Superficial Zone Chondrocytes Modulate Polyphosphate Levels In Deep Zone Cartilage Which Correlate with Increased Tissue Formation And Decreased Mineralization By Deep Zone Chondrocytes

by

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Institute of Biomaterials and Biomedical Engineering

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ABSTRACT

Loss of the superficial zone of articular cartilage is an early change in osteoarthritis and with disease progression the deep zone (DZ) of cartilage shows progressive mineralization. To date, the mechanism(s) regulating post-natal articular cartilage mineralization is poorly understood. Previously, we have shown that inorganic polyphosphate inhibits mineralization of \textit{in vitro}-formed DZ cartilage. We developed an indirect co-culture method to investigate the effect of superficial zone chondrocytes (SZC) on mineralization in DZ cartilage. Our findings suggest that SZC suppress mineralization by modulating polyphosphate levels in DZ cartilage via FGF-18. Furthermore, SZC promote glycosaminoglycan and collagen accumulation in the extracellular matrix of cartilage formed by DZ chondrocytes. This study provides insight into the interaction between chondrocyte subpopulations and possible mechanism(s) controlling post-natal articular cartilage mineralization. Moreover, the results of this study establish polyphosphate and FGF-18, separately or in combination, as therapeutic candidates for articular cartilage repair and osteoarthritis prevention.
I would like to sincerely thank several people without whom this work would not have been possible. First, I wholeheartedly thank my supervisor Dr. Rita Kandel for giving me the opportunity to be part of her research group, and for all of the guidance and support over the years. She has provided invaluable advice, continuous encouragement, and patience over the course of my degree, for which I am immensely grateful.

I would like to express my gratitude toward my Supervisory Committee members, Dr. Marc Grynpas and Dr. Robert Pilliar, for their time, insightful comments and suggestions in completing my research project.

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<td>Agc</td>
<td>aggregcan</td>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<td>ALP</td>
<td>alkaline phosphatase</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>Arp</td>
<td>actin-related protein</td>
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<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
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<td>β-GP</td>
<td>beta-glycerophosphate</td>
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<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CILP</td>
<td>Cartilage Intermediate Layer Protein</td>
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<tr>
<td>CM</td>
<td>conditioned media</td>
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<tr>
<td>Col</td>
<td>collagen</td>
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<tr>
<td>CPPD</td>
<td>calcium pyrophosphate dihydrate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'-6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DZ</td>
<td>deep zone of articular cartilage</td>
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<td>DZC</td>
<td>deep zone chondrocytes</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>fibroblast growth factor receptor</td>
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<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<td>guanosine diphosphate</td>
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<td>guanosine triphosphate</td>
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<tr>
<td>IAP</td>
<td>intestinal alkaline phosphatase</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
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<td>Ihh</td>
<td>Indian hedgehog</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>MIM</td>
<td>mineralization-inducing medium</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MZ</td>
<td>middle zone of articular cartilage</td>
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<tr>
<td>MZC</td>
<td>mid-zone chondrocytes</td>
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<tr>
<td>MV</td>
<td>matrix vesicle</td>
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<tr>
<td>NM</td>
<td>non-mineralizing medium</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NTPPHase</td>
<td>nucleotide pyrophosphohydrolase</td>
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<tr>
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<td>osteoarthritis</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Opn</td>
<td>osteopontin</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCM</td>
<td>pericellular matrix</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>PD173074</td>
<td>FGFR tyrosine kinase inhibitor (1-t-Butyl-3-(6-(3,5-dimethoxyphenyl)-2-(4-diethylaminobutylamino)-pyrido[2,3-d]pyrimidine-7-yl)urea)</td>
</tr>
<tr>
<td>polyP</td>
<td>inorganic polyphosphate</td>
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<tr>
<td>PPK</td>
<td>polyphosphate kinase</td>
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<tr>
<td>PPN</td>
<td>endopolyphosphatases</td>
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<tr>
<td>PPR</td>
<td>receptor for parathyroid hormone and parathyroid hormone–related peptide</td>
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<tr>
<td>PPX</td>
<td>exopolypophosphatase</td>
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<td>proteoglycan 4</td>
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<td>parathyroid hormone-related protein</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RQ</td>
<td>relative quantification</td>
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<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<tr>
<td>RUNX2</td>
<td>runt-related transcription factor 2</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SOX</td>
<td>Sry-type HMG box-containing transcription factor</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>SZ</td>
<td>superficial zone of articular cartilage</td>
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<td>SZC</td>
<td>superficial zone chondrocytes</td>
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<tr>
<td>SZP</td>
<td>superficial zone protein</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNAP</td>
<td>tissue non-specific alkaline phosphatase</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>ZCC</td>
<td>zone of calcified cartilage</td>
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CHAPTER 1: INTRODUCTION
Articular cartilage is a specialized form of hyaline cartilage present at the articulating ends of diarthrodial (synovial) joints. Together with synovial fluid, this unique tissue provides a low-friction interface to allow for smooth articulation and distribution of applied forces to the underlying subchondral bone (Hunziker, 2002). Throughout life, articular cartilage is able to withstand age-related changes and high cyclic loading under normal conditions. However, when damaged by disease or trauma, articular cartilage possesses a very limited capacity for self-repair. This is in part due to its avasularity and limited migration of chondrocytes to the site of injury (Buckwalter, 1998; Buckwalter, 2002; Hunziker, 2002; Pearle et al., 2005). Once articular cartilage is damaged it is unable to fully heal, and ultimately leads to the development of osteoarthritis.

Osteoarthritis (OA) is a degenerative joint disease and its impact on Canadians is ever increasing. It is the leading cause of disability in Canada today, and people with the condition experience more pain, activity restrictions and long-term disability than those with other chronic diseases (Health Canada, 2003). Currently, more than 4.4 million Canadians are living with OA, and experts project that in 30 years more than 10 million Canadians – or one in four – will have OA (Arthritis Alliance of Canada, 2011). OA is one of Canada’s most costly chronic conditions, and its toll on the health care system in 2010 was estimated to be $27.5 billion in health care expenses and cumulative indirect costs (Arthritis Alliance of Canada, 2011).

Drugs used currently reduce inflammation and pain but do not alter progression of the disease, and may even enhance it (Bhosale and Richardson, 2008). At present, the
only treatment for end-stage joint disease is replacement with a synthetic prosthesis. However, a failure rate of up to 30% has been reported after 10-20 years for total hip arthroplasty, and not all patients become pain-free after joint replacement (Grayson and Decker, 2012; Kenney and Farmer, 2012; Kremers et al., 2012). These shortcomings have prompted the demand for alternatives to current clinical approaches, which include the development of novel therapeutics and methods for bioengineering articular cartilage. The limitations in current treatment strategies identify the need for research to overcome these shortcomings. In order to develop improved therapies, a better understanding of OA disease progression is required.

Articular cartilage possesses a unique depth-dependent zonal organization through which it accomplishes its specialized function. Changes in the biomechanical properties of articular cartilage results in deterioration of the tissue and disruption of the zonal architecture (Bhosale and Richardson, 2008; Oliviero et al., 2010). An early change in OA is the loss of the superficial zone of articular cartilage and subsequent changes include mineralization of the remaining cartilage and progressive cartilage degradation (Mankin, 1982; Buckwalter and Mankin, 1997; Thambyah and Broom, 2007; Fuerst et al., 2009). Loss of the superficial zone has been attributed to mechanical forces breaking down the collagen-proteoglycan network, however, the mechanism(s) responsible for mineralization of articular cartilage have yet to be determined (Buckwalter and Mankin, 1997; Pearle et al., 2005). Interestingly, loss of the superficial zone may contribute to the advancement of the tidemark and zone of calcified cartilage into uncalcified cartilage as superficial zone
chondrocytes have been shown to regulate mineralization of the deep zone cartilage in vitro (Jiang et al., 2008).

It is well established that chondrocyte subpopulations in native articular cartilage communicate in a paracrine manner through the semi-permeable extracellular matrix (Maroudas, 1976; Quinn et al., 2000). In healthy articular cartilage, chondrocytes synthesize and secrete zone-specific growth factors and cytokines to influence cell activity and maintain the tissue (Quinn et al., 2000; Blewis et al., 2007). However, in osteoarthritic cartilage, chondrocytes undergo biochemical changes including cell necrosis and disruption of metabolic homeostasis (Pearle et al., 2005; Oliviero et al., 2010). For this reason, investigating cellular communication between chondrocytes of the different zones of articular cartilage may shed light on the mechanism(s) regulating homeostasis and post-natal articular cartilage mineralization. Elucidation of the underlying mechanism(s) and the factors involved will prove important in understanding the pathogenesis of OA and developing new approaches for the treatment of this disease.
CHAPTER 2: LITERATURE REVIEW
2.1 Role and Composition of Articular Cartilage

Articular cartilage is a specialized form of hyaline cartilage about 3-4mm thick in humans present at the articulating ends of diarthrodial (synovial) joints. Together with synovial fluid, articular cartilage provides a low-friction interface to allow for smooth articulation in joints, and distribution of applied forces to the underlying subchondral bone (Huber et al., 2000). This tissue is unique because it lacks lymphatic, neural and vascular supply, and its sole constituent cells are chondrocytes; which rely on synovial fluid for nutrition. It is the avascularity, in part, which limits the self-repair potential of articular cartilage when damaged by disease or trauma (Hunziker, 2002; Pearle et al., 2005).

Chondrocytes account for approximately 5% of the wet weight of articular cartilage. These very specialized cells are responsible for the biosynthesis and degradation of the matrix (Bhosale and Richardson, 2008; Salter, 1998). Chondrocytes synthesize and secrete appropriate molecules, including Type II collagen, large proteoglycan aggregates and noncollagenous proteins which are then assembled into the highly organized framework known as the extracellular matrix (ECM) (Bhosale and Richardson, 2008; Huber et al., 2000; Buckwalter and Mankin, 1997). Single or pairs of chondrocytes are surrounded by ECM and as a result receive their nutrition by diffusion through the semi-permeable ECM. Additionally, due to the low oxygen concentration, chondrocytes depend primarily on anaerobic metabolism (Bhosale and Richardson, 2008).
2.2 Chondrogenesis

Chondrocytes arise from mesenchymal cells derived from different sites depending on which skeletal element they contribute to – from either the neural crest (craniofacial bones), sclerotome of the paraxial mesoderm (axial elements), or lateral plate mesoderm (appendicular bones) (Pogue and Lyons, 2006). The mesenchymal cells are capable of generating progenitors with restricted developmental potential. These progenitor cells can differentiate into chondroblasts, osteoblasts, fibroblasts, and adipocytes. This is controlled by factors produced in a temporal and/or sequentially regulated manner (Hall and Miyake, 2000; Komori, 2006; Komori, 2010; Studer et al., 2012). In order for limb-bud mesenchyme to initiate chondrogenesis, the condensation of mesenchymal cells into aggregates is required (Umansky, 1966). Accordingly, at the onset of chondrogenesis, mesenchymal cells are signaled to migrate, proliferate and condense at the site of skeletogenesis. Studies have shown that these mesenchymal cells express the transcription factor Sox9 prior to condensation. Sox9 belongs to the SOX (Sry-related HMG box) family of transcription factors and is required for prechondrogenic cell condensation, prechondrocyte and chondroblast differentiation, and activation of Sox5, Sox6, and cartilage matrix genes (Akiyama, 2008). Chondrogenic condensation does not form in Sox9 mutants. In mouse chimaeras, Sox9/-/- cells are excluded from all cartilages but are present as a juxtaposed mesenchyme that does not express the chondrocyte-specific markers Col2a1, Col9a2, Col11a2 (collagen type -2, -9, -11) and Agc1 (aggrecan). Moreover, no cartilage developed in teratomas derived from Sox9/-/- embryonic stem cells (Bi et al., 1999).
Expression of Sox5, Sox6 and Sox9 promote the development of a highly proliferating pool of chondroblasts of future long bones (Lefebvre et al., 2001; Ikeda et al., 2004). The cells in the center of the condensations differentiate into chondroblasts and organize into growth plates, which are responsible for bone growth. The chondroblasts begin to proliferate and produce a specialized extracellular matrix containing collagens II, IX, and XI, Gla protein, the large chondroitin sulfate rich proteoglycan aggrecan, and link protein in addition to other components (DeLise et al., 1999; Pogue and Lyons, 2006). Sox5 and Sox6 permit formation of growth plate columnar zones by keeping chondroblasts proliferating and by delaying chondrocyte pre-hypertrophy (Smits et al., 2004). The proliferating chondrocytes and their secretion of matrix increase the size of the cartilage. The chondrocytes in the center of the cartilage stop proliferating, increase in size (hypertrophy) and begin to synthesize an increased amount of Col2a1, Agc1, and other early cartilage matrix proteins relative to chondroblasts. Upon chondrocyte pre-hypertrophy, the expression of Sox5, Sox6, and Sox9 are turned off (Lefebvre et al., 1998). These pre-hypertrophic chondrocytes sequentially activate receptors for parathyroid hormone and parathyroid hormone–related peptide (Ppr), Indian hedgehog (Ihh), and collagen type 10 (Col10a1) (Smits et al., 2004). Ihh is a master regulator of bone development, coordinating chondrocyte proliferation, chondrocyte differentiation and osteoblast differentiation (St-Jacques et al., 1999; Kronenberg, 2003; Goldring et al., 2006).

The signal for proliferating chondrocytes to exit the cell cycle and become pre-hypertrophic is under the control of the Ihh and parathyroid hormone-related protein
(PTHrP) negative feedback loop (Kronenberg, 2003; Goldring et al., 2006). Studies in Ihh−/− mice have shown normal bones at the condensation stage, but subsequently develop pronounced abnormalities of bone growth. Ihh−/− mice also showed an increase in the fraction of chondrocytes leaving the pool of proliferating chondrocytes prematurely to be converted into hypertrophic chondrocytes because the cartilage in Ihh−/− mice fails to synthesize PTHrP (Kronenberg, 2003). Ihh induces the expression of PTHrP in periarticular cells, keeping them in a proliferative state, preventing their apoptosis and inhibiting their differentiation into Ihh-expressing pre-hypertrophic cells (Pogue and Lyons, 2006; Smits et al., 2004; Goldring et al., 2006). In PTHrP−/− or PPR−/− mice, chondrocytes become hypertrophic close to the ends of bones (Lanske et al., 1996), and overexpression of PTHrP in chondrocytes delays the appearance of hypertrophic chondrocytes (Weir et al., 1996). Consequently, these two paracrine factors together control the decision of chondrocytes to leave the proliferative pool. Therefore, PTHrP secreted from cells near the ends of bone, acts on its receptor on proliferating chondrocytes to keep them proliferating. When chondrocytes are no longer sufficiently stimulated by PTHrP, as the distance from the source of PTHrP increases, they stop proliferating and synthesize Ihh (Kronenberg, 2003).

Expression of runt-related transcription factor 2 (Runx2) and Osterix (Osx), a novel zinc finger-containing transcription factor are also upregulated in pre-hypertrophic chondrocytes (Zou et al., 2006). Runx2 and Osx are required factors for terminal differentiation of pre-hypertrophic chondrocytes and osteoblast maturation (Day and Yang, 2008). Studies in Runx2−/− mice have shown a complete absence or decrease of
pre-hypertrophic and hypertrophic chondrocytes (Yoshida et al., 2004). Furthermore, the hypertrophic chondrocytes that are present fail to mineralize their matrix and have decreased or absent expression of genes normally expressed by late hypertrophic chondrocytes such as osteopontin (Opn) and matrix metalloproteinase 13 (Mmp13) (Pogue and Lyons, 2006). Similarly, Osx deficiency in chondrocytes also resulted in delayed chondrocyte maturation and impaired endochondral bone formation (Oh et al., 2012).

Next, the pre-hypertrophic chondrocytes become hypertrophic and stop expressing Col2a1, Agc1, and other early markers. The hypertrophic chondrocytes progressively down-regulate Ppr and Ihh, up-regulate expression of Col10a1, and activate vascular endothelial growth factor (Vegf) expression (Smits et al., 2004). Hypertrophic chondrocytes direct adjacent perichondrial cells to become osteoblasts, which secrete a characteristic matrix to form a bone collar (Kronenberg, 2003). As the hypertrophic chondrocytes terminally differentiate, they stop expressing Col10a1, express Opn, Mmp13 and alkaline phosphatase, and undergo apoptosis (Smits et al., 2004). The cartilage matrix provides a scaffold for osteoblasts to lay down bone matrix and the expression of VEGF allows for the invasion of blood vessels (Kronenberg, 2003; Zelzer and Olsen, 2005). VEGF is also an important regulator of chondrogenic and osteogenic differentiation and is highly expressed in osteoblastic precursor cells (Liu et al., 2012).
2.3 Development of Articular Cartilage

Growth plate chondrocytes responsible for laying down the cartilaginous anlagen are termed transient chondrocytes (Pacifici, 1995). These chondrocytes display a very dynamic phenotype; they undergo proliferation, differentiation, hypertrophy and apoptosis in the growth plate, and are terminally replaced by osteoblasts in endochondral ossification (Pacifici et al., 2000; Shum and Nuckolls, 2002). Articular chondrocytes on the other hand, possess a permanent phenotype, and remain stable throughout life. They provide the tissue with functional resilience, producing all the macromolecular components of articular cartilage including aggrecan and Type II collagen (Pacifici et al., 2000). Based on cell fate maps it is believed the cells that give rise to articular cartilage derive from a population of stem cell-like cells located in the interzone (Dowthwaite et al., 2004; Hattori et al., 2007; Pacifici et al., 2006). Cells of the interzone are also responsible for generation of joint associated structures including synovium, ligaments and capsule (Pacifici et al., 2000).

Although articular cartilage formation and growth arise from different cells than osteochondral progenitors, they display many parallels with that seen in the epiphyseal growth plate. Proliferation near the articular surface suggests that articular cartilage arises by appositional growth. Similar to growth plate cartilage, proliferating chondrocytes expand within the transitional zone, and progress to terminal differentiation in the upper and lower radial zones (Hayes et al., 2001). However, unlike growth plate cartilage, terminal differentiation is not accompanied by extensive hypertrophy since there is no requirement for longitudinal expansion (Hayes et al., 2001; Onyekwelu et al., 2009).
Interestingly, articular chondrocytes undergo hypertrophy and display phenotypes analogous to growth plate chondrocytes in osteoarthritic cartilage (Pacifici et al., 2006; Dreier, 2010). Articular chondrocytes become enlarged, and exhibit metalloprotease and alkaline phosphatase activity (Pfander et al., 2001; Goldring and Goldring, 2007; Clouet et al., 2009). These cellular changes experienced by chondrocytes are not compatible with the maintenance of articular cartilage, and are believed to promote the development of OA (Dreier, 2010; van der Kraan and van den Berg, 2012). However, the two types of cartilage are different, and therefore care must be taken when transferring knowledge from growth plate cartilage to articular chondrocytes. Nevertheless, characterization of these mechanisms may possibly lead to the development of therapeutic means to maintain normal cell phenotype in health, and restore phenotype during disease.

### 2.4 Composition of the Extracellular Matrix

The extracellular matrix (ECM) of articular cartilage is primarily composed of water and macromolecules, including collagens, proteoglycans and non-collagenous proteins. Water comprises 60-80% of the total wet weight of the tissue, and contains dissolved electrolytes, gases, small proteins and metabolites. ECM molecules account for the remaining 20-40% of wet weight primarily: collagens (50-70% of dry weight), proteoglycans (15-30% of dry weight), and non-collagenous proteins (15-20% of dry weight) (Bhosale and Richardson, 2008; Buckwalter and Hunziker, 1999). The amount and type of macromolecules are distributed depending on the joint, site in the joint and
It is the interactions between the fluid and the matrix macromolecules, which provide stiffness and resilience to mechanical forces (Huber et al., 2000).

The major constituents of the ECM are collagens and proteoglycan aggregates, which together give articular cartilage its unique physiological properties. The collagen fibrils provide elements of high tensile strength. Collagen fibrils are comprised of three \(\alpha\)-chain polypeptides. Each chain contains at least one domain of repeating glycine-X-Y, which facilitates self-assembly into a triple-helix; X is often proline and Y is hydroxyproline (Eyre, 2006). The most abundant and characteristic collagen is Type II collagen, which accounts for 80%–90% of total collagen (Eyre, 2004). Type II collagen is distributed with differing orientations and concentrations throughout the different zones of articular cartilage. In the superficial zone, the collagen fibrils are of the thinnest size in diameter and are arranged parallel to the surface. In the middle zone, the collagen fibrils are of larger diameter compared to superficial zone and its orientation is less organized. In the deep zone, the collagen fibrils are of the largest diameter and are perpendicular to the surface of the joint and cross through the tidemark into the underlying calcified cartilage (Cohen et al., 1998; Newman, 1998).

Collagen types III, V, VI, IX, X and XI are also found, but to a lesser degree (Eyre, 2004). The collagens cross-link to form fibrils and networks to entrap proteoglycans (Buckwalter and Mankin, 1997). Type III collagen is located around the chondrocytes in the pericellular matrix and is also believed to copolymerize with Type II collagen (Eyre, 2004). Type V collagen participates in the formation of fibrillar collagen network.
VI collagen accounts for less than 1 percent of collagens in cartilage and is predominantly located in the pericellular matrix. Type VI collagen interacts with chondrocytes and various ECM components (Alexopoulos et al., 2009). Collagen type II, IX, and XI are cross-linked together in a network that forms the extracellular framework, which gives the tissue its form and contributes to its tensile stiffness and strength. Type IX collagen accounts for approximately 1 percent of total collagen and its molecules are covalently linked to the surface of Type II collagen fibrils. It is suspected that it mediates fibril-to-fibril and fibril-to-proteoglycan interactions. Type XI collagen is found within or on Type II collagen as it is thought to nucleate fibril formation and control fibril diameter (Cremer et al., 1998; Eyre, 2004; Bhosale and Richardson, 2008). Type X collagen is present in the calcified zone of articular cartilage, and is also expressed by hypertrophic chondrocytes of growth plate cartilage. It has been hypothesized that Type X collagen is involved in the mineralization of cartilage (Schmid et al., 1990; Gress and Jacenko, 2000; Huber et al., 2000). However, knockout of collagen X in mice does not lead to abnormal mineralization of the growth plate (Chan and Jacenko, 1998; Gress and Jacenko, 2000). The potential function of Type X collagen in cartilage mineralization will be discussed in detail in a later section.

The second largest component of the ECM is proteoglycans. Proteoglycans exist as either monomers or aggregates joined via link proteins to hyaluronic acid filaments. These special glycoproteins, the most predominant being aggrecan (90% of total cartilage proteoglycan mass), consists of predominantly keratan sulfate and chondroitin sulfate glycosaminoglycan (GAG) chains covalently bound to a central protein core. The GAG
side chains have a high concentration of anionic charge, and bind water and small ions (Newman, 1998; Huber et al., 2000). Proteoglycans occupy a larger volume in solution than they do in native cartilage, and therefore likely are only partially hydrated and are compressed by the collagen network in vivo (Newman, 1998). The hydrophilic nature of proteoglycans is largely responsible for the high water content of articular cartilage, which gives cartilage its compressive stiffness, and ability to resist deformation and dissipate load (Salter, 1998; Hasler et al., 1999). The distribution of proteoglycans in articular cartilage increases from being the lowest in the superficial zone to highest in the deep zone (Huber et al., 2000). A wide range of small proteoglycans, including decorin, biglycan, and fibromodulin also contribute to the ECM. They make up approximately 3% of the total proteoglycan mass and bind to matrix macromolecules to help stabilize the matrix and may play a role in regulation of chondrocyte function and growth factor availability (Salter, 1998; Huber et al., 2000). Decorin and fibromodulin bind to Type II collagen and may have a role in organizing and stabilizing the Type-II collagen meshwork. Decorin can bind to collagen during fibril formation, and reduce the final diameter of the forming fibril. The collagen fibrils in the superficial zone, where decorin is concentrated, are of the thinnest diameter (Poole et al., 2001). Biglycan is concentrated in the pericellular matrix and may interact with Type VI collagen (Buckwalter and Mankin, 1997).

