimental spring setup with wire ligature
Appendix 2.

Measurement of tooth movement

1. Tooth movement was measured indirectly. Polyvinyl siloxane impressions (Imprint 3, 3M ESPE) were taken intra-orally with a special customised acrylic tray at Days 0, 7 and 14 (Figure 1). Photos of the impressions were taken under light microscopy (MZ16FA stereo microscope) and were calibrated by Image Analysis software (analySIS® a SOFT IMAGING SYSTEM product).

![Figure 1: Impression taken with special tray](image)

2. The image capturing procedure involved recording the animal number and date of impression. A millimetre rule was photographed adjacent to the set impression, and the image captured and digitised using Image analysis software.

3. Landmarks of the first molar of both sides and the central incisors were located and digitised in a specific order and their X and Y coordinates were collected (Figure 2). Four anatomical landmarks along the circumference of the clinical crown were recorded for the incisors, the first and second molars. These were the distobuccal, mesiobuccal, mesiopalatal and distopalatal points, and a centroid was constructed by using these four points (Figure 3).
5. Measurements were made between the centroids of the first and second molars, and between the centroids of the first molar and incisors, for both the left and right sides. These were recorded and tabulated at specified time points (Appendix 3).

**Figure 2:** Impression landmarks

**Figure 3:** Illustration of centroid determination
Appendix 3.

Tooth measurement results

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Table 1: Tooth movement measurements of Day 0 (* = animals which died during the experimental procedure, C = control left side, T = treated right side)
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Table 2: Tooth movement measurements of Day 7 (* = animals which died during the experimental procedure, C = control left side, T = treated right side).
### Table 3: Tooth movement measurements of Day 14 (* = animals which died during the experimental procedure, C = control left side, T = treated right side)

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Appendix 4.

Harvesting of Tissue

1. Animal Sacrifice: A second anaesthetic procedure, involving administered intra-peritoneal administration of chloral hydrate 5g/100ml H₂O (5 ml per animal), was required immediately prior to sacrifice to provide anaesthesia for intra-cardiac perfusion of fixative.

2. Perfusion of Animal: The animals were perfused at either 7 or 14 days after coil spring placement at the Animal House, Medical School. The chest cavity was opened and rib cage removed to allow open access to the heart and aorta. A wide bore needle of the perfusion apparatus was placed into the left ventricle and the right atrium was cut to allow venous blood returning to the heart to drain. Each animal was initially perfused with 200-300ml of previously prepared 0.1% sodium nitrite solution for 5 minutes to cause sufficient vasodilation to facilitate tissue perfusion with the fixative (Figure 1).

![Figure 1: Perfusion apparatus](image)

3. Perfusion with Fixative: 200ml of $p$-benzoquinone solution and 200ml of 4% paraformaldehyde were combined and perfused into the left ventricle under positive pressure for approximately 15 minutes (Figure 2). A satisfactory level of fixation was
indicated when the extremities became brown in colour. The removal of the cranial vault allowed visual inspection of the brain and the degree of tissue perfusion.

Figure 2: Animal at completion of perfusion, note the brown colour of all tissues and extremities

4. Dissection of the maxilla: The maxilla and cranial vault were dissected out and superfluous soft tissues removed. The dissected maxilla and cranium were then placed into 0.2% p-benzoquinone and 2% para-formaldehyde for approximately 2 hours.

5. Decalcification: After 2 hours of immersion in the fixative solution, the tissues were then placed in 4% EDTA solution at ph 7.0. This solution was changed every second day for 14 days and then twice a week for a further 6 weeks, giving the minimum total decalcification time of 8 weeks. These tissues were then examined radiographically to confirm decalcification prior to usage. The maxillae from the seventeen animals were stored in the EDTA solution until use in the current study.