The other constituents of the ECM are non-collagenous proteins. These proteins are involved in cell-to-matrix binding and contribute to the structural integrity of the ECM. One non-collagenous protein is anchorin CII, a collagen-binding chondrocyte
surface protein, which may be involved in the interaction of chondrocytes with collagen fibrils (Buckwalter and Mankin, 1997). Link protein, another non-collagenous protein, links aggrecan molecules to hyaluronan (Huber et al., 2000). Tenascin and fibronectin are non-collagenous proteins, which have been found in other tissues also. It has been proposed that they interact with a variety of ECM molecules and cell surface receptors affecting tissue architecture, tissue resilience and cell responses (Roughley, 2001; Patel et al., 2011). Expression of the two proteins during early limb development suggests they may be involved in chondrogenesis (Mackie and Murphy, 1998). Tenascin expression is highly restricted in healthy mature cartilage but reappears in diseased conditions such as osteoarthritis (Salter, 1993; Veje et al., 2003).

It is the precise composition of collagens, proteoglycans, and non-collagenous proteins that are responsible for the structure and mechanical properties of articular cartilage. It is important to note that the combination of proteins is not homogenous throughout the tissue. This distinguished characteristic generates stratified zones, which together contribute to the tissues functionality.

2.5 Zonal Architecture - Structure of Mature Articular Cartilage

Although articular cartilage appears to be homogenous histologically, and cells of articular cartilage are said to be of a single cell type, chondrocytes differ in size, shape, and metabolic activity depending on their position within the tissue (Huber et al., 2000). The depth-related differences in extracellular matrix composition, macromolecular
organization and mechanical properties result from metabolic specialization of the chondrocytes resident in the different zones (Schumacher et al., 1994). The difference in composition and structural organization of each zone has a discrete role in the ability of articular cartilage to articulate joints and respond to mechanical loads. Mature articular cartilage possesses a highly ordered zonal architecture, and it is accepted that the tissue is divided into four distinct zones, from the articulating surface to the subchondral bone, articular cartilage is separated into superficial, middle (or transitional), deep (or radial), and the zone of calcified cartilage (Newman, 1998).

2.5.1 Superficial Zone

The thinnest zone of articular cartilage is the superficial zone, which constitutes the uppermost 10-20%. The superficial zone together with synovial fluid gives articular cartilage the low-friction surface. The specialized mechanical properties are produced through the composition and unique structure of the zone. The joint surface is covered by the lamina splendens, which contains low amounts of polysaccharides, no cells and a layer of fine, densely packed collagen fibers aligned parallel to the articular surface (Buckwalter and Mankin, 1997). The parallel arrangement of collagen fibers gives articular cartilage tensile strength and thus the ability to resist shear forces generated during movement (Kumar et al., 2001). The dense organization of fibers also acts as a barrier to prevent the entry of large molecules such as antibodies or other proteins and the exit of large cartilage molecules (Newman, 1998; Huber et al., 2000; Poole et al., 2001). Below this acellular sheet of fibrils are flattened ellipsoid-shaped chondrocytes arranged so the cells major axis
is parallel to the articular surface. Relative to the other zones, the superficial zone has the highest cell density and water content, the highest concentration of Type II collagen, and lowest concentration of proteoglycans (Buckwalter and Mankin, 1997; Poole et al., 2001).

Although proteoglycan concentration is low in this zone, one very important proteoglycan produced by superficial zone chondrocytes is Superficial Zone Protein (SZP). SZP plays a critical role in facilitating the low-friction property of articular cartilage at the joint-cartilage interface (Schmidt et al., 2004). The fibril-associated, leucine-rich decorin and biglycan are small proteoglycans also predominantly located in the superficial zone (Poole et al., 1996). Consequently, disruption of the superficial layer not only alters the mechanical properties of articular cartilage thus contributing to the development of OA but may also release cartilage molecules that stimulate an immune or inflammatory response (Buckwalter and Mankin, 1997). The effect of loss of the superficial zone on OA disease development and cartilage mineralization will be discussed in detail in a later section.

Another protein produced solely by superficial zone chondrocytes is clusterin. Although the exact function of this glycoprotein is not known, clusterin is thought to play a role in many important biological functions. Interestingly, clusterin expression is elevated in osteoarthritic cartilage and preliminary evidence suggests that it is involved in regulating cell death (Khan et al., 2001). Superficial zone chondrocytes also produce signaling molecules that include members of the Wnts, bone morphogenetic proteins, and transforming growth factor families (Anderson et al., 2000; Pacifici et al., 2005; Yamane
et al., 2007). The Wnt/β-catenin pathway is an essential regulator of superficial zone chondrocyte function and phenotype. Activation of Wnt/β-catenin signaling in transgenic mice has been shown to increase superficial zone thickness, proliferation of superficial zone chondrocyte, and expression of SZP (Yasuhara et al., 2011; Inui et al., 2013).

2.5.2 Transitional Zone

Below the superficial zone is the middle zone, which is also referred to as the transitional or intermediate zone. The middle zone is the largest of the four zones in terms of thickness, and it constitutes 40-60% of articular cartilage’s full thickness (Newman, 1998). The cell density is lower than in the superficial zone and the chondrocytes have a spheroidal morphology. Relative to the superficial zone, chondrocytes in the middle zone have a higher concentration of synthetic organelles, endoplasmic reticulum, and Golgi membranes (Buckwalter and Mankin, 1997). As the name implies, the matrix composition of the transitional zone are intermediate between the superficial and the deep zone. The chondrocytes are embedded in abundant extracellular matrix with lower concentrations of water and collagen, and a higher concentration of proteoglycans compared to the matrix of the superficial zone. The collagen fibrils have a larger diameter compared to collagen of the articular surface, and assume a more random alignment (Pearle et al., 2005).

The high concentration of proteoglycans, the most prominent being aggrecan, provides the proper mechanical properties to withstand and absorb the compressive forces
produced by the joint. A characteristic matrix protein present in the middle zone is Cartilage Intermediate Layer Protein (CILP), termed on the basis of its localization (Lorenzo et al., 1998a). Isolation and peptide mapping of CILP revealed this protein acts as a precursor for two different proteins. The precursor protein is synthesized as a single polypeptide chain larger than expected for CILP. Cleaved intracellularly, the polypeptide chain produces two proteins, CILP and the homologous nucleotide pyrophosphohydrolase (NTPPHase) (Lorenzo et al., 1998b). The amino-terminal portion of the transcript from the CILP gene encodes CILP, while the carboxy-terminal portion encodes NTPPHase. NTPPHase is an inorganic pyrophosphate generating ectoenzyme, which has been detected in synovial fluid, and cartilage extracts from patients affected with calcium pyrophosphate dihydrate (CPPD) crystal deposition disease (Johnson et al., 2001). Interestingly, CILP is more abundant in adult and osteoarthritic than juvenile articular cartilage, but the relevance of the site- and age-related distribution to function is unknown (Lorenzo et al., 1998a; Roughley, 2001).

2.5.3 Deep Zone

The deep zone, also known as the radial zone, constitutes 30-40% of articular cartilage thickness (Newman, 1998). The chondrocytes in the deep zone are spheroidal in shape, and can be grouped in columns aligned perpendicular to the articular surface. This zone has the highest concentration of proteoglycans, and lowest water and collagen contents. However, the collagen fibrils in the deep zone are of the largest diameter (Newman, 1998; Pearle et al., 2005). Similar to the chondrocytes, the collagen fibers are also oriented
perpendicular to the articular surface and cross through the tidemark into the underlying calcified cartilage. The tidemark is a wavy, irregular line that separates the deep zone from the calcified zone and is known to contain crystals of calcium salt and hyaluronan. The nature of the tidemark remains uncertain but ultrastructurally it has been revealed that the band of fibrils may serve as a tethering mechanism for the collagen fibrils (Redler et al., 1975). This orientation of collagen fibrils allows for a strong bond between articular cartilage and the subchondral bone and also contributes to the compressive properties of cartilage. The chondrocytes in the deep zone express tissue non-specific alkaline phosphatase (TNAP), matrix metalloproteinase 13 (MMP-13) and Type X collagen, features similar to hypertrophic chondrocytes of the growth plate (Mackie et al., 2008).

2.5.4 Zone of Calcified Cartilage

The zone of calcified cartilage (ZCC) is defined as highly mineralized cartilage that lies between the deep zone of hyaline cartilage and the subchondral bone. It is approximately 100-300µm thick and accounts for 3-9% of the total articular cartilage thickness (Gupta et al., 2005; Wang et al., 2009). Articular cartilage is anchored to the subchondral bone by collagen fibers between the ZCC and hyaline cartilage termed the tidemark and between the ZCC and the subchondral bone termed the cement line (Lyons et al., 2006; Wang et al., 2009). However, Oegema et al. (1997) through transmission electron microscopic studies reported that the collagen fibers do not cross the interface into the subchondral bone, suggesting that the two tissues are held together through interdigitation rather than reinforcement by way of collagen fibrils (Oegema et al., 1997).
Studies on the ZCC have shown that the mineral component is a carbonate-substituted form of hydroxyapatite similar in composition and size to that in bone (Arsenault and Grynpas, 1988; Kandel et al., 1999). Although the mineral content in the ZCC is significantly higher than in bone, the elastic modulus of subchondral bone is one order of magnitude greater than that of calcified cartilage (Mente and Lewis, 1994; Zizak et al., 2003; Duer et al., 2009; Wang et al., 2009). This is a result of the composition of organic matrices and difference in the mineral–collagen packing at the fibrillar level. The matrix of bone consists of highly organized Type I collagen molecules whereas that of calcified cartilage consists of less organized Type II collagen (Ferguson et al., 2003; Gupta et al., 2005). In bone, Type I collagen molecules assemble in a regularly staggered manner to form fibrils with several hundred nanometers in diameter. These fibrils are impregnated with and surrounded by small nanocrystallite particles of carbonated apatite, forming a reinforced composite (Ferguson et al., 2003; Gupta et al., 2005).

It has been suggested that the ZCC functions as an intermediate stiffness layer between articular cartilage and the subchondral bone (Patel and Buckland-Wright, 1999). There exists a significant difference between the mineral content–mechanical property correlation of the ZCC and subchondral bone. The subchondral bone requires less mineral content to attain the same stiffness or hardness values as calcified cartilage (Gupta et al., 2005). The elastic modulus of compliant hyaline cartilage ranges from 1.9 to 15 MPa, while the elastic modulus of subchondral bone is about 4 GPa (Mente and Lewis; 1994; Wang et al., 2009). This stiffness allows the ZCC to transform shear stresses into tensile and compressive stresses (Patel and Buckland-Wright, 1999). In addition, the ZCC
stabilizes articular cartilage by acting as a barrier to interrupt the transfer of interstitial fluid between articular cartilage and the subchondral bone (Kovach, 1996; Oegema et al., 1997; Wang et al., 2009). Consequently, the ZCC becomes the first fracture site in experimental trauma to the joint (Wang et al., 2009). Such damage can lead to advancement of the ZCC, resulting in the decrease in thickness of the overlying articular cartilage. This thinning can exert mechanical stress in the overlying articular cartilage and could contribute to cartilage loss in OA (Patel and Buckland-Wright, 1999).

The cell density in the ZCC in normal human femoral condyle, as measured by Wang et al. (2009), was 51.25 ± 21.26 cells/mm², which is significantly lower than hyaline cartilage (152.54 ± 35.77 cells/mm²) (Wang et al., 2009). In the adult, cells in the ZCC are metabolically active, and cells in the deeper regions of ZCC become quiescent but not inactive (Revell et al., 1990; Wang et al., 2009). In OA, the quiescent chondrocytes may be reactivated and progressively calcify the unmineralized cartilage. Subsequently, thickening of the ZCC would increase the concentration of forces across the uncalcified cartilage accelerating the rate of OA progression (Oegema et al., 1997; Duer et al., 2009).

### 2.6 Organization of the Extracellular Matrix

Articular cartilage is divided into horizontal zones with circumferential variations in the matrix around individual chondrocytes resulting in compartments. These compartments are divided into three regions: the pericellular region, the territorial region, and the
interterritorial region (Buckwalter and Mankin, 1997). The three regions differ in matrix composition as well as organization resulting in each zone having a specific function. The pericellular and territorial regions appear to protect the chondrocytes from damage during loading and deformation of the tissue (Guilak and Mow, 2000). Moreover, it has been proposed that these zones also facilitate the transmission of mechanical signals to the cells when the matrix deforms during joint loading (Guilak et al., 2006). The interterritorial matrix provides the mechanical properties of articular cartilage (Buckwalter and Hunziker, 1999).

2.6.1 Pericellular Region

All chondrocytes are surrounded by a narrow region termed the pericellular matrix (PCM). The PCM together with the chondrocyte has been termed the “chondron” (Poole, 1997). The PCM is rich in proteoglycans and contains non-collagenous proteins, such as the cell membrane-associated molecule anchorin CII. The region has little to no fibrillar collagen but contains non-fibrillar collagens such as Type VI collagen, which under normal circumstances is not found elsewhere in cartilage (Buckwalter and Mankin, 1997; Alexopoulos et al., 2003). Cytoplasmic extensions from the chondrocyte project into the PCM and also through to the territorial matrix, binding the cell membrane to matrix macromolecules. Considerable evidence suggests that the PCM is involved in the transmission of mechanical and chemical signals to the chondrocytes (Alexopoulos et al., 2003; Alexopoulos et al., 2005a; Alexopoulos et al., 2005b; Guilak et al., 2006).
In order to withstand and transmit shearing and compressive forces to the subchondral bone, chondrocytes must function synergistically with the ECM. Since chondrocytes are completely surrounded by the PCM, any mechanical or chemical signals must pass through the PCM to reach the chondrocyte. Therefore, it has been hypothesized that the chondron serves as a mechanical transducer through the interaction of Type VI collagen with cell surface integrins or hyaluranan, and a chemical transducer by selectively trapping and modifying soluble factors and retaining growth factors (Alexopoulos et al., 2003; Larson et al., 2002). It has been shown that retaining native PCM following cell isolation influences the metabolic activity of chondrocytes resulting in significantly improved matrix production in vitro (Graff et al., 2003; Larson et al., 2002). It has also been postulated that the chondron provides hydrodynamic protection for chondrocytes during loading through an “adaptive water loss from the PCM proteoglycans”. During mechanical loading the lower permeability of PCM relative to the ECM inhibits fluid flux and strain within the chondron relative to the ECM by a factor of 30 (Alexopoulos et al., 2005a).

2.6.2 Territorial Matrix

The territorial matrix surrounds the pericellular matrix of chondrocytes. The collagen fibers nearest to the cell are similar in size to those in the PCM and appear to adhere to the PCM. The fibers intersect at various angles, forming a fibrillar basket around the cells (Buckwalter and Mankin, 2005). It has been suggested that the meshwork provides mechanical protection for the chondrocytes during loading and deformation of the tissue.
The collagen fibrils increase in diameter and the basket-like orientation transitions to a more parallel arrangement marking the boundary between the territorial matrix and the interterritorial matrix (Huber et al., 2000). The precise boundary between the two regions is indistinct because many collagen fibrils connect the two regions (Buckwalter and Mankin, 1997).

### 2.6.3 Interterritorial Matrix

The interterritorial matrix makes up most of the volume of mature articular cartilage, and its primary function is to provide the mechanical properties of the tissue. The collagen fibrils are of the largest diameter, and are organized depending on the zone they are in (Huber et al., 2000; Buckwalter et al., 2005). The region has a higher concentration of proteoglycans rich in keratan sulfate compared to the PCM and territorial matrix (Huber et al., 2000). Due to the densely compacted collagen fibers and high proteoglycan content, this region contributes most to the biomechanical properties of articular cartilage (Poole et al., 1984; Mow and Guo, 2002).

### 2.7 Pathology of Osteoarthritis

Osteoarthritis (OA) is the most prevalent chronic musculoskeletal disease and leading cause of pain and physical disability in the elderly population (Jüni et al., 2006). OA is characterized by the progressive degeneration of articular cartilage but changes extend to the underlying subchondral bone and the synovium (Oliviero et al., 2010). The causes of
OA are multifactorial, of which age is the strongest risk factor. It is important to identify the synergistic effects of other contributing factors such as genetic predisposition, joint misalignment, gender, race, hormonal status, joint trauma, overuse or immobilization, and obesity (Ghosh, 1999; Wieland et al., 2005; Mobasher, 2012).

With increasing age there is a reduction in hydration and chondrocyte cell numbers in the cartilage. Furthermore, chondrocytes undergo decrease in proliferative and synthetic activities, and are no longer able to regulate matrix turnover because of their inability to maintain anabolic and catabolic homeostasis (Bhosale and Richardson, 2008; Loeser, 2009). Still, OA is more complex than a simplistic age-dependent degenerative process because, despite these age-related changes and high cyclic loading, the integrity of articular cartilage can be maintained under normal conditions. Normal loading is necessary and useful to stimulate physiological changes of joint structure. On the other hand, abnormal loading of a joint leading to excessive focal stresses or trauma resulting in heavy impact to the joint surface can cause microdamage to the cartilage (Bhosale and Richardson, 2008; Oliviero et al., 2010). Trauma to articular cartilage can lead to cellular degeneration and cell death. Additionally, the location of the defect, whether in a loadbearing or unloaded region, also influences repair response. If left untreated, the mechanical factors can induce biological events that may accelerate OA onset and progression (Buckwalter, 2002; Oliviero et al., 2010).

Due to these changes the biomechanical properties of articular cartilage weaken, which results in the breakdown of ECM constituents and architecture (Pearle et al., 2005).
Changes begin in the superficial zone and advance with OA disease progression to include formation of cartilage clefts, further loss of cartilage layers, and duplication of the tidemark (Mankin, 1982; Buckwalter and Mankin, 1997; Fuerst et al., 2009; Thambyah and Broom, 2009). Biochemical changes include cell necrosis, proteolytic degradation of proteoglycans and disruption of the collagen network. Proteoglycan degradation results in reduced proteoglycan chain length, glycosaminoglycan content, and inhibition of normal formation of macromolecular complexes (Pearle et al., 2005; Oliviero et al., 2010).

Disruption of the collagen network of the superficial zone is one of the first detectable structural changes in early OA (Hollander et al., 1995; Setton et al., 1999; Silver et al., 2001; Pritzker et al., 2006; Thambyah and Broom, 2009). Deterioration of the superficial zone has been suggested to contribute to the development of the disease (Buckwalter and Mankin, 1997; Pearle et al., 2005). The densely packed collagen fibrils function to limit the entry and exit of large molecules such as antibodies, isolating cartilage from the immune system. Disruption of the collagen matrix results in decreased tensile stiffness and strength as well as a reduction in the compressive stiffness of the tissue due to an increase in water content as a result of increased permeability (Buckwalter and Mankin, 1997; Pfander et al., 2001; Pearle et al., 2005; Goldring and Goldring, 2007; Bhosale and Richardson, 2008). Therefore, changes to the superficial zone decrease the mechanical properties of articular cartilage and also stimulate an immune response causing inflammation within the synovial cavity (Buckwalter and Mankin, 1997; Balcerzak et al., 2003; Pearle et al., 2005; Pritzker et al., 2006).
Changes are also observed in the ZCC in the early stages of OA following deterioration of the superficial zone. The tidemark reduplicates and the ZCC advances into non-calcified articular cartilage, and as the disease progresses the tissue undergoes further mineralization (Oegema et al., 1997; Burr, 2004; Wieland et al., 2005; Thambyah and Broom, 2007; Thambyah and Broom, 2009). OA is not limited to articular cartilage and changes extend to the underlying subchondral bone as well as the synovium. Pathological structural changes to the subchondral bone in areas underlying the damaged cartilage areas are hallmarks of OA. There is an increase in bone formation and resorption resulting in the thickening of the cortical subchondral plate and formation of osteophyte (Pearle et al., 2005; Wieland et al., 2005; Clouet et al., 2009; Thambyah and Broom, 2009). Associated with this is synovial inflammation, which leads to over-expression of pro-inflammatory mediators such as cytokines and chemokines, and proteolytic enzymes that in turn contribute to the subsequent degeneration of articular cartilage (Benito et al., 2005; Krasnokutsky et al., 2008; Clouet et al., 2009).

Ultimate loss of joint function is the end result of the irreversible destruction of articular cartilage and bone changes associated with OA. The disease appears to be due to an imbalance between anabolic and catabolic processes as well as loss of control of the cartilage mineralization mechanisms. Elucidation of the mechanisms regulating these processes will be critical in understanding OA.
2.8 Biomineralization

Mineralization of cartilage is a characteristic change in OA. To date, the mechanism(s) controlling this complex process is not well understood. To better understand the potential mechanism(s), this section will review the factors postulated to play a role in cartilage mineralization and how they are controlled by chondrocytes.

2.8.1 Principle of crystal nucleation

The mineral component in articular cartilage is a calcium phosphate salt, Ca_{10}(PO_4)_6(OH)_2 with a molar Ca:P ratio of 1.67:1, identified as hydroxyapatite (Landis et al., 1982; Landis, 1999; Fuerst et al., 2010). Single hydroxyapatite crystals are acicular in shape, have a diameter of about 1 nm and vary in length from approximately 15 to 100 nm (Kandel et al., 1999; Fuerst et al., 2010). Single crystals are not detectable by light or polarized light microscopy, and are visible with conventional light microscopy only if they appear in clumps or aggregates. In articular cartilage, the clusters of acicular crystals have no organizational relationship with the collagen fibrils. The orientation of the crystals is completely different from that found in bone, where the longitudinal axis of the crystals is oriented parallel to the collagen fibrils (Serafini-Fracassini and Smith, 1974). Biological mineralization in articular cartilage is the result of a complex series of events occurring between specific organic and inorganic components, which have not been fully elucidated.

There is no evidence that calcification of cartilage or any tissues in which physiological calcification occurs is through one specific mechanism. In most tissues the
calcification process is similar in that they involve binding of calcium salts to a pre-existing organic molecule in which the reactive groups are arranged so that they fulfill the structural and electrostatic charge distribution required of a nucleating agent (Serafini-Fracassini and Smith, 1974). The formation of hydroxyapatite crystals is specific in the manner in which crystals are deposited, grow, and progress in number, size and mass. The first step in crystal formation is the nucleation of ions. Here ions come together in the right orientation to form a stable cluster known as the critical nucleus, which is dependent on the level of supersaturation, the interfacial free energy and temperature (Walton, 1965). If enough ions are present the critical nucleus can become sufficiently large to remain stable and grow further by ordered addition of ions (Boskey, 1991). However, if the critical nucleus is not stabilized and not enough ions are present, it cannot grow and will dissolve (Boskey, 1992). Mineral nucleation occurs outside the confines of a cell in the ECM where fluids are supersaturated in calcium, inorganic phosphate, and hydroxyl ions. The concentrations are sufficiently high to support the growth of hydroxyapatite crystals once the initial crystals are formed, but the concentrations of these ions are not high enough to precipitate spontaneously. Therefore, some seeding mechanism is required to initiate the formation of the critical nucleus (Fleisch and Neuman, 1961). Understanding the physical chemistry of crystal formation and growth in articular cartilage requires an appreciation of the effect the organic matrix has on the mineralization process.


### 2.8.2 Role of Extracellular Matrix in Mineralization

The differentiation of chondrocytes to hypertrophic chondrocytes is a key step in endochondral ossification and production of hydroxyapatite crystals. Chondrocyte hypertrophy has been described to occur in OA, and this change has been implicated in the mineralization that occurs in this disease process (Fuerst et al., 2009; Pitsillides and Beier, 2011; van der Kraan and van den Berg, 2012). However, the calcification of articular cartilage is a complicated process, and the mechanism(s) that regulates mineral formation is still not well understood.

The direction and extent of hydroxyapatite growth is governed by the concentrations of ions in solution as well as the stability of the critical nucleus. The initial formation and stabilization of the critical nucleus as well as the size, morphology, and orientation in hydroxyapatite growth are regulated by chondrocytes, its surrounding matrix and non-collagenous proteins (Boskey, 1992). Prior to the initiation of mineralization, chondrocytes reorganize the ECM to support mineral formation. The ECM may participate in the formation of hydroxyapatite in two ways; 1) Directly – by serving as a nucleator for crystals to bind, and providing oriented support for mineral deposition and growth; or 2) Indirectly – by transporting, sequestering and orienting ions to form a critical nucleus which becomes incorporated into the mineral crystals (Boskey, 1996; Boskey, 2003).

In calcifying cartilage, Type II collagen, Type X collagen, matrix vesicles, alkaline phosphatase, chondrocalcin and proteoglycans have been implicated in modulating
mineralization (Boskey, 1996; Kirsch and Wuthier, 1994; Golub, 2009). The exact mechanisms through which these proteins modulate mineral formation are not yet clear but some generalizations have come to light. Firstly, there is evidence that the many highly acidic extracellular matrix proteins bind Ca$^{2+}$, and as a result, either inhibit calcification or serve as nucleators (Hunter and Szigety, 1992). Secondly, many of the extracellular matrix proteins are highly phosphorylated, which provides phosphate-rich nucleation sites, and serves as putative substrates for matrix phosphatases such as TNAP (Golub, 2009). The exact mechanism through which the extracellular matrix constituents modulate the mineralization of cartilage is still not fully elucidated, but there is no doubt that they play key roles in the process.

2.8.3 Type II and VI Collagen

In hard connective tissues, such as bone and dentin, collagen actively controls crystal formation and is a necessary scaffold that guides the organization and growth of hydroxyapatite (Boskey, 1991; Nudelman et al., 2010). However, the evidence that collagen acts as an organic template in calcified cartilage is much less convincing because the mineral crystallites appear to bear no specific spatial or directional relationship to the collagen fibrils (Serafini-Fracassini and Smith, 1974; Kandel et al., 1999). This may be a result of the different matrix constituents within the tissues as well as the collagen itself, such as cross-linking and collagen type (Olszta et al., 2007; Price et al., 2009; Nudelman et al., 2012; Wang et al., 2012). Nonetheless, the importance of Type II collagen in calcification is supported by a previous study using transgenic mice with a partially
deleted Type II collagen gene that showed delayed mineralization (Vandenberg et al., 1991).

In bone, Type I collagen regulates two stages of mineralization: 1) infiltration of the mineral phase (amorphous hydroxyapatite) into the fibril; and 2) transformation of the mineral phase into oriented apatite crystals (Nudelman et al., 2010). Infiltration of mineral phase into the fibrils is mediated by interactions of amorphous hydroxyapatite with the C-terminus of collagen. Subsequently, clusters of charged amino acid residues present throughout collagen serve as nucleation centers to allow formation and growth of apatite crystals (Kawska et al., 2008; Silver and Landis, 2011). In cartilage, Type II collagen forms a complex with chondrocalcin, the C-terminal propeptide of Type II procollagen that is cleaved from the collagen molecule prior to the formation of fibrils (Canty and Kadler, 2005). Chondrocalcin is a calcium-binding extracellular protein with two roles: 1) assembly of the triple helix of Type II collagen; and 2) binding calcium in mineralizing cartilage (Hall, 2005). The distribution of chondrocalcin coincides with sites of mineralization, concentrated within hypertrophic and calcifying cartilage. Chondrocalcin binds avidly to hydroxyapatite and is likely involved in regulation of nucleation and crystal growth (Poole et al., 1989; Boskey, 1992). Not surprisingly, raised chondrocalcin levels are found in the synovial fluid of osteoarthritic cartilage (Lohmander et al., 1996). Type II collagen also binds to plasma membrane annexin V stimulating its Ca$^{2+}$ channel activities, resulting in an increased Ca$^{2+}$ influx into matrix vesicles. Matrix vesicles are small spherical bodies bound by a lipid bilayer (Golub, 2009). Studies have shown that annexin V mediates Ca$^{2+}$ influx regulating mineralization events (Kirsch et al.,
2000; Kim and Kirsch, 2008). The role of matrix vesicles in cartilage mineralization will be discussed in detail later in this section.

Another collagen linked to mineralization defects is Type VI collagen. It has been proposed that chondrocytes perceive biomechanical signals through the interaction of Type VI collagen with cell surface integrins or hyaluranan (Alexopoulos et al., 2003; Larson et al., 2002; Guilak et al., 2006). Significant musculoskeletal changes have been reported in Type VI collagen deficient mice (Alexopoulos et al., 2009). Mice lacking Type VI collagen developed intact pericellular matrices with significantly inferior mechanical properties compared to wild-type controls. Furthermore, Col6a1−/− mice suffered accelerated osteoarthritic joint degeneration, experienced delayed secondary ossification, and developed bone with lower mineral density (Alexopoulos et al., 2009). This study shows loss of Type VI collagen can lead to abnormal skeletal development, and development of OA.

### 2.8.4 Type X Collagen

Similar to Type II collagen, Type X collagen is also able to bind to membrane bound annexin V and mediate Ca$^{2+}$ influx into matrix vesicles (Kirsch et al., 2000). However, unlike Type II collagen, Type X collagen is exclusively expressed in the hypertrophic zone of growth plate cartilage and in osteoarthritic articular chondrocytes (Boskey, 1992; Eerola et al., 1998; Oyajobi et al., 1998; Thouverey et al., 2009). As a result of its spatio-temporal association with hypertrophic chondrocytes in growth-plate and osteoarthritic
cartilage, Type X collagen has been linked to processes such as mineralization, matrix stabilization or remodeling, and vascular invasion (Gress and Jacenko, 2000). One hypothesis is that Type X collagen replaces the more densely packed Type II collagen fibrils to provide stability and room for chondrocytes to hypertrophy (Wallis, 1993). Another hypothesis suggests that Type X collagen binds Type II collagen to direct calcification away from Type II collagen since it ultimately has to be removed (Poole and Pidoux, 1989; Alini et al., 1992). An in vitro study showed parallel mineralization with the expression of Type X collagen, whereas other studies found that it did not have a direct effect on crystal formation or growth (Boskey, 1992). It has also been hypothesized that Type X collagen may compartmentalize proteoglycans and matrix vesicles within hypertrophic cartilage (Gress and Jacenko, 2000).

Due to the consequences of Type X collagen mutations in both mouse and man, it is predicted that Type X collagen is involved in skeletal development but its role in mineralization of articular cartilage is harder to determine. Several syndromes and mutations illuminate the role of Type X collagen. Type X collagen null mice are described to have compression of the growth-plate (particularly in the region of chondrocyte hypertrophy), decreased deposition of trabecular bone and variable skeleto-haematopoietic phenotypes (Wallis, 1993; Kwan et al., 1997; Grskovic et al., 2012). Interestingly, mineralization appeared to be normal. Furthermore, perinatal lethality was seen in approximately 10.8% of null mutants (Wallis, 1993; Gress and Jacenko, 2000). In two human diseases resulting from Type X collagen mutations, Schmid metaphyseal chondrodysplasia and spondylometaphyseal dysplasia, individuals had short stature, bow
legs, coxa vara (a deformity of the femoral neck in which the angle between the neck and shaft is decreased) and a waddling gait due to growth plate abnormalities (Gress and Jacenko, 2000). To date, there are no conclusive in vivo results suggesting that Type X collagen is essential in the mineralization process as only subtle changes in mineralization were observed in Type X collagen null studies (Rosati et al., 1994; Kwan et al., 1997; Grskovic et al., 2012). Instead, it is proposed that Type X collagen functions to stabilize the ECM of the hypertrophic growth plate by interacting with proteoglycan monomers or Type II collagen (Schmid et al., 1990; Chen et al., 1992; Gress and Jacenko, 2000; Grskovic et al., 2012).

2.8.5 Proteoglycans

Proteoglycans, like the majority of non-collagenous matrix proteins, are anionic (Newman, 1998; Huber et al., 2000). The highly negatively charged chondroitin sulfate chains of proteoglycans attract and are able to bind $\text{Ca}^{2+}$ in solution and on the surface of hydroxyapatite (Hunter, 1987; Hunter and Szigety, 1992). At the time of calcification, these proteoglycans become focally concentrated in sites where mineral is deposited. It may be that calcium binds more readily to chondroitin-6-sulfate than to chondroitin-4-sulfate indicated by the observation that in the hypertrophic zone there is a much higher ratio of chondroitin-6-sulfate to chondroitin-4-sulfate (Poole et al., 1989). Boskey (1992) reported high affinity of epiphysis proteoglycans for calcium allowing them to serve as $\text{Ca}^{2+}$ reservoirs (Boskey, 1992).
It appears though that proteoglycans have multifunctional properties *in vitro*. When immobilized on agarose beads, proteoglycans are able to promote hydroxyapatite deposition (Linde et al., 1989). On the other hand, when in solution, proteoglycans are capable of inhibiting hydroxyapatite formation and growth (Boskey, 1992). Studies have shown that proteoglycans undergo changes before calcification begins; there is a decrease in the quantity, size and charge density of proteoglycans (Buckwalter et al., 1987; Yoshioka and Yagi, 1989). These modifications to proteoglycans are necessary to facilitate mineralization since large proteoglycan aggregates and small monomers can inhibit the growth of hydroxyapatite by adsorbing onto the surface of a mineral crystal and sterically block growth sites (Boskey, 1992). It is clear that the role proteoglycans play in the mineralization of articular cartilage is not fully understood and more research needs to be completed.

It is important to note that similar extracellular matrices to articular cartilage are found in non-mineralizing tissues such as skin and lung (Boskey, 1992). This suggests that the extracellular matrix is not solely responsible for mineral crystal formation, suggesting that other molecules or structures such as matrix vesicles, alkaline phosphatase, and matrix metalloproteinases, are important for extracellular matrix mineralization.

### 2.8.6 Matrix Vesicles

Degradation of the ECM and deep zone articular chondrocytes undergoing terminal differentiation into hypertrophic chondrocytes, similar to that in growth plate cartilage, are
characteristic of OA (Kirsch et al., 2000). The terminally differentiated hypertrophic chondrocytes may initiate mineralization by releasing matrix vesicles (MVs) (Thouverey et al., 2009). MVs are small (20-200 nm) spherical bodies bounded by a lipid bilayer derived from the plasma membrane of chondrocytes (Golub, 2009). However, the membrane composition of MVs differs from the parent membrane. Relative to the cell membrane from which they derived, the membrane of MVs is enriched in tissue non-specific alkaline phosphatase (TNAP), nucleotide pyrophosphatase phosphodiesterase, annexins (principally annexins II, V and VI) and phosphatidyl serine (Kirsch et al., 2000; Golub, 2009). MVs also contain matrix metalloproteinases (MMPs), which play a role in the digestion of triple helical collagens at neutral pH (Davidson et al., 2006).

Although similar numbers of MVs may be present in normal cartilage and in arthritic cartilage, it is in the latter that there is high TNAP activity and therefore able to induce mineralization (Ali, 1992). Another explanation by Kirsch et al. (2000) suggested that cartilage mineralization is under cellular control and only post-hypertrophic chondrocytes release mineralization competent MVs containing TNAP, annexin II, V and VI, and Type X collagen. It is believed that healthy and hypertrophic chondrocytes release vesicles that do not contain the necessary components to initiate hydroxyapatite formation (Ali, 1992; Kirsch et al., 2000).

Matrix vesicles are involved in the initial step of mineralization by promoting the formation of small crystals of calcium phosphate mineral in their lumen which are then released into the ECM where they are postulated to both mineralize collagen fibrils and
seed additional crystals (Kirsch et al., 2000; Golub, 2009; Thouverey et al., 2009). As discussed earlier, the influx of \( \text{Ca}^{2+} \) into the MVs are mediated by plasma membrane bound annexin V binding to Type II and X collagens. Blocking of annexin channel activities by 1,4-benzothiazepine derivative K-201 (JTV519) inhibits calcium uptake and subsequently inhibits mineralization (Kirsch et al., 2000). Along with calcium uptake, uptake of phosphate is critical for intravesicular crystal formation. It has been shown that inorganic phosphate enters through the type III \( \text{Na}^+ \) dependent phosphate transporter Glvr-1. However, transportation of phosphate is not strictly sodium dependent (Huitema and Vaandrager, 2007). The membrane of MVs is enriched in several phosphatases but most importantly TNAP. TNAP facilitates the increase of inorganic phosphate via hydrolysis of phosphates from various organic substrates (Golub, 2009; Orimo, 2010).

In summary, interactions of matrix vesicle phospholipids (which serve as nucleation sites), \( \text{Ca}^{2+} \), \( \text{PO}_4^{3-} \) and some MV proteins coalesce to form a molecular architecture that nucleates intravesicular apatite crystallization. These crystals then migrate to collagen, become inserted into the aligned hole zones of the fibrils, and undergo maturation in the presence of extracellular \( \text{Ca}^{2+} \) and \( \text{PO}_4^{3-} \) ions.

### 2.8.7 Alkaline Phosphatase

In 1924, Robison and Soames hypothesized that precipitation of calcium phosphate mineral was due to increased local inorganic phosphate concentrations mediated by the
hydrolysis of organic phosphates by alkaline phosphatase (ALP) (Robison and Soames, 1924). Almost a century later, this hypothesized role for ALP still holds true.

Alkaline phosphatase is a membrane-bound ectoenzyme that hydrolyzes monophosphate esters at a high pH (pH 8-10). There are 4 isoforms of human ALP: 1) tissue-nonspecific; 2) intestinal; 3) placenta; and 4) germ cell (Orimo, 2010). Tissue-nonspecific alkaline phosphatase (TNAP) is an isoenzyme of ALP, which is expressed in many tissues including liver, kidney and skeletal tissues such as hypertrophic chondrocytes and osteoblast cells (Tesch et al., 2003; Omelon et al., 2009). Almost 80% of the alkaline phosphatase activity of growth plate cartilage is present in matrix vesicles and only 20% in the cells (Ali, 1992). TNAP is notably increased at the site of calcification, and due to its temporal and spatial location, it is presumed to be involved in cartilage mineralization. Its postulated roles in the mineralization process are: 1) elevation of inorganic phosphate concentrations via hydrolysis of organic phosphate esters to facilitate precipitation of calcium phosphate; and 2) destruction of physiological crystal growth inhibitors such as pyrophosphate and polyphosphates (Register et al., 1986; Golub, 2009). Through studies using specific inhibitors for phosphatase activity the amount of calcification and mineral formation could be controlled under specific experimental conditions (Ali, 1992). Addition of levamisole, a specific inhibitor of TNAP activity prevented beta-glycerophosphate-induced mineralization in vitro (Tenenbaum, 1987).

The importance of TNAP in skeletal development is evident in human hypophosphatasia, a rare metabolic disorder where reduced TNAP activity results in
hypomineralization of the skeleton, and depending on the severity of the disease, leads to multiple dental and skeletal aberrations (Tesch et al., 2003). TNAP deficiency in mice also resulted in a significant overall decrease of mineral density in cartilage and bone matrix (Anderson et al. 2004). Normal mineral crystal formation took place within MVs but the proliferation and growth of mineral crystals once released by the MVs was inhibited. This finding suggests that in TNAP-deficient mice, hypomineralization results primarily from an inability of initial mineral crystals within MVs to self-nucleate and to proliferate beyond the protective confines of the MV membrane. The failure of the second stage of mineral formation may be caused by an excess of the mineral inhibitors pyrophosphate or polyphosphate in the extracellular fluid, which in normal circumstances would be hydrolyzed by TNAP (Anderson et al. 2004). Another study using TNAP knockout mice revealed developmental arrest of chondrocyte differentiation in epiphyses and in growth plates with diminished or absent hypertrophic zones (Fedde et al., 1999). These observations underline the sophistication of the temporal and spatial location of TNAP, and the selective location of enzyme enriched MVs in the calcification of growth plate cartilage.

2.8.8 Matrix Metalloproteinases

Matrix metalloproteinase (MMP) are a family of 25 zinc-dependent and calcium-dependent endopeptidases that are structurally and functionally related, and selected ones have been implicated in mineralization. A number of MMPs are secreted by resident cells of joint tissue, including MMP-1 (collagenase-1), MMP-2 (Gelatinase A), MMP-3
(stromelysin-1), MMP-8 (collagenase -2), MMP-9 (gelatinase B), and MMP-13 (collagenase-3) (Martel-Pelletier et al., 2001; Golub, 2009). MMPs are active around neutral pH and have the combined ability to degrade all the components of the extracellular matrix including the collagens, proteoglycans, fibronectin and laminin (Martel-Pelletier et al., 2001; Murphy et al., 2002). Many reports have implicated MMPs in pathological mineralization associated with arthritis, but they also play a significant role in the development and remodeling of skeletal tissues (Mort et al., 1993; Beertsen et al., 2002; Wu et al., 2002; Inada et al., 2004). In growth plate cartilage, MMPs are required for the developmental processes which result in calcification of the growth plate, and its subsequent replacement by endochondral bone (Golub, 2009).

Of the matrix metalloproteinases, MMP-13 is of particular interest because its expression pattern correlates with the presence of pathological chondrocytes that undergo hypertrophic differentiation in the early stage of OA development (Takaishi et al., 2008). Furthermore, it is associated with matrix vesicles, and appears to be under control of the transcriptional regulator, Cbfa1, the master regulator of bone formation (Golub, 2009). Its known substrates are collagens Type I, II, III and IV, aggrecan, gelatin, plasminogen activator inhibitor 2, perlecan, and tenascin (Murphy et al., 2002; Mackie et al., 2008). MMP-13 is thought to be most important for degradation of collagen within the cartilage because it cleaves Type II collagen 40-fold more effectively than MMP-1 (Takaishi et al., 2008; Golub, 2009). Triple-helical collagen molecules (Type I, II and III) are resistant to proteolysis by ordinary proteases and instead require specific collagenases such as MMP-13 to degrade these molecules (Golub, 2009). MMP-13 cleaves native triple-helical
fibrillar collagens at a single bond, generating characteristic one-quarter and three-quarter fragments (Murphy et al., 2002; Mackie et al., 2008). Its role in the development of skeletal tissues has been studied through MMP-13 null mice. Work by Inada et al. (2004) concluded that a null mutation resulted in abnormalities in growth plate development and delayed invasion of the ossification front. Studies with MMP-13 null mice confirm its requirement for normal removal of extracellular matrix and progression of the ossification front (Stickens et al., 2004).

It is suggested that MMP-13 predominates in OA where chondrocytes drive cartilage destruction (Murphy et al., 2002; Takaishi et al., 2008). In human osteoarthritic cartilage, MMP-1, MMP-8, and MMP-13 have been identified to increase in expression. MMP-1 and MMP-8 are located preferentially in the superficial zone of osteoarthritic cartilage, whereas MMP-13 is found mainly in the lower middle and deep zone (Martel-Pelletier et al., 2001). In the progression of OA, MMP-1 expression in chondrocytes increases steadily in the superficial zone, whereas MMP-13 levels reach a plateau at the moderate stage of disease (Martel-Pelletier et al., 2001). Another study by Takaishi et al. (2008) discovered co-localization of MMP-13 and Type X collagen in the area adjacent to the destructive lesion of osteoarthritic cartilage (Takaishi et al., 2008). This confirms the close connection between hypertrophic differentiation and MMP-13 production by chondrocytes. These findings suggest a close relationship between MMP-13 and the destruction of articular cartilage in OA.
2.8.9 In vitro Mineralization Induced by Beta-glycerophosphate

Beta-glycerophosphate (β-GP) has been shown to successfully stimulate cells in culture to mineralize (Bellows et al., 1991; Coe et al., 1992; Boskey et al., 1996; Yu, et al., 1997; Kandel et. al, 1999). Although other molecules containing phosphate, such as phosphethanolamine and adenosine triphosphate are capable of inducing mineralization, β-GP is most commonly used in culture studies (Anderson and Reynolds, 1973; Boskey et al., 1994; Kandel et al., 1999). In spite of this, the mechanism through which mineralization is induced by β-GP has not been fully elucidated. In the wide range of culture systems in which the effects of β-GP on gene expression and protein production have been studied, the results have been contradictory (Boskey et al., 1996).

While β-GP is predominantly cleaved by alkaline phosphatase (ALP), which leads to increased inorganic phosphate concentrations, several studies surprisingly reported decreasing ALP expression with mineralization (Tenenbaum, 1987; Sun and Kandel, 1999; Chung et al., 1992). Conflicting with these findings, other reports demonstrated increased levels of ALP in mineralizing chondrocyte culture systems (Gerstenfeld and Landis, 1991; Iwamoto et al., 1993; Kergosien et al., 1998). One explanation with respect to a role for ALP is that it plays an important role in the initiation, but not progression of organic phosphate-induced mineralization (Register and Wuthier, 1984; Bellows et al., 1991). However, more recent studies showed an increase in ALP activity with respect to culture duration (Azari et al., 2008; Abrahamsson et al., 2010; Teixeira et al., 2010). There is also a possibility that other phosphatases with increased affinity for β-GP are present, and can replace ALP (Tenenbaum et al., 1989). In addition to being a phosphate
source, β-GP has been shown to decrease the level of tyrosine kinase pp60c-src in hypertrophic chondrocytes (Coe et al., 1992), and affect phosphoprotein phosphatase and casein kinase II activity in chick limb-bud mesenchymal cells (Boskey et al., 1996). Moreover, treatment of CPO cultures with β-GP resulted in decreased incorporation of thymidine into the DNA; however, cellular proliferation was unaffected (Tenenbaum et al., 1989).

In a study by Sun and Kandel (1999), the addition of β-GP to deep zone chondrocyte cultures significantly inhibited expression of Type II and X collagen, and aggrecan core protein. Similar changes in gene expression were observed when phosphoethanolamine was used to induce mineralization (Sun and Kandel, 1999). The authors proposed the observed changes were related to the ongoing mineralization process and not the direct effect of β-GP addition. More importantly, the mineral formed in \textit{in vitro} grown tissue had similar characteristics in terms of size and appearance to the hydroxyapatite crystals present in \textit{in vivo} calcified cartilage, suggesting that the mineralization was not a dystrophic or pathological process (Kandel et al., 1997; Kandel et al., 1999). In support of this, addition of β-GP to superficial zone chondrocytes, chondrocytes that do not have the potential to form calcified tissue, formed tissue without mineral. Likewise, the changes in gene expression observed in deep zone chondrocyte were not seen in superficial zone chondrocytes (Sun and Kandel, 1999).
2.9 Inorganic Polyphosphate

Inorganic polyphosphates (polyPs) are linear polymers consisting of many tens or hundreds of orthophosphate residues linked by energy-rich phosphoanhydride bonds (Kawazoe et al., 2004). They are synthesized by the condensation of two orthophosphates, one orthophosphate and one polyP, or two polyP molecules through dehydration processes such as heating or enzymatic condensation by select kinases (Kornberg et al., 1999; Shiba et al., 2000; Omelon et al., 2009). The phosphoanhydride bonds are hydrolyzed by endopolyphosphatases resulting in the formation of two shorter polyP chains, or, more commonly by exopolyphosphatases resulting in an orthophosphate ion and a shorter polyP chain (Omelon et al., 2009).

Polyphosphates have been found in a wide range of organisms, including bacteria, fungi, algae, mosses, insects, and protozoa, and also in the tissues of higher plants and animals (Kulaev et al., 1999). The presence of polyP has been established in mammalian nuclei, mitochondria, lysosomes, and plasma membranes, and in human peripheral blood mononuclear cells, erythrocytes, gingival fibroblasts, osteoblasts (relatively high levels), and blood plasma (Gabel and Thomas, 1971; Kumble and Kornberg, 1995; Leyhausen et al., 1998; Kawazoe et al., 2004). Studies by Omelon et al. (2009) revealed that polyP is also present in growth plate cartilage (Omelon et al., 2009). In their study, they detected polyP in the ECM of hypertrophic murine growth plate cartilage using the fluorescent reporter DAPI (4',6-diamidino-2-phenylindole). Moreover, a quantitative analysis of the Ca:P molar ratio of growth plate cartilage showed that the ratio increased with proximity to the calcified cartilage. The proliferating cartilage cells exhibited a molar ratio of
approximately 0.8±0.05 corresponding to calcium polyphosphate (0.5) whereas the Ca:P ratio of calcified cartilage was up to 1.51±0.09, which is towards the value for hydroxyapatite (1.67) (Landis et al., 1982; Omelon and Grynpas, 2008).

2.9.1 Metabolism of Inorganic Polyphosphates

Although polyPs were first found in living organisms more than 100 years ago (Liebermann, 1888), its biological function remains uncertain. To better understand the function and pathways mediating polyP, its metabolism will be reviewed. Maintenance of polyP homeostasis is modulated through its synthesis by polyphosphate kinases and its degradation by polyphosphatases.