6. Cryo-protection: In preparation for cryo-protection, the maxilla and the brain stem were dissected further into two portions, separating the maxilla from the brainstem with the trigeminal ganglion attached. The tissues were impregnated with embedding medium (O.C.T. Sakura Finetek USA Inc Torance, CA 90504 USA), via a gradual
increase in concentration and gentle agitation. Over an 8 day period, the concentration of the embedding medium was steadily increased in a 30 \% sucrose solution until 100\% embedding medium was obtained by day 8.

7. Sectioning: Specimens were sectioned horizontally at 25 \(\mu \text{m}\) on a Leitz 1720 cryostat at -20\(^\circ\)C with the blade set at 6\(^\circ\). Sections were collected directly onto pre-coated slides. Each maxilla was orientated so that the crowns of the teeth were uppermost. The initial transverse sections through to the pulpal regions were removed and discarded. The slides were placed into a slide box, and left over night at room temperature to allow the sections to fully dry and adhere to the slide. The following day the slide box was sealed into a plastic bag, labelled and placed into a -20\(^\circ\)C refrigerator until required.

8. Immunohistochemical Staining Protocol: After cryosectioning, the sample was incubated with blocking agents. 1\% \(\text{H}_2\text{O}_2\) (in 70\% methanol in 0.1M PBS) was used to block endogenous peroxidase and then rinsed in PBS. The sections were then incubated in 3\% NGS (normal goat serum) in Milli Q water for one hour, to prevent non-specific binding. After blocking and rinsing, the sample was incubated with goat polyclonal anti-mouse SOST antibodies and rinsed. The sample was then incubated with a biotin labelled secondary antibody (Biotin conjugated affinity purified anti-rabbit IgG (Goat), which acts to bind to the primary antibody, to amplify the resultant amount of visual staining.

An enzyme-based immuno-staining method was used, involving the secondary antibody that is tagged with biotin. This Secondary antibody labeling and detection were accomplished using the Vectastain Universal Quick kit (Vector Labs, Inc. Cat N.o PK-7800). Diaminobenzidine (DAB) was used as the chromogen, resulting in a brown precipitate forming where the antibody has bound. To enhance visualisation prior to
viewing under the light microscope the immunolabeled sections were counterstained with haematoxylin and then coverslipped.

Positive immunohistochemistry controls were provided by sections of rat tibia which had been perfused and placed in fixative according to the same protocol as the maxillary tissue.
Appendix 5

Avidin-Biotin Peroxidase Complex immunolabelling protocol

1. Sections were collected on APT coated slides to increase adhesion.
2. The slides were wiped and the sections were encircled with a liquid repellent slide marker pen to retain the staining solutions on the specimens.
3. Endogenous peroxidase was blocked by rinsing slides using 1% H₂O₂ (in 100% methanol) for 10 minutes.
4. The slides were rinsed in PBS three times for 2 minutes.
5. A solution of normal goat serum was pipetted onto the sections and the slides were incubated for 30 minutes.
6. A solution of 1:75 polyclonal goat anti-mouse SOST primary antibodies in PBS was pipetted onto the sections and then incubated overnight in a wet chamber within a fridge at a temperature of 4 degrees.
7. The slides were rinsed in PBS three times for 2 minutes.
8. A prediluted biotinylated panspecific universal secondary antibody (anti-rabbit goat immunoglobulin G) was pipetted onto the sections and then incubated for 30 minutes at room temperature.
9. The slides were rinsed thoroughly in 1M PBS three times for 2 minutes.
10. A ready-to-use solution of streptavidin-peroxidase preformed complex was pipetted into the sections and incubated for 30 minutes at room temperature on a shaker.
11. The slides were rinsed thoroughly in 1M PBS three times for 2 minutes.
Appendix 6

AEC and counter-staining protocol

1. A ready-made AEC solution was placed on the slides for 18 minutes. The solution was kept in the dark to ensure the reaction did not proceed further.

2. Sections were washed for 5 minutes with Milli-Q water.

3. The sections were stained with haematoxylin for 10 seconds and washed in tap water to remove the excess stain. They were dipped in lithium carbonate for 30 seconds, followed by a wash in tap water.

4. The sections were mounted using aquamount and covered with large coverslips.