2.9.1.1 Synthesis of Inorganic Polyphosphate

Discovering and characterizing the enzymes that synthesize and degrade polyP is essential in determining the multiple functions of polyP. Insights into the biosynthesis of polyP were first discovered in protein extracts from *Escherichia coli* (Kornberg et al., 1956). The enzyme, later named polyphosphate kinase 1 (PPK1), catalyzes the formation of highly polymerized polyPs from the terminal (γ) phosphate of adenosine-5'-triphosphate (ATP) with a more favored reverse reaction (Kornberg, 1957). PPK is a homotetramer of 80-kDa subunits bound to the inner cell membrane (Kornberg et al., 1999). Over 100 homologs of PPK1 have been found in prokaryotic species (Rao et al., 2009). A homolog
of the enzyme was also found in a eukaryote, *Dictyostelium discoideum* (Zhang et al., 2005).

PPK1 insertion and null mutants resulted in lowered levels of polyP, whereas PPK1 overexpression resulted in increased levels of polyP (Crooke et al., 1994; Kuroda et al., 1997). Despite the fact that polyPs were found in yeast and higher eukaryotes, sequence comparisons with several genome databases showed no significant PPK homologies (Hooley et al., 2008). Furthermore, lysates of yeast and animal cells did not detect PPK-like activity (Kornberg et al., 1999). Due to the absence of PPK in these species it has been suggested that synthesis of polyPs involve other polyphosphate kinases (Kumble and Kornberg, 1995; Pavlov et al., 2010). In addition, a study by Cowling and Birnboim (1994) using 32P-labeled orthophosphate proposed that polyP synthesis occurs independent of ATP (Cowling and Birnboim, 1994). To date, it is not known how PPK1 converts ATP to polyPs of 700-800 orthophosphate residues since intermediate polyP chain-lengths have not yet been detected (Kornberg et al., 1999).

Although PPKs have been extensively studied in *E. coli*, they have also been purified from many other prokaryotes. Examination of the bacterial genomes revealed a high degree of PPK conservation (Kornberg et al., 1999). Similar to PPK1, PKK2 identified in *Pseudomonas aeruginosa* is also able to catalyze the formation of polyP from ATP in a reversible reaction (Ishige et al., 2002). Moreover, PPK2 is able to convert guanosine diphosphate (GDP) to guanosine triphosphate (GTP) in a reversible reaction using polyP as the orthophosphate donor and acceptor. Interestingly, GDP is preferred
over adenosine diphosphate (ADP) as the orthophosphate acceptor. A homolog of PPK2 has been identified in another prokaryote, *Corynebacterium glutamicum*, but has not been identified in higher organisms (Lindner et al., 2007). A study by Gomez-Garcia and Kornberg (2004) identified a previously unrecognized PPK in *Dictyostelium discoideum*, the only eukaryote known to have PPK1 activity similar to bacterial PPK1 (Gomez-Garcia and Kornberg, 2004). This third class of enzyme with PPK activity, designated PPK3, is likely a complex of three actin-related proteins (Arp). Analysis of the single 43kDa band revealed the proteins to be Arp1, Arp2, and Actin 28, which show an amino acid identity of 60%, 72% and 62% with human Arp1, Arp2 and Actin, respectively (Hooley et al., 2008). PPK3 assembles into sedimentable filaments in the presence of ATP and simultaneously synthesizes polyPs in a fully reversible reaction (Gomez-Garcia and Kornberg, 2004).

Most research exploring the synthesis of polyP has been performed in microorganisms and simple eukaryotes. Although these findings may be representative models and potential candidates of polyP synthesis in vertebrates little is known about the pathways involved, and therefore the information must be interpreted with caution.

### 2.9.1.2 Degradation of inorganic polyphosphate

Determining the enzymes involved in the degradation of polyP will help establish a better understanding of the functions of polyP and the pathways modulating their levels. The phosphoanhydride bonds of polyP are hydrolyzed by a group of enzymes called
polyphosphatases. These enzymes can be divided into 3 categories: 1) transferases and
kinases, which phosphorylate a molecule using polyP as the orthophosphate donor; 2)
exopolyphosphatases, which hydrolyze polyP at the end of the chain to produce a shorter
chain and an orthophosphate ion; and 3) endopolyphosphatases, which hydrolyze polyP in
the middle to produce two shorter chains.

Like exopolyphosphatases, transferases and kinases attack the terminal residue of
polyP or ATP (Kornberg et al., 1999; Omelon et al., 2009, Rao et al., 2009). These
enzymes have been studied in prokaryotes but have yet to be studied in higher organisms.
Polyphosphate:adenosine monophosphate phosphotransferase, first purified from
Acinetobacter (Bonting et al., 1991), utilizes polyP as the donor where as nicotinamine
adenine dinucleotide kinase and glucokinase can utilize both polyP and ATP (Rao et al.,
2009).

In extracts of E. coli, the exopolyphosphatase (PPX) activity levels were similar to
those of PPK and vary little in response to large fluctuations in polyP levels (Kornberg et
al., 1999). Interestingly, the gene encoding PPX is immediately downstream of the gene
encoding PPK (Akiyama et al., 1992). These observations suggest that PPX and PPK
closely regulate polyP homeostasis. PPX of E. coli has a preference for long chain polyPs
and cleaves the terminal residue of polyP to orthophosphate almost to completion
(Akiyama et al., 1993). Similarly, the PPX of Saccharomyces cerevisiae (designated
scPPX1) also has preference for longer chained polyPs. scPPX1 is more powerful and has
a broader size range for polyP compared to PPX of E. coli. It is able to hydrolyze polyPs
ranging in length from 3-1000 residues, but is unable to hydrolyze pyrophosphates, trimetaphosphates and ATP (Wurst and Kornberg, 1994). This difference in preferred substrate is evident in the many enzymes with exopolyphosphatase activity purified from the cell envelope, cytosol, vacuoles, mitochondria, and nucleus of *S. cerevisiae* (Andreeva and Okorokov, 1993; Andreeva et al., 1998a; Andreeva et al., 1998b; Lichko et al., 1998; Lichko et al., 2000; Lichko et al., 2002; Lichko et al., 2003). These exopolyphosphatases not only differ in location within the cell but also physiochemical properties, polyP chain length substrate preference, metal cation requirements, and optimal pH. These findings reveal exopolyphosphatases display substrate and compartment specificity, which aids to uncover the biological roles of polyP.

Like exopolyphosphatases in yeast, different enzymes with exopolyphosphatase activity have been identified in mammalian tissues. Of the mammalian exopolyphosphatases, alkaline phosphatase (ALP) is the most frequently referenced enzyme and provides more pertinent information to this study. In humans, four isoforms of ALP exist: 1) tissue-nonspecific; 2) intestinal; 3) placenta; and 4) germ cell (Orimo, 2010). Due to its expression in hypertrophic chondrocytes and osteoblast cells, tissue-nonspecific alkaline phosphatase (TNAP) is of particular interest (Tesch et al., 2003; Omelon et al., 2009).

TNAP from bovine epiphyseal cartilage hydrolyzes a variety of phosphate esters, ATP and inorganic pyrophosphate at pH 10.5, 10.0 and 8.5, respectively (Fortuna et al., 1979). Omelon et al. (2009) observed that TNAP hydrolyzes polyP with an average chain
length of 28 orthophosphate residues at a pH of 9.0, and in the absence of divalent metal cations (Omelon et al., 2009). Intestinal alkaline phosphatase (IAP), like TNAP, also hydrolyzes polyPs in the absence of divalent cations. However, the optimal pH for IAP is 9.5 and it is capable of hydrolyzing inorganic pyrophosphate as well as polyPs with chain lengths up to 800 orthophosphate residues (Lorenz and Schroder, 2001). A novel enzyme whose major enzymatic function is hydrolysis of polyP in animals is h-prune (Tammenkoski et al., 2008). Experiments on substrate specificity, metal cofactor requirements and optimal pH revealed h-prune has preference for short chain polyPs and does not hydrolyze pyrophosphate or ATP, favors Mg$^{2+}$ or Co$^{2+}$ as the metal cofactor and its enzymatic activity is optimal at near neutral pH. This study also revealed that h-prune does not possess endopolyphosphatase activity.

Since polyPs are characterized by a bimodal chain length distribution in vertebrate cells (Pisoni and Lindley, 1992; Lorenz et al., 1997), cells containing short-chain exopolyphosphatases require enzymes to metabolize longer-chain polyPs. Endopolyphosphatases (PPN) responsible for hydrolyzing long polyPs in to shorter polyPs have been identified in mammalian cells (Kumble and Kornberg, 1996). PPN in S. cerevisiae favor Mn$^{2+}$ or Mg$^{2+}$ as the metal cofactor and its enzymatic activity is optimal near pH of 7.5. Experiments on substrate specificity showed PPN hydrolyzes polyPs with chain lengths up to 750 orthophosphate residues to produce predominantly polyPs with 60 orthophosphate residues in length and tripolyphosphates, as well as orthophosphates as final digestion products. Endopolyphosphatase producing similar digestion products as
PPN identified in *S. cerevisiae* have been partially purified from rat and bovine brain (Kumble and Kornberg, 1996).

### 2.9.2 Inhibition of Mineralization by Inorganic Polyphosphate

Polyphosphates have been widely used as industrial and domestic water softeners to prevent deposition of calcium carbonate (Larson, 1957). They are able to inhibit the conversion of slurry amorphous calcium phosphate in solution to crystalline hydroxyapatite. In 1961, Fleisch and Neuman observed increased amounts of calcium and phosphate required for spontaneous formation of hydroxyapatite crystal in solutions of collagens supplemented with polyPs (Fleisch and Neuman, 1961). Their study established that polyPs are able to inhibit hydroxyapatite formation, even at concentrations as low as $10^{-7}$ M. Inhibition of apatite formation at such low concentrations of polyP cannot be explained simply by lowering of the ionized calcium concentration by chelation, although this may be part of the inhibitory process. They proposed that polyP is able to stabilize the amorphous calcium phosphate, not by affecting its dissolution, but by adsorbing to the apatite growth sites preventing its growth. Their conclusion was supported by other reports that suggest polyP regulates mineralization by poisoning previously formed crystals to prevent their growth (Blumenthal et al., 1977; McGaughey and Stowell, 1977). These studies proposed polyPs inhibit apatite formation by: 1) poisoning heteronuclear growth sites; 2) poisoning the growth of heterogeneously and/or homogeneously formed hydroxyapatite nuclei before their critical size is reached; or 3) poisoning both. Poisoning
of the hydroxyapatite nuclei would drive it to redissolve back into solution, preventing hydroxyapatite crystal formation.

A study by Fleish et al. (1965) showed that vitamin D-induced aortic calcification in a rat can be prevented through subcutaneous injection of pyrophosphate or polyPs (Fleish et al., 1965). Furthermore, the effectiveness of polyP in inhibiting tissue mineralization was dependent on dose concentration. This notion was supported by a later study that showed the amount of mineral deposited in the bone of *in vitro* maintained embryonic chicken femurs was significantly reduced when grown in medium containing high concentrations of polyP (4 and 16µg of phosphorous per milliliter) (Fleish et al., 1966). In contrast, embryonic chicken femurs maintained in medium containing 1µg of phosphorous per milliliter resulted in higher amounts of mineral deposited in the bone.

Similar findings were seen in more recent work by St-Pierre et al. (2010), which demonstrated that continuous presence of polyP in culture inhibited cartilage mineralization induced by β-GP in a concentration and chain length dependent manner (St-Pierre et al., 2010). Exogenous addition of polyPs ranging in concentration from 0.01 – 1.0mM had an increased inhibitory effect on mineralization with increased polyP concentration. This positive correlation appeared to plateau at 0.5mM at which point increasing the polyP concentration did not have a further effect on the inhibition of mineralization. This supports the proposed hypothesis that inhibition of hydroxyapatite crystal formation occurs through adsorption of polyP to growth sites of the crystals instead of calcium chelation. Furthermore, exogenous addition of polyPs with average chain
lengths of 45 and 75 phosphate units exhibited significantly better inhibition of mineralization compared to shorter chain length polyPs (5 phosphate units) even when correcting for phosphate concentration (St-Pierre et al., 2010).

Interestingly, polyPs are found in vertebrate cells in two distinct classes of polymers: longer chain (high molecular) and shorter chain (low molecular) factions (Pisoni and Lindley, 1992; Lorenz et al., 1997). A study with human promyelocytic leukemia HL-60 cells showed that loss of longer chain polyPs induced cell apoptosis (Lorenz et al., 1997). This study also showed a decrease in the amount of long-chain polyPs in brain tissue during ageing. These findings, along with the study on cartilage mineralization by St-Pierre et al. (2010), suggest that polyphosphate chain length influences its biological function (Lorenz et al., 1997; St-Pierre et al., 2010).

2.9.3 Induction of Mineralization by Inorganic Polyphosphate

Contrary to what was observed in the studies cited in the previous section, Kawazoe et al. (2004) showed that polyP induced mineralization in MC3T3-E1 pre-osteoblast cells grown in monolayer. Polyphosphate treated MC3T3-E1 had more apatite crystal formation than MC3T3-E1 treated with β-GP, as demonstrated by alizarin red staining (Kawazoe et al., 2004). Another study was able to promote the regeneration of alveolar bone in rats by treating with polyP (Hacchou et al., 2007). Similar to rats, the promotion of alveolar bone regeneration was also observed in polyP-treated beagle dogs (Usui et al., 2010). In addition to stimulating mineralization, polyP enhanced differentiation of
MC3T3-E1 into mature osteoblasts and increased osteocalcin, osteopontin, osterix, bone sialoprotein, and TNAP gene expression (Kawazoe et al., 2004). These findings suggest that polyP affects mineralization in ways other than directly modulating calcium and orthophosphate levels and crystal poisoning. Revisiting experiments by Fleish’s group, while they were able to inhibit vitamin D-induced aortic calcification in rats, they were not able to inhibit calcifications in the kidney (Schibler et al., 1968). The inability to inhibit calcification within the kidney was believed to be due to the high TNAP activity in the kidney, which hydrolyzed polyPs to levels that favoured tissue mineralization. This clarifies the importance of maintaining a steady level of polyP to facilitate the inhibition of mineralization (Schibler et al., 1968). Furthermore, these findings allude to the dual ability of polyphosphate to affect cellular processes that activate or inhibit tissue mineralization.

### 2.9.4 Proposed Mechanism by which Inorganic Polyphosphate Modulates Mineralization

The presence of polyPs in mammalian tissues has been well established (Gabel and Thomas, 1971; Kumble and Kornberg, 1995; Leyhausen et al., 1998; Kawazoe et al., 2004). Presence of polyPs in osteoblasts, resorption pits and growth plate cartilage are of particular interest because of the ability of the cells to promote mineralization. Polyphosphates are more highly concentrated in the hypertrophic zone of growth plate cartilage, just above the calcifying cartilage (Omelon et al., 2009). Due to the temporal and spatial location of polyP, it is believed to play an important role in modulating apatite biomineralization.
Omelon et al. (2009) have proposed a simplified mechanism for apatite mineral formation modulated by polyP. When biomineralization is undesirable, for example during bone resorption or within articular cartilage, polyPs are maintained at a steady level or synthesized which in turn reduces the free orthophosphate concentrations. Furthermore, the condensed phosphate polymers sequester free calcium ions reducing the amount of free orthophosphate and calcium ions to ultimately prevent formation of apatite crystals. Additionally, as discussed in a previous section, polyPs also adsorb to apatite crystals and inhibit their growth. In contrast, when biomineralization is required, for example during endochondral ossification and bone remodeling, destruction of the apatite mineralization inhibitor—polyphosphate is necessary. TNAP, a recognized exopolypophosphatase, hydrolyzes polyP releasing orthophosphate ions and calcium ions from their chelation with polyP. Degradation of polyP results in increased free orthophosphate and calcium concentrations, which promotes formation of apatite crystals (Omelon et al., 2009).

The proposed mechanism is supported by a more recent study, which through DAPI staining visualized the presence and absence of polyP in non-mineralizing and mineralizing in vitro-formed deep-zone cartilage, respectively (St-Pierre et al., 2010). Currently, little is known about the regulation of enzymes involved in the metabolism of polyP and the mechanism(s) by which polyP levels are maintained. A better understanding of how polyP modulates mineralization and the pathways involved are essential in understanding tissue homeostasis, mineralization and osteoarthritis.
2.9.5 Biological Functions of Inorganic Polyphosphate

The biological functions of polyP have been investigated mostly in microorganisms; there have been relatively few studies in eukaryotic cells, particularly in mammals. The following functions for polyP have been proposed: 1) alternate source of energy to ATP; 2) orthophosphate reservoir; 3) chelator of divalent cations; 4) blood coagulation and fibrinolysis; 5) an antibacterial agent; 6) structural element in competence for DNA entry and transformation; 7) a regulatory factor of gene expression; and 8) have recently been linked to apatite biomineralization processes (Kumble and Kornberg, 1995; Kornberg et al., 1999; Kulaev et al., 1999; Schroder et al., 1999; Shiba et al., 2000; Omelon et al., 2009; St-Pierre et al., 2010).

Inorganic polyphosphate has been associated with the modulation of mammalian cell proliferation in vitro. A study by Shiba et al. (2003) revealed a novel function of polyP in connection with the modulation of mitogenic activity of acidic and basic fibroblast growth factors (FGFs), respectively, FGF-1 and FGF-2. In this study, the group determined that exogenous addition of polyP enhanced the proliferation of normal human fibroblast cells. Through physical interaction with FGF-2, polyP physically and functionally stabilized the growth factor. Polyphosphate was shown to enhance the functions of the growth factor by strengthening the affinity and facilitating binding between FGF-2 and its cell surface receptors (Shiba et al., 2003). Interaction between polyP and FGF-2 was verified in a study by Kawazoe et al. (2008), where the addition of polyP enhanced proliferation of FGF-2-producing human dental pulp cells (Kawazoe et al., 2008).
The combined effect of FGF-2 and polyP was also studied on MC3T3-E1 pre-osteoblast cells (Yuan et al., 2009). Treatment of these cells with FGF-2 and polyP (average chain length of 60 phosphate residues) resulted in increased cell proliferation, ALP activity and calcification compared to non-treated or treated with FGF-2 or polyP alone. Another study showed an increase in exopolyphosphatase activity in in vitro-cultured deep zone chondrocytes treated with polyP (average chain length of 45 phosphate residues) (St-Pierre et al., 2010). Furthermore, polyP administered to full thickness articular cartilage grown in vitro promoted the accumulation of GAG and collagen in the extracellular matrix (St-Pierre et al., 2012). This anabolic effect of polyphosphate was positively correlated with increased concentration and chain length.

The previous studies show a clear interaction between polyP and FGFs, however, the mechanisms by which they modulate cell proliferation, extracellular matrix production and tissue mineralization remain poorly understood and have to be investigated further.

### 2.10 Fibroblast Growth Factor Signaling and Cartilage

Fibroblast growth factors (FGFs) have been shown to be involved in the earliest stages of limb development (Martin, 1998). The FGF family consists of 22 genes that encode structurally related proteins. Mammalian FGFs can be divided into three groups: intracellular (FGF11-14), hormone-like (FGF15/19, 21, 23) and canonical (FGF1-10, 16-18, 20, 22) (Itoh and Ornitz, 2008). FGFs are ligands for four high-affinity FGF receptor (FGFR) tyrosine kinases (FGFR1 to FGFR4) (Ornitz and Marie, 2002). Canonical FGFs
are paracrine factors that when secreted bind and activate FGFRs. They affect proliferation and differentiation of many cell types during development and tissue repair, including chondrocytes (Degnin et al., 2010). Point mutations in Fgfr1, Fgfr2, and Fgfr3 result in several human skeletal dysplasia syndromes suggesting that FGFR signaling is an essential component of the regulatory cascades governing endochondral ossification and skeletal growth (Naski and Ornitz 1998). Research to identify endogenous ligands for these receptors expressed in the epiphyseal growth plate and perichondrium remain elusive, however, studying limb development may reveal some clues.

Two members of the canonical family, FGF-2 and FGF-18, are of particular interest since they are involved in maintaining cartilage homeostasis (Osborn et al., 1989; Stewart et al., 2007; Ellsworth et al., 2002; Moore et al., 2005; Ellman et al., 2008). The first FGF to be isolated from growth plate chondrocytes was FGF-2 (Sullivan and Klagsbrun, 1985). Targeted inactivation of FGF-2 in mice resulted in decreased bone growth and bone density (Montero et al., 2000). Moreover, cultures of extracted bone marrow stromal cells from FGF-2 knockout mice exhibited decreased mineralization compared to wild-type extracted bone marrow stromal cells. However, contradictory effects were observed in another study in which non-targeted over-expression of Fgf2 in mice also resulted in decreased endochondral and intramembranous bone formation (Sobue et al., 2005). Correspondingly, FGF-2 was shown to inhibit the terminal differentiation of isolated growth plate chondrocytes and down-regulate ALP activity, which is known to induce mineralization (McCarthy et al., 1989; Rodan et al., 1989; Hill et al., 1991; Hurley et al., 1993). Yet still, in another study, exogenous addition of FGF-2
to *in vitro* grown fetal rat metatarsals accelerated endochondral ossification and inhibited longitudinal bone growth (Mancilla et al., 1998).

In articular cartilage, chondrocytes produce FGF-2, store it in the ECM and release it upon damage to the tissue (Vincent et al., 2002). Studies show FGF-2 to be a potent mitogen of articular chondrocytes, however, the anabolic effects of FGF-2 on the production of ECM molecules have been contradictory, as studies from a variety of species have yielded opposing results. FGF-2 was shown to stimulate chondrocyte proliferation and enhance the production of ECM by adult bovine (Sah et al., 1994), murine (Chia et al., 2009), and lapine (Kaul et al., 2006; Madry et al., 2010) articular chondrocytes. In contrast, although FGF-2 exerted a mitogenic effect on human adult articular cartilage, it is believed that this is a sign of degeneration rather than regeneration as it failed to increase ECM production (Loeser et al., 2005; Ellman et al., 2008). The inconsistent results may be connected to the concentration of FGF-2 present. A study by Mancilla et al. (1998) showed that exogenous addition of low concentrations (10 ng/ml) of FGF-2 stimulated cartilage matrix production, whereas high concentrations (1000 ng/ml) inhibited matrix production (Mancilla et al., 1998).

A more recent study by Yan et al. (2011) suggests the contradictory findings are a result of the receptor activated by FGF-2. Human adult articular chondrocytes dynamically express FGFR1 to FGFR4, with FGFR1 and FGFR3 being the predominant isoforms expressed (Yan et al., 2011). The study demonstrated that FGF-2 selectively activates FGFR1 to enhance chondrocyte proliferation and exert catabolic effects by
stimulating MMP-13 expression and reducing aggrecan accumulation. Inhibiting FGFR1 signaling reversed the catabolic effects of FGF-2 (Yan et al., 2011). These findings are consistent with an in vivo murine study showing deletion of the Fgfr1 gene had a chondroprotective effect due to decreased expression of MMP-13 and up-regulation of FGFR3 (Weng et al., 2012). FGFR3-related pathways are believed to be chondroprotective because they exert anabolic effects, which stimulate ECM formation by increasing proteoglycan synthesis and downregulating MMP-13, and inhibit cell proliferation (Ellman et al., 2008; Vincent, 2012). Interestingly, FGF-2 levels are abnormally elevated in synovial fluid of osteoarthritic patients (Im et al., 2007). Furthermore, in osteoarthritic chondrocytes FGFR1 expression is significantly upregulated relative to FGFR3 expression, which is down regulated (Yan et al., 2011). The catabolic effects of FGFR1 and anabolic effects of FGFR3 seem to be essential for articular cartilage homeostasis and the balance between FGFR1 and FGFR3 signaling, and their cognate ligands may contribute to the pathophysiology of OA.

Another FGF found to play a prominent regulatory role in skeletal development and maintenance of articular cartilage homeostasis is FGF-18 (Marie, 2003; Moore et al., 2005; Haque et al., 2007; Liu et al., 2007). In the growth plate, FGF-18 signals through FGFR1 and IIIc splice variants of FGFR2 and FGFR3 to regulate chondrocyte proliferation and differentiation as well as vascular invasion (Shimoaka et al., 2002; Liu et al., 2002; Ohbayashi et al., 2002; Ellsworth et al., 2002; Davidson et al., 2005; Marie et al., 2005; Ellman et al., 2008). In growth plate cartilage, expression of FGFR1 is restricted to prehypertrophic and hypertrophic chondrocytes where its activation by FGF-
18 regulates cell survival, differentiation, and apoptosis (Barnard et al., 2005). Activation of FGFR2 by FGF-18 in growth plate cartilage is associated with osteogenesis and bone formation (Eswarakumar et al., 2002; Haque et al., 2007). FGF-18 acts through its preferential receptor, FGFR3, which is expressed by chondrocytes in the resting and proliferating zone to negatively regulate chondrocyte proliferation and differentiation (Shimoaka et al., 2002; Liu et al., 2002; Ohbayashi et al., 2002; Ellsworth et al., 2002 Davidson et al., 2005; Haque et al., 2007). Activation of FGFR3 by FGF-18 inhibits Indian hedgehog (Ihh) which acts through a negative feedback loop with parathyroid hormone related peptide (PTHrP) to control proliferation and maturation of growth plate chondrocytes and the mineralization of growth plate cartilage (Lanske et al., 1996; Vortkamp et al., 1996; Naski et al., 1998; Karp et al., 2000; Chung et al., 2002; Kobayashi et al., 2002). The Ihh-PTHrP negative feedback loop is of particular interest because it has been reported to be the mechanism also regulating mineralization in articular cartilage (Jiang et al., 2008). This will be discussed in greater detail in a subsequent section.

Studies on other cartilaginous tissues apart from the growth plate show FGF-18 to have significant anabolic effects (Ellsworth et al., 2002; Ohbayashi et al., 2002; Shimoaka et al., 2002; Moore et al., 2005). FGF-18 delivered to the pinnae of nude mice increased the number of chondrocytes and production of extracellular matrix molecules (Ellsworth et al., 2002). Similarly, FGF-18 has been shown to stimulate chondrocyte proliferation and production of extracellular matrix molecules in mature articular cartilage (Ellsworth et al., 2002). Furthermore, intra-articular injections of FGF-18 to a rat meniscal tear model of osteoarthritis stimulated de novo cartilage formation and reduced cartilage degeneration.
in a dose-dependent manner (Moore et al., 2005). A study by Yan et al (2011) demonstrated that FGF-18 selectively signals through FGFR3 in human articular cartilage to promote cartilage-protective responses (Yan et al., 2011). Furthermore, the study also found that FGFR3 was significantly reduced in OA suggesting FGF-18 and FGFR3 have a cartilage-protective role during the development of OA. Taken together with its involvement in regulating chondrocyte maturation and bone formation, FGF-18 could be a therapeutic candidate for the repair of cartilage defects and OA prevention.

A more recently identified FGF shown to be involved in chondrocyte maturation and mineralization is FGF-23. FGF-23 protein has been localized to the hypertrophic chondrocytes of growth plate cartilage where it acts as a negative regulator of chondrogenesis and may induce premature hypertrophy (Sanchez and He, 2007; Orfanidou et al., 2009). Unlike most FGFs that act in paracrine and autocrine manner, FGF-23 acts as an endocrine factor and can be detected in normal serum (Yu et al., 2005). This circulating phosphaturic factor is essential in regulating serum phosphorous homeostasis and Vitamin D3 metabolism (Shimada et al., 2004; Quarles, 2008), which are both closely associated with mineral homeostasis (Orfanidou et al., 2012). High concentrations of phosphate induce terminal chondrocyte maturation and subsequent mineralization (Mansfield et al. 1999; Magne et al. 2003; van Donkelaar et al., 2007; Orfanidou et al., 2012). Elevated levels of phosphate and Vitamin D3 were detected in FGF-23 null mice that developed skeletal abnormalities and increased mineralization, as well as excessive tissue calcification in the heart, kidney and lung (Sitara et al., 2004; Razzaque et al., 2006).
Interestingly, FGF-23 expression is low in normal articular chondrocytes and significantly elevated in osteoarthritic chondrocytes (Orfanidou et al., 2009). The study by Orfanidou et al. (2009) showed that FGF-23 activates RUNX-2 expression, which in turn up-regulates MMP-13 expression. A later study by the same group showed that FGFR1c mRNA and protein levels were also significantly higher in osteoarthritic chondrocytes (Orfanidou et al., 2012). Although FGF-23 binds and activates the c splice isoform of FGFr1-3 and FGFr4 (Yu et al., 2005), Orfanidou et al. (2012) observed that FGF-23 exhibits greater binding to FGFR1c in osteoarthritic chondrocytes than in normal chondrocytes, suggesting FGFr1c to be the biologically relevant receptor (Orfanidou et al., 2012). An *in vivo* study by Gattineni et al. (2009) also showed that FGFR1 is the predominant receptor for the hypophosphatemic action of FGF23 (Gattineni et al., 2009). Given that altered mineral-ion homeostasis contribute to chondrocyte hypertrophy and mineralization, elucidation of the pathway by which the novel phosphaturic hormone regulates mineral-ion levels and vitamin D metabolism is essential to understand OA development and progression.

### 2.11 Role of Superficial Zone Chondrocytes in Osteoarthritis

In OA, tensile, compressive and shear behaviors dramatically diminish with time. Many studies suggest that this is due to thinning and deterioration of the superficial zone (SZ) along with advancement of the zone of calcified cartilage into uncalcified cartilage (Guilak et al., 1994; Oegema et al., 1997; Setton et al., 1999; Silver et al., 2001; Pritzker et al., 2006; Duer et al., 2009). Damage to the SZ of articular cartilage, either with age or
disease, is one of the first observed macroscopic changes and leads to changes in the mechanical properties of the tissue and with disease progression the deep zone (DZ) of articular cartilage shows progressive mineralization (Buckwalter and Mankin, 1997; Balcerzak et al., 2003; Pearle et al., 2005; Pritzker et al., 2006; Fuerst et al., 2009).

Breakdown of the densely packed collagen matrix in the SZ is the first observed change in both aging and osteoarthritic articular cartilage (Hollander et al., 1995; Stoop et al., 2001; Hayami et al., 2006; Pritzker et al., 2006). Fibrillation of the collagen network extends progressively down to the deeper zones with gradual denaturation. It has been suggested that superficial zone chondrocytes (SZC) become catabolically more active in the earlier stages of OA, which initiates cartilage degeneration at the articulating surface (Hollander et al., 1995; Lark et al., 1997). In sites where Type II collagen degradation is observed, SZC express MMP-1 and MMP-13 as well aggrecanases (Lark et al., 1997; Chambers et al., 2001; Wu et al., 2002). Intense immunostaining for these catabolic enzymes along with their degradation products were detected in the pericellular matrix of SZC (Hollander et al., 1995; Chambers et al., 2001; Wu et al., 2002). In the later stages of OA, SZC begin to express alkaline phosphatase, annexin II, annexin V and Type X collagen; all proteins normally expressed by hypertrophic chondrocytes (Fukui et al., 2008). Furthermore, these changes are accompanied by SZC cell death, which has been associated with mineralization (Kirsch et al., 2000). These observations provide evidence that degradation of matrix constituents is mediated by SZC at the denaturation front. The catabolic enzymes responsible for degradation of the ECM originate within the
pericellular matrix of SZC and with disease development extend to the territorial and interterritorial matrix (Hollander et al., 1995; Wu et al., 2002).

The collagen network of the SZ is responsible for isolating cartilage from the immune system and providing tensile strength to withstand shear forces (Buckwalter and Mankin, 1997; Poole et al., 2001; Pearle et al., 2005; Bhosale and Richardson, 2008). Deterioration of the collagen network results in decreased mechanical properties as well as an immune response causing inflammation within the synovial cavity (Buckwalter and Mankin, 1997; Pearle et al., 2005; Bhosale and Richardson, 2008). Degradation of the collagen network as observed in osteoarthritic cartilage can be induced \textit{in situ} by culturing healthy human articular cartilage with interleukin 1 (IL-1) (Dodge and Poole, 1989; Mort et al., 1993). The studies confirm denaturing of Type II collagen is mediated by chondrocytes and can be induced \textit{in vitro} and \textit{in vivo}. Elevated levels of cytokines such as IL-1 and tumor necrosis factor-alpha (TNF-α) have been reported in damaged and diseased articular cartilage (Coates and Fisher, 2010). It has been shown that cytokines increase the production of nitric oxide (NO) and MMPs, and induce cell death (Fukuda et al., 1995; Hayashi et al., 1997; Häuselmann et al., 1998; Tagi et al., 1998). Interestingly, SZC produce two to three times more NO compared to deep zone chondrocytes (DZC) (Häuselmann et al., 1998). Enhanced NO production leads to more severe inhibition of proteoglycan synthesis. Therefore, these studies draw attention to the elevated sensitivity of SZC to cytokines. SZC respond to lower amounts of IL-1 by producing higher levels of NO, which correspond to more severe inhibition of proteoglycan synthesis (Häuselmann et al., 1998).
Moreover, SZC decrease proteoglycan 4 (PRG4) expression in response to IL-1 and TNF-α (Jones and Flannery, 2007; Schmidt et al., 2008). The PRG4 gene encodes for superficial zone protein (SZP) as well as a highly homologous protein named lubricin, which are produced by SZC and synovial fibroblasts, respectively (Ikegawa et al., 2000; Jay et al., 2001). Abundant at the articulating surface of articular cartilage and in synovial fluid, SZP functions to prevent wear, lubricate the articular surface and prevent cartilage-cartilage adhesion (Jay et al., 1998; Schaefer et al., 2004; Rhee et al., 2005). PRG4 knockout mice showed loss of SZC, articular surface deterioration, increase in the coefficient of friction, synoviocyte overgrowth and ultimately joint failure (Rhee et al., 2005). Recent studies have shown that intra-articular administration of PRG4 in a rat model of OA was therapeutically effective in reducing cartilage degeneration and preventing disease progression (Flannery et al., 2009; Jay et al., 2010; Teeple et al., 2011). The expression of PRG4 by SZC can be upregulated by treatment with growth factors and morphogens such as transforming growth factor-beta (TGF-β), insulin-like growth factor-1 (IGF-1), and bone morphogenetic protein (BMP-2, -4, and -7) (Lee et al., 2008; Darling and Athanasiou, 2005; Jones and Flannery, 2007; Schmidt et al., 2008; Khalafi et al., 2007; Niikura and Reddi, 2007). An increase in PRG4 secretion and expression was also observed when SZC were cultured in the presence of ascorbic acid (Schmidt et al., 2004), and when co-cultured with middle zone chondrocytes (Blewis et al., 2007). These findings demonstrate that expression of PRG4 by SZC is important for maintaining cartilage homeostasis and TGF-β, IGF-1, BMP, ascorbic acid and cellular interactions may have implications for prevention of osteoarthritic disease.
Interestingly, cells with stem or progenitor capacity have been isolated from the SZ of articular cartilage (Dowthwaite et al., 2004; Hattori et al., 2007). Dowthwaite et al. (2004) isolated a progenitor cell population with chondrogenic potential and the ability to form large numbers of colonies from an initially low seeding density. Hattori et al. (2007) isolated cells with progenitor characteristics for both superficial and middle zone chondrocytes. What is more interesting is that mesenchymal progenitor cells were isolated from human osteoarthritic cartilage and in synovial fluid of osteoarthritic patients (Fickert et al., 2004; Jones et al., 2004). Isolation of cells with progenitor capacity suggests that deterioration of the SZ not only results in decreased mechanical properties but also loss of potential self-renewal capacity and articular cartilage maintenance.

The ECM of articular cartilage is semi-permeable to facilitate cellular interactions between chondrocytes through soluble factors, and the diffusion of nutrients (Maroudas, 1970; Maroudas, 1976; Quinn et al., 2000). Chondrocyte subpopulations synthesize and secrete zone-specific growth factors and cytokines to influence cell activity in a paracrine manner (Quinn et al., 2000; Blewis et al., 2007). Co-culture systems can be used to study these cellular interactions between chondrocyte subpopulations and determine the soluble factors involved. Co-culture studies have shown that SZC regulate DZC proliferation and promote the biosynthetic activity of the DZC to produce engineered tissue with enhanced mechanical properties (Sharma et al., 2007; Ng et al., 2009) as well as regulate deep zone calcification (Jiang et al., 2008). These findings highlight the importance of SZC in maintaining articular cartilage since proliferation of chondrocytes and degeneration of the
ECM are hallmark changes in OA (Setton et al., 1999; Silver et al., 2001; Quintavalla et al., 2005).

Numerous theories have been proposed to explain the advancement of the calcification front but to date the mechanism controlling chondrocyte mineralization remains unknown. SZC have been implicated in being an important signaling center involved in the maintenance of articular cartilage and suppressing mineralization by DZC. Determining the role of SZC in regulating DZ mineralization and the mechanism by which polyphosphate regulates cartilage mineral formation could have significant implications in understanding the pathology of OA. Elucidation of the biochemical process responsible for mineralization of articular cartilage is critical in the treatment and eventual prevention of OA.

2.12 Hypothesis

Loss of the superficial zone of articular cartilage is the earliest macroscopic change in the development of osteoarthritis and is followed by degradation of the extracellular matrix, duplication of the tidemark and advancement of the mineralization front into uncalcified cartilage (Hollander et al., 1995; Buckwalter and Mankin, 1997; Oegema et al., 1997; Setton et al., 1999; Silver et al., 2001; Stoop et al., 2001; Pearle et al., 2005; Hayami et al., 2006; Pritzker et al., 2006; Duer et al., 2009; Fuerst et al., 2009; Thambyah and Broom, 2009). The mechanism(s) regulating the advancing calcification of hyaline cartilage has not been elucidated. Superficial zone chondrocytes have been implicated in the
maintenance of articular cartilage and suppressing mineralization by deep zone chondrocytes (Jiang et al., 2008). It is hypothesized that superficial zone chondrocytes modulate polyphosphate levels in deep zone cartilage via a soluble factor to promote tissue formation and suppress mineralization by deep zone chondrocytes.

2.13 Specific Aims

I. Characterize the effect of superficial zone chondrocytes on mineral formation in cartilage formed in vitro by deep zone chondrocytes.

II. Investigate if superficial zone chondrocytes modulate endogenous inorganic polyphosphate levels in in vitro-formed deep zone cartilage.

III. Determine if FGF-18 modulates mineral formation and endogenous inorganic polyphosphate accumulation in in vitro-formed deep zone cartilage.
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CHAPTER 3: SUPERFICIAL ZONE CHONDROCYTES PRODUCE SOLUBLE FACTOR(S) THAT MODULATE POLYPHOSPHATE LEVELS AND SUPPRESS MINERALIZATION OF DEEP ZONE CARTILAGE

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As first author of this multi-author contribution, I helped design and conducted all the experiments. Histological sections were prepared by others. I also analyzed the results and wrote the manuscript.

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SUMMARY

Loss of the superficial zone of articular cartilage is an early change in osteoarthritis and with disease progression the deep zone (DZ) of cartilage shows progressive mineralization. To date, the mechanism(s) regulating post-natal articular cartilage mineralization is poorly understood. Previously, we have shown that inorganic polyphosphate inhibits mineralization of in vitro-formed DZ cartilage. We developed an indirect co-culture method to investigate the effect of superficial zone chondrocytes (SZC) on mineralization in DZ cartilage. Our findings suggest that SZC suppress mineralization by modulating polyphosphate levels in DZ cartilage via FGF-18. Furthermore, SZC promote glycosaminoglycan and collagen accumulation in the extracellular matrix of cartilage formed by DZ chondrocytes. This study provides insight into the interaction between chondrocyte subpopulations and possible mechanism(s) controlling post-natal articular cartilage mineralization. Moreover, the results of this study establish polyphosphate and FGF-18, separately or in combination, as therapeutic candidates for articular cartilage repair and osteoarthritis prevention.
3.1 Introduction

Articular cartilage is found at the articulating ends of diarthrodial (synovial) joints. Together with synovial fluid, this tissue provides a low-friction interface to allow for smooth articulation and distribution of applied forces to the underlying subchondral bone (Huber et al., 2000). Articular cartilage fulfills these functions through a complex depth-dependent zonal architecture. Cartilage can be divided into 4 zones: the superficial zone (SZ), the middle zone (MZ), the deep zone (DZ), and the zone of calcified cartilage (ZCC) (Buckwalter and Mankin, 1997; Newman, 1998; Poole et al. 2001). Although all zones contain aggrecan (proteoglycan), Type II collagen and water, each zone is characterized by a distinct composition and extracellular matrix (ECM) organization (Huber et al., 2000; Roughley, 2006; Cremer et al., 1998; Eyre et al., 2006).

The SZ comprises the first 10-20% of articular cartilage (Pearle et al., 2005). The collagen content in the SZ is the highest of any zone in articular cartilage and proteoglycan content is low. It is rich in Type I and II collagen fibrils, which are oriented parallel to the joint surface (Huber et al., 2000; Poole et al. 2001; Pearle et al., 2005). The chondrocytes produce molecules specific to this zone such as Wnts, BMP and TGF-β family members, lubricin/superficial zone protein (encoded by the gene PRG4) and clusterin (Anderson et al., 2000; Yamane et al., 2007; Schumacher et al., 1994; Khan et al., 2001). The MZ represents 40-60% of articular cartilage and is composed of larger, randomly oriented collagen fibrils (Pearle et al., 2005; Buckwalter and Mankin, 1997; Buckwalter et al., 2005; Hasler et al., 1999) and the proteoglycan concentration is higher than in the SZ, providing it with the ability to withstand compressive forces (Poole et al.
The DZ has the largest collagen fibrils leading to the lowest water content of any of the zones of articular cartilage, despite the highest proteoglycan content and lowest collagen content (Poole et al. 2001; Pearle et al., 2005; Buckwalter et al., 2005). The collagen fibrils are oriented perpendicular to the joint surface (Pearle et al., 2005; Buckwalter and Mankin, 1997; Buckwalter et al., 2005; Hasler et al., 1999). Deep zone chondrocytes (DZC) share phenotypic similarities with growth plate hypertrophic chondrocytes, such as expression of Type X collagen, MMP-13, tissue non-specific alkaline phosphatase (TNAP), and other proteins that have been implicated in the regulation of cartilage calcification (Gannon et al., 1991; Miao and Scutt, 2002). The ZCC is the mineralized region of the DZ of articular cartilage and anchors hyaline cartilage with subchondral bone (Wang et al., 2009). At the interface between the hyaline and calcified cartilage is the tidemark. Cells in the DZ and ZCC contribute to this calcification process (Revell et al., 1990). As ZCC thickness remains relatively constant through life in healthy cartilage, a control mechanism, not yet elucidated, must be present to ensure that cartilage calcification occurs at the same rate as its replacement by bone (Bullough and Jagannath, 1983).

Osteoarthritis (OA) is characterized by loss of the articular cartilage and is the leading cause of disability in people aged 65 years or older worldwide. People with this disease experience pain, activity restrictions, and long-term disability (Arden and Nevitt, 2006). Loss of the SZ of articular cartilage is an early change in the development of OA and is followed by degradation of the ECM, advancement of the ZCC and duplication of the tidemark (Pearle et al., 2005; Revell et al., 1990; Oegema et al., 1997; Setton et al.,
1999; Silver et al., 2001; Stoop et al., 2001; Hayami et al., 2006; Duer et al., 2009; Fuerst et al., 2009; Thambyah and Broom, 2009). The mechanism(s) regulating the advancing calcification of hyaline cartilage has not been elucidated; one potential mechanism is via cross talk between the zones of cartilage. Superficial zone chondrocytes (SZC) are responsive to chondrocytes in other zones as co-culture of SZC and mid-zone chondrocytes (MZC) led to upregulation of PRG4 by SZC (Blewis et al., 2007).

Furthermore, Jiang et al. (2008) demonstrated that SZC inhibit DZC mineralization when grown in direct co-culture. This study showed that SZC suppressed DZC alkaline phosphatase activity and mineral deposition as determined by Alizarin Red staining (Jiang et al., 2008). Nevertheless, the mechanism by which SZC regulate mineralization of DZ articular cartilage remains poorly understood.

In previous studies we developed a method to form cartilage tissue by DZC. This tissue develops a ZCC when grown in the presence of β-glycerophosphate, a known mineralization-inducing agent (Kandel et al., 1997; Yu et al., 1997). The mineral is poorly crystalline hydroxyapatite comparable to that present in native articular cartilage (Kandel et al., 1999), making it a suitable model to study the mechanism(s) regulating cartilage calcification. Moreover, St-Pierre et al. (2010) showed previously that exogenously administered inorganic polyphosphate inhibits calcification of the in vitro-formed DZ cartilage in a concentration and chain length dependent manner (St-Pierre et al., 2010). Inorganic polyphosphates are linear polymers consisting of greater than 2 orthophosphate residues linked by energy-rich phosphoanhydride bonds (Kulaev et al., 1999). Polyphosphates have been found in a wide range of organisms, as well as in the tissues of
mammals including cartilage and bone (Kulaev et al., 1999; Kumble and Kornberg, 1995; Kornberg et al., 1999; Kawazoe et al., 2004; Pisoni and Lindley, 1992; Leyhausen et al., 1998; Omelon et al., 2009). Polyphosphate can either inhibit mineralization by mineral poisoning or contribute to mineralization by serving as a phosphate source (Omelon et al., 2009; St-Pierre et al., 2010).

Little is known about the enzymes and cellular pathways involved in the metabolism of polyphosphate in vertebrates even though it has been extensively studied in lower organisms. Enzymes identified to date exhibiting exopolyphosphatase activity in eukaryotes include TNAP, intestinal alkaline phosphatase and h-prune (Schroder et al., 2000; Omelon et al., 2009; Tammenkoski et al., 2008). Interestingly, polyphosphate is present in the DZ of native articular cartilage. Because the SZ is lost in early OA, we hypothesized that SZC regulate mineralization in the DZ of articular cartilage through soluble factors, which modulate the levels of polyphosphate produced by DZC. Confirming the effect of SZC on DZ cartilage mineralization and the mechanism by which polyphosphate regulates this process could advance our understanding of the pathogenesis of OA and lead to the development of novel treatment approaches and ultimately the prevention of OA.
3.2 Materials and Methods

3.2.1 Cell Isolation and Culture

Articular cartilage was harvested from the metacarpal-phalangeal joint of 9-12 month-old calves. Due to the differences in cartilage thickness between animals and isolation sites, the upper 20% (by height of full thickness of articular cartilage) closest to the articulating surface was considered to be the superficial zone, the middle 50% was designated the middle zone, and the 30% closest to the subchondral bone was considered the deep zone. Cartilage from several animals was pooled together to obtain sufficient number of cells for each experiment. Chondrocytes were then isolated by sequential enzymatic digestion using 0.5% protease (Sigma Chemical Co., St. Louis, MO, USA) in Ham’s F-12 medium for 90 minutes followed by 0.1% collagenase (Roche Diagnostics GmbH, Mannheim, Germany) in Ham’s F-12 medium supplemented with 25mM HEPES for 15-17 hours (Kandel et al., 1997; Yu et al., 1997). The superficial zone chondrocytes (SZC) and deep zone chondrocytes (DZC) were seeded separately on 12mm diameter membrane inserts (Millicell™) coated with Type II collagen (Sigma Chemical Co.) at a density of either $1 \times 10^6$ or $2 \times 10^6$ cells per construct in Ham’s F-12 medium supplemented with 25mM HEPES, 5% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 1% antibiotics (Penicillin G, Streptomycin sulphate, and Amphotericin B; Invitrogen Co., Auckland, NZ). On Day 3, the medium was changed to mineralization-inducing medium (MIM) composed of Dulbecco’s modified Eagle medium (DMEM) supplemented with 25mM HEPES, 20% FBS, 10mM β-glycerophosphate (β-GP) (Sigma Chemical Co.) and ascorbic acid (100
µg/mL, Sigma Chemical Co.). Cultures were grown for up to 10 days following the initiation of mineralization.

In selected experiments, the MIM was supplemented with human recombinant FGF-18 (10ng/mL, PeproTech Inc., Rocky Hill, NJ, USA) and grown for up to 7 days following the initiation of mineralization.

### 3.2.2 Side-By-Side Co-Culture Model of Zonal Populations of Chondrocytes

To evaluate cellular interactions between chondrocytes of the different zones, side-by-side co-cultures were established consisting of DZ (1×10^6 DZC) cultured together with either 2×10^6 SZC or DZC inside a single well (6-well plate) containing 11.5mL of Ham’s F-12 medium supplemented with 25mM HEPES, 5% FBS (HyClone, Logan, UT, USA) and 1% antibiotics (Penicillin G, Streptomycin sulphate, and Amphotericin B; Invitrogen Co., Auckland, NZ). On Day 3, the medium was changed to MIM and grown for another 7 days. The culture medium was changed every 2 days. The co-culture ratio of SZC to DZC (2:1) was chosen to account for the dilution of soluble factors released by SZ chondrocytes into the media.

### 3.2.3 Conditioned Media Culture

To determine if SZC produced a soluble factor, cultures of either 4×10^6 SZ or DZ chondrocytes were seeded onto Millipore filters at a density of 2×10^6 cells per filter (2 Millipore filters total per condition) in serum-free media containing high-glucose DMEM,
1 × ITS cell culture supplement (BD Biosciences, Bedford, MA), 100 nM dexamethasone, 40 µg/mL proline, 100 mM sodium pyruvate, and 100 µg/mL ascorbic acid. After 48 hours, and then daily thereafter, half the media was collected and centrifuged at 600×g for 4 minutes to remove any cells. The serum-free conditioned media from the SZC or DZC was supplemented with 10% FBS, 10mM β-GP and 100 µg/mL ascorbic acid and then added individually to DZC (1×10^6 cells). The DZC had been grown in DMEM supplemented with 25 mM Hepes, 20% FBS and 100µg/mL ascorbic acid for 7 days prior to treatment with the conditioned media. The culture media was changed every 24 hours and the cultures were harvested on Day 7 for analysis.

In selected experiments, the serum-free conditioned media from SZC or DZC were either heated for 1 hour at 90°C or treated with trypsin (250µg/mL, Invitrogen Life Technologies, Burlington, Canada) for 1 hour at 37°C to digest proteins. The treated conditioned media was then supplemented with 10% FBS, 10mM β-GP and 100 µg/mL ascorbic acid, added individually to DZC (1×10^6 cells) and cultured as described above.

To investigate the role of fibroblast growth factor signaling in regulating DZC mineralization, the serum-free conditioned media from SZC or DZC was supplemented with 10% FBS, 10mM β-GP, 100 µg/mL ascorbic acid and PD173074 (60nM, EMD Biosciences Inc., San Diego, CA, USA), a pharmacological inhibitor of FGF receptor (FGFR) signaling (Koziczak et al., 2004), added individually to DZC (1×10^6 cells) and cultured as described above.
3.2.4 Analysis of Gene Expression

Total RNA was extracted from freshly isolated chondrocytes from the three zones (SZ, MZ and DZ) and *in vitro*-formed cartilage using TRIzol (GIBCO BRL, Rockville, MD, USA), according to the manufacturers instructions. The purity of the RNA was confirmed spectrophotometrically (260/280 nm ratio) and ranged between 1.8 and 1.9. Invitrogen Superscript II reverse transcription kit was used to reverse transcribe 1.0µg of total RNA in 20µL of total reaction volume in FirstStrand buffer with 0.1M dithiothreitol for 50 minutes at 42°C followed by a 15 minute extension period at 72°C (Invitrogen, Carlsbad, CA). Each reaction contained 40 U/mL of recombinant ribonuclease inhibitor RNase OUT (Invitrogen, Carlsbad, CA), 50 mg/mL of random hexamers, 10mM dNTPs, and 200 units of SuperScript II enzyme. All samples in an experiment were subjected to reverse transcription at the same time to avoid variability and the resulting cDNA was diluted 5 times and subjected to real-time PCR.

Using primers specific for the gene of interest, the cDNA underwent real-time PCR. The PCR reaction mixture contained 4.0µL of cDNA, 0.6µL each of forward and reverse primer, and 4.8µL of Bio-Rad iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) for a total reaction volume of 10µL. Reaction mixtures were subjected to a 10 minute enzyme activation step at 95°C followed by a 15 second denaturation step at 95°C and a 30 second annealing step at 60°C (59°C for Proteoglycan 4, and 61°C for h-prune and CILP) (primer sequences are listed in Table 1). Amplification and detection were performed using an iCycler Thermal Cycler (Bio-Rad). Each reaction was performed in triplicate. Mean relative quantification (RQ) values were calculated using 18S rRNA as endogenous control. Calibrators were specific for each experiment. PCR amplification specificity was
ensured by examining the melting curve (dF/dT versus temperature) for non-specific peaks. Only primer pairs with efficiency greater than 90% were used.

**Table 1**: Gene-specific primer sequence and amplification condition

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
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<tr>
<td>Proteoglycan 4</td>
<td>fwd: 5'-ATG CCTGAACCGACTCCTAC-3’</td>
</tr>
<tr>
<td></td>
<td>rev: 5’-CGCCGACTCCTACCTTGAG-3’</td>
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<td></td>
<td>rev: 5’-CAGCAACCACACAGATTTC-3’</td>
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<tr>
<td>h-prune</td>
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<td></td>
<td>rev: 5’-GTGGAGGCCAACAGACACC-3’</td>
</tr>
</tbody>
</table>

### 3.2.5 Dry Weight of Cartilaginous Tissue

Cartilage tissues formed in vitro on membrane inserts were harvested on Day 7, washed twice in PBS and removed from the membrane. The tissues were then lyophilized.
overnight and weighed using an electrical balance (Explorer, Ohaus Corp., Florham Park, NJ, USA).

### 3.2.6 Mineral Content of Cartilaginous Tissue

To quantify the mineral content of *in vitro*-formed cartilage the calcium and phosphate contents were measured. Briefly the tissues were lyophilized, weighed, and then wet washed as described previously (Allan et al., 2007). The tissues were placed in nitric acid/perchloric acid (4:1 vol/vol) and heated at 150°C for 90 minutes in a closed Teflon beaker. The acid mixture was evaporated to a small drop (approximately 100 µL), and then the mixture was diluted with ddH₂O (2 mL). The pH of the solution was then adjusted to between 2.5 and 2.9 and ddH₂O added to a final volume of 2.5 mL. To measure calcium content, an aliquot was mixed with 0.01% cresolphthalein complexone (Sigma Chemical Co.) buffered with 0.25 M sodium borate in a 1:4 vol/vol ratio and the resulting color quantified spectrophotometrically at 570 nm (Titertek Multiscan) within 5 minutes. The standard curve was generated using a calcium standard. To measure phosphate content, an aliquot of sample was mixed with a solution containing 1:6 vol/vol ratio of 10% ascorbic acid and 0.42% ammoniummolybdate (Sigma Chemical Co.) in 1 NH₂SO₄ (1:3 vol/vol ratio) at 37°C for 1 hour and the resulting color quantified spectrophotometrically at 620 nm. The standard curve was generated using a sodium phosphate dibasic solution (stock = 0.5mM; pH 7.5).
3.2.7 Histological Evaluation

*In vitro*-formed cartilage constructs were harvested and fixed in 10% buffered formalin and embedded in paraffin. Sections (5 µm) were cut and stained with Von Kossa and toluidine blue to demonstrate the presence of apatite mineral deposits and sulphated proteoglycans, respectively, and examined by light microscopy.

To visualize the presence of polyphosphate in the tissues, 5 µm sections were cut, dewaxed in xylene and stained with 4’,6-diamidino-2-phenylindole (DAPI) (10 µg/mL, Pierce Biotechnology, Inc., Rockford, IL, USA). Approximately 250 µL of DAPI staining solution was added to each slide preparation making certain that the tissue section was completely covered. The slides were then incubated for 5 minutes and rinsed in distilled deionized water. The fluorescence was visualized with a Zeiss Axioplan epifluorescence microscope using a wide pass DAPI filter. Inorganic polyphosphate specifically shifts the emission peak of DAPI from 456 to 526 nm, permitting its visualization in the yellow-green spectrum rather than the blue spectrum associated with nucleic acids or glycosaminoglycans (Allan and Miller, 1980; Tijssen et al., 1982).

3.2.8 Extraction of Polyphosphate

Cartilage tissues formed *in vitro* were harvested, washed twice in PBS, removed from the filter and sectioned into small pieces (approximately 2 mm³). Tissues were resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl₂, 0.5 mM EDTA and 150 mM NaCl (homogenization buffer; 180 µL per sample) and digested with proteinase K (20 µL per sample, Ambion, Inc., Austin, Texas, USA) at 37°C for 3 hours. Samples were
vortexed for 15 seconds every 30 minutes to promote complete digestion of tissues. DNase I (2 µL per sample, Ambion, Inc., Austin, Texas, USA) was added to each sample, vortexed for 10 seconds and then incubated at 37°C for 1 hour. Samples were vortexed for 10 seconds after 30 minutes of incubation. Polyphosphate was extracted using TRIzol (500 µL per sample, GIBCO BRL, Rockville, MD, USA). 100 µL of chloroform was added to the extract, and the resulting solution was centrifuged (14,000rpm for 15 minutes at 4°C). Polyphosphate was recovered in the upper aqueous phase, precipitated with 500 µL isopropanol (4°C for 3 hours) and then centrifuged at 14,000rpm for 30 minutes at 4°C. The pellet was washed with 1mL of 70% ethanol, and resuspended in 100µL homogenization buffer. 5µL of calf intestine alkaline phosphatase and 75µL buffer (50mM Tris, pH 9.4) was added to an aliquot (20µL) of the extract. To correct for phosphate content of the extracts, aliquots containing 20µL of sample and 80µL of buffer (no enzyme) were also prepared. The reaction mixtures were incubated at 37°C for 30 minutes and the phosphate released was measured spectrophotometrically at 620nm. The standard curve was generated using a sodium phosphate dibasic solution (stock = 0.5mM; pH 7.5). Extraction efficiency was calculated by preparing inorganic polyphosphate mixtures containing known concentrations (1.0mM and 2.5mM) of sodium phosphate glass (average chain lengths of 45 phosphate units; Sigma Chemical Co.). For each concentration, duplicates were either frozen at -30°C or processed for polyphosphate extraction. Extraction yield was calculated by comparing inorganic polyphosphate which was frozen at -30°C and the inorganic polyphosphate spiked sample processed for extraction.
3.2.9 Quantitation of Alkaline Phosphatase Activity

Proteins were extracted and assessed for alkaline phosphatase (ALP) activity according to a protocol modified by Allan et al. (2007). Briefly, chondrocytes re-suspended in Ham’s F-12 media with 5% FBS were split into two equal aliquots and then centrifuged at 4°C (600×g) for 5 minutes to pellet the cells. One pellet was re-suspended in Buffer A (0.1% Triton X, 0.2 M Tris, pH 7.4, 45mM NaCl) and frozen at -30°C, while the other pellet was processed as described above to quantify DNA content.

ALP Quantification

To quantify ALP activity, the cells in Buffer A were freeze thawed-twice and centrifuged at 4°C for 20 min (833×g) to pellet the cell membranes. Aliquots (100µL) of the supernatant were mixed with equal volumes of p-nitrophenol phosphate (0.02M PNP (Sigma Chemical Co.) in Buffer B (0.2M Tris, pH 9.3, 45mM NaCl) for 1 hour at 37°C and then analyzed spectrophotometrically at 405 nm (Titertek Multiscan Interscience, Markham, ON). The standard curve was generated using 10mM p-nitrophenol (Sigma Chemical Co.) diluted in Buffer B. To determine ALP activity in the *in vitro*-formed cartilage, the tissue was removed from the Millipore membrane and cells were isolated by digestion in 0.1% collagenase (1mL, Roche Diagnostics GmbH, Mannheim, Germany) for 6 hours at 37°C. The cells were processed as described above.
3.2.10 Statistical Analysis

A minimum of three independent sets of experiments was performed, and each condition was done in triplicate. The data were pooled and presented as mean ± standard error of the mean. To evaluate statistical significance, results were analyzed using a one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Statistical significance was assigned at p < 0.05.

3.3 Results

3.3.1 Differential Cartilage Dissection Generates Zonal Populations of Cells

To verify the quality of zonal separation, gene expression of freshly isolated chondrocytes from the different zones of bovine articular cartilage was analyzed (Figure 1). Superficial zone protein (SZP), and cartilage intermediate layer protein (CILP) were predominantly expressed in the SZC and MZC, respectively. Alkaline phosphatase (ALP), Type X collagen (Col-X), and matrix metalloproteinase 13 (MMP-13) were primarily expressed in DZC.

To further assess the quality of zonal separation, ALP activity assay was performed. DZC had significantly higher ALP activity compared to SZ and MZC (p < 0.01) with average ALP activities of 206.7 ± 10.4, 27.6 ± 2.8, and 19.4 ± 1.9 uM/ug DNA/hr, respectively.
3.3.2 Superficial Zone Chondrocytes Inhibit Mineralization in Cartilage Formed by Deep Zone Chondrocytes

Side-by-side co-cultures were established to evaluate the inhibitory effect of SZC on mineral deposition in cartilage formed \textit{in vitro} by DZC. DZC co-cultured with SZC (SZ-DZ, Figure 2A and C) had significantly less mineral deposition in the DZ tissue compared to DZC co-cultured with DZC (DZ-DZ, Figure 2B and D). Quantification of calcium and phosphate contents showed that DZ tissue co-cultured with SZC (SZ-DZ) had approximately 70\% less mineral deposition than DZ-DZ cultures (Figure 2E).

3.3.3 Superficial Zone Chondrocytes Influence Polyphosphate Metabolism by Deep Zone Chondrocytes

The DZ cultures were stained with DAPI to visualize the presence and localization of polyphosphate after 7 days of culture in MIM (Figure 3). DZ cartilage co-cultured with SZC exhibited more yellow-green DAPI staining suggesting increased polyphosphate accumulation within the tissues (Figure 3C) compared to DZC co-cultured with DZC (Figure 3D). DAPI staining was also seen in DZC grown in media that was not supplemented with $\beta$-GP (Figure 3A) but not in DZC grown in the presence of MIM with $\beta$-GP (Figure 3B).

Quantification of polyphosphate from the \textit{in vitro}-formed cartilage confirmed the results obtained by DAPI staining. DZ cartilage co-cultured with SZC accumulated significantly higher polyphosphate compared to DZ cartilage co-cultured with DZC (Figure 3E).
3.3.4 Superficial Zone Chondrocytes Influence Alkaline Phosphatase Activity of In Vitro-Grown Deep Zone Chondrocytes

To determine whether SZC influence ALP activity of DZC, the ALP activity of DZC co-cultured with either SZC or DZC was quantified over time. As shown in Figure 4, ALP activity of DZC co-cultured with either DZC or SZC were similar until Day 6 when ALP activity was significantly less in the DZ tissues grown in the presence of DZC compared to co-culture with SZC. This difference was maintained at Day 8 even though ALP activity levels are decreasing.

3.3.5 Media Conditioned by Superficial Zone Chondrocytes Inhibits Deep Zone Chondrocyte Mineralization

To determine if SZC secrete a soluble factor that inhibits mineralization, DZC were cultured with media conditioned by SZC. This media significantly inhibited mineralization as demonstrated by quantification of calcium and phosphate contents in the tissue (Figure 5). The mineral type did not change as the calculated calcium-to-phosphorous ratio remained unchanged and was similar to apatite (see Figure 11B in Appendix). This suggests that SZC secrete a soluble factor that can inhibit mineralization in DZC.

To determine if the factor(s) secreted by SZC is a protein, media conditioned by SZC or DZC were either heat treated or digested by trypsin to denature or degrade proteins, respectively. SZC conditioned media that had undergone heat or trypsin treatment lost its ability to inhibit mineralization of DZC, as there was significantly higher mineral
content compared to DZC cultured in the presence of untreated SZ conditioned media (Figure 6). This suggested that the factor(s) released by SZC is a protein.

3.3.6 Effect of Fibroblast Growth Factor Signaling on Mineralization in Deep Zone Cartilage

As FGF-18 can regulate mineralization and SZC express FGF-18 (Figure 7A), the effect of FGF-18 on DZ mineralization was evaluated. In vitro-formed DZ cartilage cultured in MIM supplemented with FGF-18 resulted in a significant decrease in mineral deposition compared to DZ cartilage grown in MIM alone (Figure 7B). To determine if FGF signaling was responsible for the inhibition of mineralization by SZC, SZC or DZC conditioned media was supplemented with PD173074, a FGFR signaling inhibitor, and then added to DZC. As shown in Figure 7C, addition of PD173074 to SZC conditioned media prevented its inhibitory effect on mineral deposition.

3.3.7 Effect of Fibroblast Growth Factor Signaling on Polyphosphate Metabolism in Deep Zone Cartilage

To determine if FGF-18 modulates polyphosphate metabolism, in vitro-formed DZ cartilage was cultured in MIM supplemented with FGF-18 and polyphosphate quantified. Polyphosphate levels in DZ cartilage grown in MIM treated with FGF-18 were significantly higher than DZC grown in MIM alone (Figure 8A), and similar to DZC grown in non-mineralizing medium (without β-GP). The effect of FGF-18 on polyphosphate levels could be prevented by treating the cells with PD173074 (Figure 8B).
Figure 1: Differential gene expression in chondrocytes freshly isolated from the different zones of bovine articular cartilage (SZ: superficial zone; MZ: middle zone; DZ: deep zone). Levels were normalized to 18S rRNA and expressed relative to full thickness cartilage (calibrator). Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates a significant difference in expression for the selected gene by the different chondrocytes subpopulations. SZP = superficial zone protein, CILP = cartilage intermediate layer protein, ALP = alkaline phosphatase, Col X = collagen Type X, and MMP13 = matrix metalloproteinase 13.
Mineralization Inhibition (% of DZ(DZ) Tissue)

Deep Zone Tissue

E

- Calcium
- Phosphate

DZ(SZ) DZ(DZ)
Figure 2: Superficial zone chondrocytes inhibit mineralization by deep zone chondrocytes. Phase contrast images of DZ cartilage after 7 days of culture in mineralization-inducing medium showing (A) inhibition of mineralization of tissue formed by DZC when co-cultured with SZC, and (B) mineralization of tissue formed by DZC when co-cultured with DZC. (C-D) Histological appearance of DZ cartilage visualized by light microscopy (toluidine blue and von Kossa) which had been co-cultured with either (C) SZC or (D) DZC. The arrow indicates mineral deposits. * Indicates the membrane insert. (E) Quantification of calcium and phosphate contents in DZC tissues after 7 days of co-culture with either SZC (DZ(SZ)) or DZC (DZ(DZ)). Each condition was done in triplicate and the experiment was repeated 5 times. The results were pooled and expressed as mean ± standard error of the mean. # Indicates significant difference.
Figure 3: Inorganic polyphosphate accumulation within *in vitro*-formed cartilage. DAPI staining was used to demonstrate inorganic polyphosphate distribution within *in vitro*-formed DZ cartilage grown in mineralization-inducing medium for 7 days. (A) DZ cultured without β-GP, (B) DZ cultured in mineralization-inducing medium with β-GP, (C) DZ co-cultured with SZ, (D) DZ co-cultured with DZ. Epifluorescence microscopy; DAPI staining. The arrow indicates mineral deposits. * Indicates the membrane insert. The scale bars represent 100µm. (E) Polyphosphate was quantified in DZ cultures after 7 days of co-culture with either SZC or DZC. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. # Indicates significantly higher polyphosphate content.
Figure 4: Alkaline phosphatase activity of DZC co-cultured with either SZC or DZC at 2, 4, 6 and 8 days in mineralization-inducing media. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates significant difference in ALP activity for selected day.
**Figure 5:** Calcium and phosphate contents of cartilage formed by DZC *in vitro* on membrane inserts after 7 days in media conditioned by either SZC (SZ CM) or DZC (DZ CM). Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates significant difference.
Figure 6: Calcium and phosphate contents of tissue formed by DZC in vitro on membrane inserts after 7 days in ITS media conditioned by either SZC (SZ CM) or DZC (DZ CM) subjected to heat treatment (H.T.) or trypsin. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates significant difference in mineral content compared to untreated SZ conditioned media.
Figure 7: (A) Differential gene expression of FGF-18, FGFR2c and FGFR3c in chondrocytes freshly isolated from the different zones of bovine articular cartilage (SZ: superficial zone; MZ: middle zone; DZ: deep zone). Levels are expressed relative to full thickness cartilage (calibrator). Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled (n = 9) and expressed as mean ± standard error of the mean. * Indicates a significant difference in expression for the selected gene. FGF-18 = fibroblast growth factor 18, FGFr2c = fibroblast growth factor receptor 2c, and FGFr3c = fibroblast growth factor receptor 3c.

(B) Calcium and phosphate contents of DZ cartilage formed in vitro on membrane inserts after 7 days in the presence of mineralization-inducing medium supplemented with human recombinant fibroblast growth factor 18 (FGF-18; 10ng/mL). Cells were grown in Non-mineralizing medium (NM, without β-GP) as a control.

(C) Calcium and phosphate contents of DZ cartilage formed in vitro on membrane inserts after 7 days in the presence of media conditioned by SZC (SZ CM) or DZC (DZ CM) supplemented with the FGFR tyrosine kinase inhibitor (PD173074; 60nM). Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates significant difference in mineral content.
Figure 8: Inorganic polyphosphate content in DZ cartilage formed in vitro on membrane inserts after 7 days in (A) mineralization-inducing medium alone (MIM) or supplemented with human recombinant fibroblast growth factor 18 (FGF-18; 10ng/mL). Cells were grown in Non-mineralizing medium (NM, without β-GP) as a control. (B) Deep zone chondrocytes (DZ) were grown in media conditioned by SZC (SZ CM) or DZC (DZ CM) alone or supplemented with FGFR tyrosine kinase inhibitor (PD173074; 60nM). Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates significant difference in polyphosphate content.
3.4 Discussion

This study demonstrated that SZC released a soluble factor that was sensitive to heat denaturation and trypsin digestion, and inhibited mineralization in deep zone cartilage formed in vitro. This effect correlated with increased levels of polyphosphate in the tissue. This factor may be FGF-18 as it was shown to inhibit mineralization in in vitro-formed DZ cartilage and treatment of DZ cartilage with FGF-18 resulted in increased accumulation of polyphosphate. Furthermore, the FGF receptor inhibitor, PD173074, reversed the inhibitory effect of SZ conditioned media on mineralization in DZ cartilage and also decreased polyphosphate accumulation by DZC in the presence of SZ conditioned media. Based on these findings we propose that SZC regulate mineralization in DZ cartilage by modulating DZ polyphosphate levels through the FGF-18 signaling pathway. Interestingly, FGF-18 is preferentially expressed by SZC in native articular cartilage compared to DZC. This is in agreement with other studies that have shown chondrocytes secrete factors that influence middle and deep zone chondrocytes (Maroudas, 1976; Quinn et al., 2000; Blewis et al., 2007; Jiang et al., 2008). To the best of our knowledge, this is the first report showing that FGF-18 modulates polyphosphate levels.

FGF-18 has been shown by others to have divergent effects on mineralization as it binds and activates multiple receptors. It is a ligand for FGFr1 and FGFr2 to which it binds modestly, and FGFr3 to which it binds with high affinity (Shimoaka et al., 2002; Ohbayashi et al., 2002; Ellsworth et al., 2002; Liu et al., 2002; Eswarakumar et al., 2002; Barnard et al., 2005; Davidson et al., 2005; Haque et al., 2007; Naski et al., 1998; Yan et
In the developing growth plate, FGF-18 interacts with FGFr2 to promote osteogenesis and bone formation (Davidson et al., 2005; Haque et al., 2007), while interaction with FGFr3 negatively regulates chondrocyte proliferation and hypertrophic maturation (Lanske et al., 1996; Naski et al., 1998; Karp et al., 2000; Ellsworth et al., 2002; Kobayashi et al., 2002; Minina et al., 2002; Ornitz, 2005; Ozasa et al., 2005; Liu et al., 2007). Interaction of FGF-18 with FGFr3 indirectly modulates chondrogenesis by inhibiting the Indian hedgehog (Ihh) signaling pathway (Davidson et al., 2005; Haque et al., 2007; Naski et al., 1998). The Ihh pathway functions via a negative feedback loop with parathyroid hormone related peptide (PTHrP) to regulate chondrocyte proliferation and growth plate cartilage mineralization (Lanske et al., 1996; Naski et al., 1998; Karp et al., 2000; Ellsworth et al., 2002; Kobayashi et al., 2002; Minina et al., 2002; Ornitz, 2005; Ozasa et al., 2005; Haque et al., 2007). Interestingly, the PTHrP-Ihh negative feedback loop has also been reported to be involved in regulating mineralization of articular cartilage (Jiang et al., 2008). It may be that SZC communicate with DZC via the PTHrP-Ihh signaling pathway to inhibit mineralization. Interestingly, our results showed increased FGFr3 expression in DZC co-cultured with SZC, whereas DZC co-cultured with DZC expressed significantly higher FGFr2 (data not shown). Based on these results it may be possible that SZC up-regulate FGFr3 expression in DZC to inhibit the expression of Ihh and promote PTHrP signaling, preventing hypertrophy of DZC and subsequently suppress mineralization. Furthermore, selective activation of FGFr3 by FGF-18, which has been shown to exert cartilage-protective responses in human articular cartilage, is significantly reduced in the development of OA (Yan et al., 2011). An alternative mechanism to explain how SZC inhibit mineralization in the DZ is that SZC up-regulate
FGF receptor levels in DZC via an unknown process and this makes them more sensitive to existing FGF-18 already in the DZ. The role FGF-18 and its receptors play in regulating mineralization of articular cartilage remain unclear and require further investigation.

Along with its inhibitory effect on DZ cartilage mineralization, SZC also increased accumulation of polyphosphate within DZC. Polyphosphate has been well established as an important modulator of apatite biomineralization. Polyphosphate has been shown to inhibit mineralization in cell-free crystal growth assays (Fleisch and Neuman, 1961; Francis, 1969; Omelon and Grynpas, 2008), as well as biological systems (Omelon et al., 2009; St-Pierre et al., 2010; Fleisch et al., 1965; Fleisch et al., 1966; Irving et al., 1966; Schibler et al., 1968; Hoac et al., 2013). The authors propose that although the mechanisms by which polyphosphate inhibits mineralization is likely multifactorial including calcium chelation by polyphosphate, the principal method is by binding directly to the surface of hydroxyapatite crystals and preventing its growth. Interestingly, polyphosphate has also been shown to induce mineralization in pre-osteoblast cells (Kawazoe et al., 2004), promote regeneration of alveolar bone (Hacchou et al., 2007; Usui et al., 2010), and stimulate osteoblastic differentiation (Usui et al., 2010; Kawazoe et al., 2008; Morimoto et al., 2010). Omelon et al. (2009) has suggested that this biphasic response may be a result of increased polyphosphate synthesis and sequesteration of calcium when mineralization is undesirable and these calcium-polyphosphate complexes are degraded when mineralization is required (Omelon et al., 2009). The ratio of orthophosphate to polyphosphate may be critical to inducing the appropriate response.
Previous work by St-Pierre et al. (2010) showed that inhibition of cartilage mineralization is specific to condensed phosphates rather than orthophosphate as similar concentrations of phosphate to those used for polyphosphate did not have any effect on inhibiting mineralization in this culture system (St-Pierre et al., 2010).

The effects of FGF-18 and polyphosphate on mineralization have been well documented, however, this is the first report to show that FGF-18 modulates polyphosphate levels. Treatment of in vitro-formed DZ cartilage with FGF-18 resulted in increased accumulation of polyphosphate while inhibiting FGF receptor signaling reversed this effect. Although the mechanism by which FGF-18 modulates polyphosphate levels are not known, our results suggest that FGF-18 prevents polyphosphate degradation by down-regulating exopolyphosphatase activity. This is supported by the observed decrease in TNAP activity with respect to polyphosphate levels in DZC co-cultured with SZC. Interestingly, Hatch et al. (2005) showed that another fibroblast growth factor, FGF-2, also inhibits TNAP expression (Hatch et al., 2005). Furthermore, through the zones of native articular cartilage a gradient of TNAP expression exists which is opposite that of FGF-18. TNAP is predominantly expressed in the DZ while FGF-18 expression is highest in the SZ suggesting that FGF-18 may suppress TNAP in the upper zones, restricting its activity to the deeper zones.

In addition, our findings show that polyphosphate stimulates TNAP activity in DZ cartilage indicating a feedback mechanism exists to closely regulate the orthophosphate-to-polyphosphate ratio. Correspondingly, in native articular cartilage, polyphosphate
levels and TNAP activity are highest in the DZ. Another possible mechanism by which FGF-18 may inhibit degradation of polyphosphate is through direct interaction with polyphosphate. Shiba et al. (2003) determined that FGF-2 forms a complex with polyphosphate to stabilize the growth factor, which results in increased half-life and facilitates binding to its cell surface receptors (Shiba et al., 2003). It is possible that a similar complex forms between FGF-18 and polyphosphate to activate the FGF-18 signaling pathway or prevent the degradation of polyphosphate by TNAP. Suppression of TNAP prevents removal of the mineralization inhibitor as well as release of orthophosphate residues shifting the orthophosphate-to-polyphosphate ratio to favour inhibition of hydroxyapatite crystal formation. Since the enzyme(s) responsible for polyphosphate synthesis in vertebrates are not known, our current understanding of the mechanism by which polyphosphate levels are regulated is limited to enzymes capable of polyphosphate catalysis. Our data suggests that the effect of polyphosphate may be bimodal and dictated by the orthophosphate-to-polyphosphate ratio with higher levels of polyphosphate favouring inhibition.

Our findings may explain why with the progressive loss of SZC during OA, the mineralization front advances into DZ cartilage. The SZ is an important signaling center in healthy articular cartilage. SZC are responsible for the production and maintenance of the surface layer and superficial zone protein (encoded by the gene PRG4), which respectively give articular cartilage tensile strength to resist shear forces and assists in boundary lubrication during cartilage-on-cartilage articulation (Schumacher et al., 1994; Jay et al., 1998; Newman, 1998; Huber et al., 2000; Poole et al., 2001; Schaefer et al.,
2004). Interestingly, PRG4 knockout mice exhibited characteristics similar to OA cartilage including articular surface deterioration, increase in the coefficient of friction, loss of SZC and ultimately joint failure (Rhee et al., 2005). SZC also produce signaling molecules including members of the Wnts, bone morphogenetic proteins, and transforming growth factor families (Anderson et al., 2000; Pacifici et al., 2005; Yamane et al., 2007). The Wnt/β-catenin pathway is an important regulator of SZC function and phenotype as demonstrated in transgenic mice where activation of Wnt/β-catenin signaling increased SZ thickness, proliferation of SZC and expression of SZP (Yasuhara et al., 2011; Inui et al., 2013).

Given the avascularity of articular cartilage, chondrocytes depend on solute transport through the ECM for delivery of nutrients, oxygen and growth factors, as well as removal of wastes (Maroudas, 1970; Maroudas, 1975). Chondrocytes synthesize and secrete growth factors and cytokines to influence cell activity to maintain articular cartilage. The semi-permeable ECM of articular cartilage facilitates the movement of solutes and allows the chondrocyte subpopulations to communicate in a paracrine manner (Quinn et al., 2000; Vincent et al., 2002; Blewis et al., 2007). The transport of these signaling molecules is dependent on the organization and composition of ECM constituents, as well as the mechanical load as these factors dictate the pore size and charge of the ECM. Due to the zonal differences in the structure and properties of articular cartilage, the diffusion coefficients for all solutes vary with depth from the articular surface (Maroudas, 1976; Leddy and Guilak, 2003; Evans and Quinn, 2005). Changes to the ECM such as disruption of the collagen network in the SZ or loss of
proteoglycans lead to altered diffusive properties (Maroudas, 1970; Torzilli, 1993; Chin et al., 2013). Moreover, the size, structure and charge of the solute also influence the movement of the solute through the ECM (Maroudas, 1976; Evans and Quinn, 2005). In general, the diffusion coefficients of macromolecules are inversely proportional to the molecular size, with globular proteins larger than 69kDa unable to penetrate into the deeper zones of articular cartilage (Maroudas, 1976; Torzilli, 1987). In contrast, FGF-18 is a globular protein with a molecular mass of approximately 23kDa and therefore, as a secreted protein can diffuse through the ECM into the DZ of articular cartilage without limitations (Zhu et al., 1991; Hu et al., 1998). Furthermore, FGF-18 exhibits a net positive charge at physiological pH, and thus is attracted to negatively charged proteoglycans and hydroxyapatite, which are highest in the ECM of the DZ (Maroudas, 1976; Evans and Quinn, 2005; Chin et al., 2013). This interaction promotes penetration of FGF-18 to the DZ and may also enhance its activity.

3.5 Conclusions

This study demonstrates that SZC suppress DZ cartilage mineralization by modulating polyphosphate levels via a soluble factor such as FGF-18. Although our understanding of this mechanism remains limited, our results indicate FGF-18 may modulate TNAP activity within DZ cartilage to generate a polyphosphate level that favours inhibition of mineralization. These data suggest that polyphosphate and FGF-18, separately or in combination, may be therapeutic candidates for the treatment of OA. Elucidation of the interaction between the chondrocyte subpopulations and the signaling pathway(s) involved
will likely prove vital in understanding the pathogenesis of OA and developing new methods for cartilage repair.
3.6 References


CHAPTER 4: GENERAL DISCUSSION
The purpose of the study presented in this thesis was to determine the effect of superficial zone chondrocytes on deep zone cartilage matrix accumulation and mineral formation. The findings provided insights into the mechanism by which superficial zone chondrocytes suppress mineralization in deep zone cartilage and the involvement of inorganic polyphosphate and FGF-18 signaling in this process.

4.1 **Superficial zone chondrocytes suppress mineral formation in deep zone cartilage**

Our group has previously developed a method to form cartilage tissue by deep zone chondrocytes and induce calcification by growing the cartilage in the presence of β-glycerophosphate (Kandel et al., 1997; Yu et al., 1997). The mineral formed is poorly crystalline hydroxyapatite comparable to that found in native articular cartilage, making it a suitable model to study the mechanism(s) regulating cartilage calcification (Kandel et al., 1997). Interestingly, Jiang et al. (2008) demonstrated superficial zone chondrocytes (SZC) inhibit deep zone chondrocyte (DZC) mineralization when grown in direct co-culture (Jiang et al., 2008). This study supports the concept that cellular communication between cartilage layers may play a role in regulating cartilage mineralization, however, the mechanism by which SZC regulates mineralization in deep zone cartilage remains poorly understood. Previous studies have reported that chondrocytes synthesize and secrete growth factors and cytokines to influence cell activity in a paracrine manner to maintain articular cartilage (Quinn et al., 2000; Vincent et al., 2002; Blewis et al., 2007). The side-by-side co-culture and conditioned media culture systems are ideal models to
study cellular interaction and identify the soluble factor(s) involved in ECM maintenance and mineralization.

In Chapter 2 of this thesis, experiments were carried out to verify the hypothesis that SZC regulate mineralization in DZC. The inhibitory effects of SZC on DZ cartilage mineralization in direct co-culture were confirmed (Jiang et al., 2008), however, the work presented in this thesis was the first to demonstrate the inhibitory effects of SZC on DZ cartilage mineralization in a tissue culture system. Our findings establish that SZC cross-communicate in a paracrine manner through soluble factor(s) with DZC to suppress mineralization in cartilage formed in vitro by DZC. The effects were demonstrated by setting up side-by-side (not-in-contact) co-cultures of SZC and DZC as well as treatment of DZC with media conditioned by SZC. Furthermore, we determined that SZC did not require the presence of FBS in the culture media to produce the soluble factor(s) responsible for inhibiting mineralization in in vitro-formed DZ cartilage. It was also established that the soluble factor(s) released by SZC is a protein as the media conditioned by SZC following heat treatment to denature the molecule or trypsin digestion to degrade the protein was unable to inhibit mineralization by the DZ chondrocytes. This confirms that SZC suppress mineralization in DZ cartilage through soluble factor(s).

The results obtained using these culture systems have contributed to the understanding of the effect of SZC on mineralization in DZ cartilage. These findings are in keeping with the changes seen in osteoarthritic articular cartilage where changes in loss of the SZ is accompanied by advancement of the ZCC and duplication of the tidemark
(Pearle et al., 2005; Revell et al., 1990; Oegema et al., 1997; Setton et al., 1999; Silver et al., 2001; Duer et al., 2009). Although an in vitro study, the findings of this work establish that cellular communication between chondrocytes of the different zones can regulate articular cartilage mineralization. Nevertheless, the signaling pathway(s) and paracrine factor(s) by which SZC suppress mineralization in DZ cartilage has not been fully elucidated.

4.2 **Superficial zone chondrocytes suppress mineralization in DZ cartilage by modulating endogenous DZ polyphosphate levels via FGF-18**

The limited cell-to-cell contact permitted by the side-by-side co-culture system supports the notion that the chondrocyte subpopulations cross-communicate in a paracrine manner to regulate mineralization. The SZC produce and maintain the surface layer to give articular cartilage tensile strength to resist shear forces, and produce superficial zone protein (encoded by the gene PRG4), which assists in boundary lubrication during cartilage-on-cartilage articulation (Schumacher et al., 1994; Jay et al., 1998; Newman, 1998; Huber et al., 2000; Poole et al., 2001; Schaefer et al., 2004). The importance of superficial zone protein can be seen in PRG4 knockout mice, which exhibited characteristics similar to OA cartilage including articular surface deterioration, increase in the coefficient of friction, loss of SZC and ultimately joint failure (Rhee et al., 2005). Furthermore, SZC synthesize and secrete signaling molecules including members of the Wnts, bone morphogenetic proteins, and transforming growth factor families (Anderson et al., 2000; Pacifici et al., 2005; Yamane et al., 2007). The Wnt/β-catenin pathway is an important regulator of SZC proliferation, expression of SZP and SZ thickness (Yasuhara
et al., 2011; Inui et al., 2013). Recent studies also suggest that FGF18 is produced by SZC (Delve et al., unpublished data). All of these molecules have the potential to influence mineralization (Harris et al., 1994; Talley-Ronsholdt et al., 1995; Anderson et al., 2000; Ornitz and Marie, 2002; Marie, 2003; Rhee et al., 2005; Haque et al., 2007; Xiao et al., 2007; Yamane et al., 2007), so there are a number of potential molecules that could be involved. Based on the results presented in this study, we propose this soluble factor to be FGF-18. Addition of FGF-18 to DZ cultures inhibited mineralization and addition of SZ conditioned media in the presence of an FGF receptor inhibitor reversed the effect of SZ conditioned media. Furthermore, treatment of DZC with FGF-18 and culturing DZC in media conditioned by SZC increased the accumulation of polyphosphate within in vitro-formed DZ cartilage. Taken together, our findings suggest that SZC inhibit mineralization in cartilage formed in vitro by DZC by regulating DZ polyphosphate levels through FGF-18.

Due to the avascularity of articular cartilage, the chondrocyte subpopulations synthesize and secrete zone-specific growth factors and cytokines to maintain articular cartilage and influence cell activity in a paracrine manner (Maroudas, 1976; Quinn et al., 2000; Vincent et al., 2002; Blewis et al., 2007). One important growth factor is FGF-18, which is an essential in skeletal development and growth, and has been shown to have anabolic effects on articular cartilage (Ellsworth et al., 2002; Ohbayashi et al., 2002; Shimoaka et al., 2002; Marie, 2003; Moore et al., 2005). Interestingly, FGF-18 is predominantly expressed by SZC and is limited in MZC and DZC. Considering the responsibility of SZC, and the spatial location of FGF-18 suggests that it plays a
physiological role in articular cartilage homeostasis. However, for FGF-18 to influence cell activity it must be able to penetrate into the deeper zones of articular cartilage to interact with chondrocytes, which is facilitated by the semi-permeable ECM. Solute transport through the ECM is dependent on the pore size and charge of the ECM, which is determined by ECM organization and composition, as well as the mechanical load on cartilage (Maroudas, 1976; Leddy and Guilak, 2003; Evans and Quinn, 2005). Due to the depth-related differences in composition, macromolecular organization and mechanical properties of the ECM, the diffusion coefficients for all solutes vary with depth from the articular surface. Therefore, any changes to the ECM such as disruption of the collagen network in the SZ or loss of proteoglycans lead to altered diffusion properties (Maroudas, 1970; Torzilli, 1993; Chin et al., 2013).

Movement of the solute is also dependent on the size, structure and charge of the solute. The diffusion coefficients of globular proteins are inversely proportional to the molecular size, and macromolecules larger than 69kDa are unable to penetrate deeper zones of articular cartilage (Maroudas, 1976; Torzilli, 1987; Evans and Quinn, 2005). Considering that FGF-18 has a molecular mass of approximately 23kDa, as a secreted protein it should be able to diffuse through the ECM into the DZ of articular cartilage without limitations (Zhu et al., 1991; Hu et al., 1998). Moreover, FGF-18 exhibits a net positive charge at physiological pH and as a result is attracted to negatively charged proteoglycans and hydroxyapatite, which are in abundance in the deeper zones (Maroudas, 1976; Evans and Quinn, 2005; Chin et al., 2013). Interaction between these
macromolecules not only increases the diffusion coefficient of FGF-18 but also may stabilize and enhance its activity to inhibit chondrocyte hypertrophy and mineralization.

The effects of FGF-18 on articular cartilage mineralization has not yet been elucidated, however, knowledge from studies of the growth plate can be used to better understand its function in articular cartilage. FGF-18 has been shown to be an essential factor in chondrogenesis and osteogenesis during skeletal development and growth (Marie, 2003; Moore et al., 2005; Haque et al., 2007). In the growth plate, FGF-18 acts through FGFr1, and IIIc splice variants of FGFr2 and FGFr3 to exert divergent effects on chondrogenesis and bone formation (Shimoaka et al., 2002; Liu et al., 2002; Ohbayashi et al., 2002; Ellsworth et al., 2002; Marie et al., 2005). Prehypertrophic and hypertrophic chondrocytes express FGFr1, which when activated by FGF-18 is believed to regulate cell survival, differentiation and apoptosis (Barnard et al., 2005). Activation of FGFr2 by FGF-18 in growth plate cartilage has been linked to osteogenesis and bone formation (Eswarakumar et al., 2002; Ohbayashi et al., 2002; Haque et al., 2007). FGFr3 is expressed by chondrocytes in the resting and proliferating zone and interaction of FGF-18 with FGFr3 has been shown to regulate chondrocyte proliferation and differentiation by inhibiting the Indian hedgehog (Ihh) signaling pathway (Naski et al., 1998; Iwata et al. 2000; Liu et al., 2002; Ellsworth et al., 2002; Marie et al., 2005; Haque et al., 2007). Ihh forms a feedback loop with parathyroid hormone related peptide (PTHrP) to regulate proliferation and maturation of growth plate chondrocytes and growth plate cartilage mineralization (Karp et al., 2000; Kobayashi et al., 2002). Ihh promotes chondrocyte hypertrophy and is a master regulator of bone development (St-Jacques et al., 1999;
Kronenberg, 2003), whereas PTHrP maintains chondrocytes in a proliferative state (Pogue and Lyons, 2006; Smits et al., 2004). Interestingly, the PTHrP-Ihh pathway has also been reported to be the mechanism regulating mineralization in articular cartilage (Jiang et al., 2008). This study by Jiang et al. (2008) proposes that SZC communicate with DZC via the PTHrP-Ihh signaling pathway to inhibit articular cartilage mineralization.

Interestingly, chondrocytes in mature articular cartilage express FGFr1 and FGFr2 to which FGF-18 binds with low affinity, and FGFr3 to which FGF-18 binds with high affinity (Ellsworth et al., 2002; Ohbayashi et al., 2002; Davidson et al., 2005; Yan et al., 2011). Our findings coincide with what is observed in growth plate cartilage. The results of our co-culture study shows that SZC continue to express FGF-18 in vitro and DZC co-cultured with SZC exhibited higher FGFr3 gene expression compared to DZC co-cultured with DZC, which expressed significantly higher levels of FGFr2. Based on these findings it is possible that SZC releases FGF-18, which binds FGFr3 on DZC and initiates a cascade of events to down regulate Ihh expression and up regulate PTHrP signaling. This would inhibit hypertrophy of DZC and subsequently suppress DZ cartilage mineralization. Interestingly, studies have shown that FGF-18 selectively signals via FGFr3 in articular cartilage to exert cartilage-protective responses, and expression of FGFr3 is significantly reduced in OA (Ellsworth et al., 2002; Ohbayashi et al., 2002; Yan et al., 2011). In contrast, it is possible that FGFr2 is activated when DZC are co-cultured with DZC, which prompts the initiation of a different pathway that promotes hydroxyapatite deposition as observed in growth plate cartilage (Eswarakumar et al., 2002; Haque et al., 2007). To confirm that FGF-18 is the soluble factor responsible for the inhibition of mineralization,
DZC were cultured in mineralization-inducing media supplemented with FGF-18, which resulted in the inhibition of mineralization. Additionally, DZC were cultured in SZ conditioned media supplemented with an FGF receptor inhibitor, which did not inhibit mineralization. Alternatively, SZC may inhibit mineralization in the DZ by up-regulating FGF receptor levels in DZC, making them more sensitive to existing FGF-18 present in the DZ. Taken together, the results of our experiments establish FGF-18 as an inhibitor of DZ cartilage mineralization. Along with its inhibitory effect on mineralization of DZ cartilage, we determined that FGF-18 also increased the accumulation of inorganic polyphosphate within DZ cartilage. To the best of our knowledge, this is the first study to show that FGF-18 modulates polyphosphate levels in DZ cartilage.

Inorganic polyphosphates are of particular interest to our proposed mechanism by which SZC inhibit mineralization in DZ cartilage because polyphosphates have been shown to inhibit DZ cartilage mineralization (St-Pierre et al., 2010). Polyphosphate has also been shown to inhibit mineralization in other biological systems (Omelon et al., 2009; Fleisch et al., 1965; Fleisch et al., 1966; Irving et al., 1966; Schibler et al., 1968; Hoac et al., 2013), as well as in cell-free crystal growth assays (Fleisch and Neuman, 1961; Francis, 1969; Omelon and Grynpas, 2008). These studies establish that although the mechanism by which inorganic polyphosphate inhibits mineralization is likely multifactorial, the principal method is by binding directly to the surface of hydroxyapatite crystals and preventing its growth. The increased polyphosphate staining observed in unmineralized DZ cartilage that was co-cultured with SZC supports this notion. The
intensity of the fluorescence was highest at the cartilage-substrate membrane interface where apatite crystal nucleation commonly begins.

Interestingly, studies determining the effects of polyphosphate on different cell types have reported contradictory results. Polyphosphate has been shown to induce mineralization in pre-osteoblast cells (Kawazoe et al., 2004) and promote regeneration of alveolar bone (Hacchou et al., 2007; Usui et al., 2010). These seemingly contradictory effects of polyphosphate on tissue mineralization was explained by Omelon et al. (2009) who proposed that polyphosphate has a dual ability to induce or inhibit tissue mineralization. When biomineralization is undesirable, polyphosphates are maintained at a steady level or synthesized which in turn reduces the free orthophosphate concentrations. The condensed phosphate polymers are also believed to sequester free calcium ions which may also prevent apatite crystal nucleation, however, studies have shown that calcium chelation is not the main mechanism by which polyphosphate inhibits mineralization (St-Pierre et al., 2010). In contrast, when biomineralization is required these calcium-polyphosphate complexes are degraded releasing free orthophosphate and calcium ions, which promote formation of apatite crystals (Omelon et al., 2009). Fundamentally, the authors proposed crystal nucleation is determined by the ratio of orthophosphate to polyphosphate. It is important to note that inhibition of cartilage mineralization is specific to condensed phosphates only as similar concentrations of phosphate to those used for polyphosphate did not inhibit mineralization of in vitro-formed DZ cartilage (St-Pierre et al., 2010).
In our study, we observed elevated levels of polyphosphate in DZ cartilage co-cultured with SZC as well as DZ cartilage grown in the presence of FGF-18. These findings show that both SZC and FGF-18 modulate polyphosphate levels in DZ cartilage. To better understand the biological mechanism by which SZC modulate endogenous polyphosphate levels in DZC our study looked at the metabolism of polyphosphate. However, because the enzyme(s) responsible for polyphosphate synthesis are not known in mammalian tissues, this was limited to the enzymes responsible for polyphosphate degradation. In our co-culture study, we observed a decrease in TNAP activity with respect to polyphosphate levels in DZC co-cultured with SZC. This suggests that SZC may prevent polyphosphate degradation by down-regulating TNAP activity in DZ cartilage via FGF-18. Interestingly, a gradient of TNAP expression exists through the zones of native articular cartilage, which supports our proposed mechanism since it is opposite that of FGF-18. FGF-18 expression is highest in the SZ while TNAP is predominantly expressed in the DZ. This may suggest that FGF-18 restricts TNAP activity to the deeper zones by suppressing TNAP in the upper zones. Therefore, in DZ cartilage containing high levels of polyphosphate, such as DZ cartilage co-cultured with SZC and DZ cartilage treated with FGF-18, TNAP is unable to sufficiently hydrolyze polyphosphate and promote mineralization. Incomplete hydrolysis of polyphosphate is unable to shift the orthophosphate-to-polyphosphate ratio and results in the formation of calcium-polyphosphate complexes that inhibit apatite mineral formation (Omelon et al., 2009). The calculated calcium-to-phosphorus ratios of these DZ tissues support this hypothesis. These unmineralized DZ tissues exhibited calcium-to-phosphorus ratios that ranged from 0.5-0.8, which corresponds with that of linear polyphosphate ((Ca(PO3)2)n;
0.50) (Landis et al., 1982). In comparison, the calcium-to-phosphorus ratio of mineralized DZ cartilage was between 1.3-1.6, which corresponds with that of hydroxyapatite (1.62) (Landis et al., 1982).

We also observed increased levels of TNAP activity in DZ cartilage containing high concentrations of polyphosphate. This suggests that a feedback mechanism exists which elevates TNAP activity in the presence of high concentrations of polyphosphate to closely regulate the orthophosphate-to-polyphosphate ratio. The positive correlation between TNAP activity and polyphosphate levels was unexpected because TNAP activity would need to be down regulated in order to maintain elevated concentrations of polyphosphate and inhibit mineralization. However, polyphosphate levels and TNAP activity in native articular cartilage correspond with our findings, as both are highest in the DZ. Another possible mechanism by which FGF-18 inhibits the degradation of polyphosphate may be through direct interaction with polyphosphate. Polyphosphate has been shown to bind FGF-2 to form a stable complex that increases the half-life and facilitates binding of the factor to its cell surface receptors (Shiba et al., 2003). It is possible that FGF-18 forms a similar complex with polyphosphate to stabilize it and physically prevent its degradation by TNAP. This would prevent the hydrolysis of polyphosphate as well as orthophosphate residues maintaining the orthophosphate-to-polyphosphate ratio to favour inhibition of hydroxyapatite crystal formation. Interestingly, another fibroblast growth factor, FGF-2, has also been shown to inhibit TNAP expression (Hatch et al., 2005).
Taken together, the effect of FGF-18 on mineralization and endogenous polyphosphate levels within DZ cartilage have added to the current understanding of articular cartilage mineralization. Furthermore, the expression of FGF-18 by SZC suggests that suppression of mineralization in DZ cartilage is regulated by SZC. This study represents the first reported investigation showing that FGF-18 regulates polyphosphate levels within DZ cartilage. The findings have significant implications for the mechanisms that control articular cartilage mineralization and understanding the pathology of osteoarthritis disease progression. Nevertheless, further studies are needed to establish the role of each factor and fully understand the mechanism regulating post-natal articular cartilage mineralization.

### 4.3 Superficial zone chondrocytes regulate deep zone chondrocyte proliferation and promote matrix accumulation in deep zone cartilage

Loss of extracellular matrix and proliferation of chondrocytes are characteristic of OA disease progression. It has been suggested that these changes take place after the superficial zone diminishes (Lark et al., 1997; Silver et al., 2001; Wu et al., 2002; Balcerzak et al., 2003; Pearle et al., 2005; Goldring and Goldring, 2007). Therefore, this study investigated the effect of SZC on the proliferation of DZC and accumulation of extracellular matrix constituents by DZC. Our results show that SZC regulate DZC proliferation and promote collagen and glycosaminoglycan production by DZC.

Many growth factors have been shown to have anabolic effects on cartilage as reviewed by Getgood et al. (2009). Some of these factors include insulin-like growth
factor 1, transforming growth factor β-1, FGF-2, FGF-18, bone morphogenetic protein 2, -4, -5 and -7 (Getgood et al., 2009). Of particular interest to this thesis is FGF-18 because in addition to being capable of inhibiting DZ cartilage mineralization, it has been shown to have significant anabolic effects on articular cartilage (Ellsworth et al., 2002; Ohbayashi et al., 2002; Shimoaka et al., 2002; Moore et al., 2005). Intra-articular administration of FGF-18 to a rat meniscal tear model of OA reduced cartilage degeneration and promoted generation of new cartilage (Moore et al., 2005). FGF-18 has also been shown to exert cartilage-protective responses in the development of OA including stimulation of chondrocyte proliferation and increased production of proteoglycan and Type II collagen (Ellsworth et al., 2002; Ohbayashi et al., 2002; Yan et al., 2011).

Although our findings also showed increased proteoglycan and Type II collagen accumulation, a decrease in DZC proliferation was observed. The contradictory results may be explained by the ability of FGF-18 to bind and activate multiple receptors (Shimoaka et al., 2002; Liu et al., 2002; Ohbayashi et al., 2002; Ellsworth et al., 2002). FGF-18 has been shown to interact with FGFr3 to negatively regulate growth plate chondrocyte proliferation (Naski et al., 1998; Minina et al., 2002; Ornitz et al., 2005; Ozasa et al., 2005), and with FGFr3 in human articular chondrocytes and mice to suppress cellular proliferation (Ellman et al., 2008). Interestingly, activation of the FGFr3 signaling pathway is significantly reduced in the development of OA (Yan et al., 2011). Moreover, the absence of signaling through FGFr3 in mice resulted in loss of aggrecan, proteolysis of Type II collagen and increased expression of MMP-13 (Valverde-Franco et al., 2006). Similar overexpression of MMP-13 was also reported in FGF-18 knockout
mice (Ellman et al., 2008). In our study, DZC co-cultured with SZC exhibited higher FGFr3 expression compared to DZC co-cultured with DZC, which may explain the increased matrix accumulation and decreased proliferation.

Interestingly, polyphosphate has also been shown to negatively regulate chondrocyte proliferation and increase glycosaminoglycan and collagen accumulation within *in vitro*-formed DZ cartilage (St-Pierre et al., 2012). St-Pierre et al. (2012) demonstrated that polyphosphate promotes ECM accumulation in a concentration and chain length dependent manner and that continuous polyphosphate presence is required to obtain full stimulatory effects. This function of polyphosphate could explain our findings since a significant increase in polyphosphate accumulation was observed in DZ cartilage when co-cultured with SZC. In our proposed mechanism we have accounted this increase to the ability of FGF-18 to down regulate ALP activity and reduce the degradation of polyphosphate.

Taken together, the results suggest that SZC may release FGF-18 to regulate DZC proliferation and matrix accumulation in two ways; 1) Directly – by interacting with FGFr3 to inhibit DZC proliferation and stimulate the production of matrix; or 2) Indirectly – by preventing the degradation of polyphosphate in DZ cartilage, facilitating polyphosphate to inhibit proliferation and deliver its full stimulatory effects on matrix production. Alternatively, it is possible that SZC directly influences retention of glycosaminoglycans and collagens by DZ cartilage; however, this requires further investigation.
This study demonstrated that SZC interact with DZC to regulate DZC proliferation and increase matrix accumulation. However, more work is required to uncover the mechanism(s) involved. The results confirm the regulatory control of SZC and its role in maintaining articular cartilage homeostasis. The effects of SZC observed strengthen the biological and mechanical properties of in vitro-formed DZ cartilage and highlight the importance of intact articular cartilage. Finally, our findings implicate both FGF-18 and polyphosphate, separately or in combination, as therapeutic candidates for the treatment of degraded articular cartilage and OA.

4.4 Conclusions

In summary, this study demonstrates:

I. Superficial zone chondrocytes suppress mineralization in cartilage formed in vitro by deep zone chondrocytes through a soluble factor.

II. Superficial zone chondrocytes promote the accumulation of endogenous inorganic polyphosphate within in vitro-formed deep zone cartilage.

III. Fibroblast growth factor 18, which is preferentially expressed by SZC, inhibits mineralization in cartilage formed in vitro by deep zone chondrocytes by promoting the accumulation of endogenous inorganic polyphosphate levels. This suggests that the soluble factor produced by SZC may be FGF-18.

IV. Superficial zone chondrocytes negatively regulate deep zone chondrocyte proliferation and stimulates the production of glycosaminoglycan and collagen by deep zone chondrocytes cultured in vitro.
4.5 References


CHAPTER 5: FUTURE DIRECTIONS
The work presented in this thesis confirm the study hypothesis and establish the role of superficial zone chondrocytes in regulating mineralization and promoting matrix production in cartilage formed by deep zone chondrocytes \textit{in vitro}. However, the findings have also generated new questions for future work. Success in current efforts towards understanding OA disease progression and developing therapeutic treatment options depend on a better understanding of the regulatory mechanism(s) involved in the deterioration of the extracellular matrix and mineralization of articular cartilage.

We observed that signals derived from SZC suppress mineralization and promote matrix production in cartilage formed by DZC. We have also shown that freshly isolated and \textit{in vitro} cultured SZC express FGF-18; the paracrine factor we believe is responsible for the observed effects. Culturing DZC in media supplemented with FGF-18 had the same inhibitory effects on mineralization as produced by SZC. These experiments suggest FGF-18 is the factor responsible for inhibiting mineralization, however, its production and release by SZC need to be verified. Media conditioned by SZC and DZC will be collected during the first week of culture and analyzed by tandem mass spectrometry. The resulting raw mass spectra from each fraction will be analyzed using Mascot (Matrix Science) and X!Tandem (Global Proteome Machine Manager) search engines on the non-redundant International Protein Index (IPI) database. Proteins will be evaluated by Ingenuity Pathways Analysis software to identify global functions of the proteins. Once the protein(s) are identified, the SZC and DZC will be examined for differential expression of this protein by real time PCR and western blotting. The expression of this protein in native bovine and human cartilage will be examined by immunohistochemical staining to
demonstrate the distribution of gene expression in \textit{in vivo} tissue. Inhibiting and up-regulating the expression of this protein in DZC will then confirm its effect on tissue mineralization.

Our data also suggests inhibition of mineralization is mediated via regulation of endogenous polyphosphate levels by SZC and FGF-18. We show that SZC and FGF-18 increase the accumulation of polyphosphate within \textit{in vitro}-formed DZ cartilage. For this reason, the pathway(s) by which FGF-18 regulates cartilage mineralization by modulating polyphosphate levels must be examined further. An in depth investigation into the role FGF-18 plays in controlling exopolyphosphatases and polyphosphate kinases is necessary to elucidate the mechanism(s). ALP is an exopolyphosphatase that has been implicated to be involved in the mechanism regulating mineralization in DZ cartilage but its role should be further investigated through the addition of Levamisole, a non-competitive and reversible inhibitor of liver, kidney, spleen and bone alkaline phosphatase. FGF-18 signaling has also been shown to inhibit Ihh expression, which functions via a negative feedback loop with PTHrP. The Ihh/PTHrP feedback loop governs chondrocyte proliferation and maturation and PTHrP has been reported to be involved in the mechanism by which SZC inhibit articular cartilage mineralization. The role of this signaling pathway should be investigated as it may give insight into the mechanism(s) regulating chondrocyte proliferation and mineralization.
Co-culturing SZC with DZC produced DZ cartilage with higher glycosaminoglycan and collagen content, and negatively regulated DZC proliferation compared to DZC co-cultured together. Interestingly, both polyphosphate and FGF-18 have been shown to increase ECM accumulation and regulate cellular proliferation. In order to determine the regulatory mechanism involved we need to establish whether the DZC are induced to produce higher amounts of ECM matrix constituents or are able to better retain them. Additionally, experiments need to be set up to investigate if matrix accumulation in DZ cartilage is achieved by preventing the catabolic events that degrade the constituents.

In conclusion, the work presented in this thesis provides a basis for future studies investigating interactions between zone-specific chondrocytes. This study provides insight into probable mechanisms for post-natal regulation of articular cartilage mineralization and ECM metabolism. Our findings may contribute to the development of bioengineered articular cartilage with improved functional properties. Finally, our work implicates polyphosphate and FGF-18, as therapeutic candidates for repair of degraded articular cartilage.
APPENDIX: SUPPLEMENTARY METHODS, RESULTS

AND TABLES
METHODS

Preparation of Membrane Inserts

Membrane inserts (Millicell-CM®, Millipore Corp., Bedford, MA, USA) were coated with Type II collagen (0.5mg/ml in 0.1N acetic acid; Sigma Chemical Co., St.Louis, MO, USA) and dried overnight. The membrane inserts were UV sterilized for 30 minutes and soaked in Ham’s F12 for 30 minutes prior to cell culturing.

Determination of Total Proteoglycan, Collagen, and DNA Content

Cartilage tissues were harvested on Day 7, washed twice in PBS, removed from the membrane and digested by papain (Sigma Chemical Co., 40 mg/mL in 20mM ammonium acetate, 1mM ethylenediaminetetraacetic acid, and 2mM dithiothreitol) for 48 hours at 65°C and stored at -20°C until analysis. The proteoglycan content was estimated by determining the amount of sulfated glycosaminoglycans using a dimethylmethylene blue dye binding assay (Polysciences, Washington, PA, USA) and spectrophotometry (wavelength 525nm) (Goldberg and Kolibas, 1990). Standard curves were generated using bovine trachea chondroitin sulfate A (Sigma Chemical Co.). DNA content was quantified using the Hoechst 33258 dye assay (Polysciences) and fluorometry (emission wavelength 460nm and excitation wavelength 355nm) (Kim et al., 1988). Standard curves were generated using calf thymus DNA (Sigma Chemical Co.). Collagen content was estimated from the determination of the hydroxyproline content. Aliquots of the papain digest were hydrolyzed in 6N HCl at 110°C for 18 hours. Hydroxyproline content of the
hydrolysate was determined using chloramine-T/Ehrlich’s reagent assay and spectrophotometry (wavelength 560 nm) (Reddy and Enwemeka, 1996). The standard curve was generated using cis-hydroxy-L-concentration. Collagen content was determined from the hydroxyproline content by multiplying by 10, as hydroxyproline constitutes approximately 10% of collagen by weight (Woessner, 1961).

**Quantifying Mineral Content of Cartilaginous Tissue via HCl Digestion**

To quantify the mineral content of *in vitro*-formed cartilage the calcium and phosphate contents were measured. The tissues were washed 2 times with PBS --/-, lyophilized, weighed, and then digested.

**TISSUE DIGESTION**

1. Wearing gloves and safety glasses, place sample in 15mL conical tube and add 500 µL of 3N Hydrochloric acid
2. Tightly secure lid and place tubes in 90°C hot water bath for 90 minutes. Vigorously vortex tubes every 20 minutes to promote complete digestion of tissues.
3. Add 1.5mL of ddH_{2}O and adjust pH of the solution to between 2.5 and 2.9 using NaOH and HCl.
4. Top up with ddH_{2}O to a final volume of 2.5 mL.
PHOSPHATE QUANTIFICATION

The phosphate content was measured as outlined by Allan et al., (2007) without any deviations. Briefly, mix an aliquot of sample with a solution containing 1:6 vol/vol ratio of 10% ascorbic acid and 0.42% ammoniummolybdate (Sigma Chemical Co.) in 1 NH$_2$SO$_4$ (1:3 vol/vol ratio) at 37°C for 1 hour and then quantify the resulting color spectrophotometrically at 620 nm. The standard curve was generated using a sodium phosphate dibasic solution (stock=0.5mM; pH 7.5).

CALCIUM QUANTIFICATION

The calcium content was measured as outlined by Allan et al., (2007) with an adjustment made to the standard to correct for the 3N HCl digestion process. To measure calcium content, an aliquot was mixed with 0.01% cresolphthalein complexone (Sigma Chemical Co.) buffered with 0.25 M sodium borate in a 1:4 vol/vol ratio and the resulting color quantified spectrophotometrically at 570 nm (Titertek Multiscan) within 5 minutes. The standard curve was generated using a calcium standard.

Modification to Standard for Calcium Assay

*Prepare solution to contain the exact same volumes of 3N HCl and NaOH as the digested tissue samples.

1. Add 500 µL of 3N Hydrochloric acid and 1.5mL of ddH$_2$O to a 15mL conical tube
2. Adjust pH of the solution to between 2.5 and 2.9 using NaOH and HCl.
3. Top up with ddH$_2$O to a final volume of 2.5 mL.
4. Add 8 µL of this solution to all standard wells to correct for the 3N HCl digestion process. Prepare the standard on the 96-well plate as follows:

**Table A1:** Preparation of calcium standard from 2mM Calcium Chloride stock solution

<table>
<thead>
<tr>
<th>Calcium Concentration</th>
<th>2mM Calcium Standard</th>
<th>ddH₂O</th>
<th>Correction Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>0 µL</td>
<td>72 µL</td>
<td>8 µL</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>20 µL</td>
<td>52 µL</td>
<td>8 µL</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>40 µL</td>
<td>32 µL</td>
<td>8 µL</td>
</tr>
<tr>
<td>1.25 mM</td>
<td>50 µL</td>
<td>22 µL</td>
<td>8 µL</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>60 µL</td>
<td>12 µL</td>
<td>8 µL</td>
</tr>
<tr>
<td>1.75 mM</td>
<td>70 µL</td>
<td>2 µL</td>
<td>8 µL</td>
</tr>
</tbody>
</table>

**Detecting orthophosphate, pyrophosphate and linear polyphosphate species separated by PAGE**

Separation, hydrolytic degradation and visualization of orthophosphate (Pi), pyrophosphate (PPi) and linear polyphosphate species (PP5, PP45, and PP65) by PAGE was performed as described by Omelon and Grynpas (2007). Briefly, vertical slab gels (16 × 12.5 × 0.15 cm) were cast with 16% polyacrylamide (19:1 bisacrylamide/acylamide ratio, 7M urea, and 1× TBE (90mM Tris, 90mM borate, and 2.7mM EDTA, pH 8.3)). Gels were pre-electrophoresed for approximately 45 minutes at 280V. The wells were loaded with 20µL of phosphate species containing 0.5mM of Pi per species and 5µL of loading dye solution (1x TBE buffer, 10% sucrose, 0.05% bromophenol blue).
The electrophoresis was run at 283V for approximately 1 hour or until the bromophenol blue migrated halfway through the gel. After electrophoresis, hydrolytic degradation of the polyphosphate species was carried out by coating the gel with 15 mL/cm$^2$ of 4°C 5M HCl saturated with NaCl for 10 minutes. To visualize the Pi degradation products, the gel was soaked in a 1% w/v ammonium molybdate, 0.25% w/v methyl green, and 1M HCl solution for 4 minutes. The gel was then removed from the solution and encased in plastic wrap for the colour to develop overnight.
RESULTS

Alkaline Phosphatase Activity to evaluate quality of cartilage zonal separation

To verify quality of zonal separation, alkaline phosphatase assay was performed on freshly isolated chondrocytes. ALP activity normalized to DNA content was significantly higher in DZC compared to SZC and MZC.

Figure A1: Alkaline phosphatase activity of chondrocytes extracted from the different zones of bovine articular cartilage (SZ: superficial zone; MZ: middle zone; DZ: deep zone). Activity was normalized to DNA content. DZ chondrocytes had significantly higher alkaline phosphatase activity compared to SZ and MZ chondrocytes. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates significantly higher alkaline phosphatase activity.
Figure A2: Differential expression of fibroblast growth factor signaling genes as determined by qPCR after normalization to 18S rRNA in chondrocytes freshly isolated from the different zones of bovine articular cartilage (SZ: superficial zone; MZ: middle zone; DZ: deep zone). Levels expressed relative to full thickness cartilage (calibrator). Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates a significant difference in expression for the selected gene. FGF-1 = fibroblast growth factor 1, FGF-2 = fibroblast growth factor 2, FGF-18 = fibroblast growth factor 18, FGFr2c = fibroblast growth factor receptor 2c, and FGFr3c = fibroblast growth factor receptor 3c.
Figure A3: Superficial zone chondrocytes inhibits mineralization in cartilage formed in vitro by deep-zone chondrocytes. (A) Quantification of calcium and phosphate contents and (B) calcium-to-phosphorus ratio of DZC cultures after 7 days in medium without β-glycerophosphate (Neg. C), mineralization-inducing medium (Pos. C) and co-culture with either SZC (DZ(SZ)), MZC (DZ(MZ)) or DZC (DZ(DZ)). Each condition was done in triplicate and the experiment was repeated 5 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates a significant difference.
Dry weight of cartilaginous tissue

Following 7 days of culture the tissues were lyophilized and weighed. The average weight of the control constructs (No β-GP) was significantly lower than DZ constructs co-cultured with DZ. The average weight of DZ constructs co-cultured with SZC as well as MZC was lower than DZ constructs co-cultured with DZ, however, the results were not significant. This outcome is expected as the negative control does not mineralize and as a result should have a lower weight compared to the DZ-DZ constructs.

Table A2: Dry weight of tissue formed by DZ chondrocytes cultured in the presence of SZ, MZ or DZ chondrocytes for 7 days. The results from all experiments were pooled and expressed as mean ±SEM. * indicates a significant difference in weight. (1-way ANOVA: N=14, α = 0.05)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>AVERAGE (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZ – β-GP</td>
<td>1.9 ± 0.10 *</td>
</tr>
<tr>
<td>DZ + β-GP</td>
<td>2.1 ± 0.11</td>
</tr>
<tr>
<td>DZ(SZ)</td>
<td>2.1 ± 0.14</td>
</tr>
<tr>
<td>DZ(MZ)</td>
<td>2.1 ± 0.18</td>
</tr>
<tr>
<td>DZ(DZ)</td>
<td>2.5 ± 0.17</td>
</tr>
</tbody>
</table>
Superficial Zone Chondrocytes Suppress Deep Zone Chondrocyte Proliferation and Stimulate Matrix Accumulation in In Vitro-Formed Deep Zone Cartilage

SZC suppressed DZC proliferation and enhanced matrix accumulation by DZC cultured in mineralization-inducing medium for 7 days. The total DNA content of DZC co-cultured with SZC was significantly lower than when co-cultured with DZC (Figure A4). After normalization for DNA content, the accumulation of glycosaminoglycan and collagen within DZ cartilage co-cultured with SZC was significantly higher compared to DZC co-cultured with DZC (Figure A5).

![Graph](image)

**Figure A4:** Superficial zone chondrocytes suppresses deep zone chondrocyte proliferation *in vitro*. Total DNA content of deep zone chondrocyte co-cultured with SZ, MZ and DZ chondrocytes on membrane inserts for 7 days in mineralization-inducing medium. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean.

* Indicates significant difference in DNA content.
Figure A5: Effect of superficial zone chondrocytes on accumulation of glycosaminoglycan and collagen by deep zone chondrocytes. Glycosaminoglycan and collagen content of DZ cartilage co-cultured with either SZC (DZ(SZ)), MZC (DZ(MZ)) or DZC (DZ(DZ)) on membrane inserts for 7 days in mineralization-inducing medium. The data were normalized to DNA content. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates significantly higher GAG and collagen content than in DZ(DZ) cultures.
Differential gene expression of mineralization related genes in chondrocytes co-cultured for 7 days in mineralization-inducing medium. DZC were either co-cultured with SZC (DZ(SZ)) or DZC (DZ(DZ)). Differential gene expression was determined by the ∆∆Ct method after normalization to 18S rRNA. Levels are expressed relative to DZ(DZ) construct (calibrator). Each condition was done in triplicate and the experiment was repeated 4 times. The results were pooled and expressed as mean ± standard error of the mean. ALP = tissue non-specific alkaline phosphatase; COL X = collagen Type X; h-PRUNE = short-chain exopolyphosphatase; MMP-13 = matrix metalloproteinase 13; RUNX2 = runt-related transcription factor 2.
Figure A7: Differential gene expression of fibroblast growth factor signaling in chondrocytes co-cultured for 7 days in mineralization-inducing medium. DZC were either co-cultured with SZC (DZ(SZ)) or DZC (DZ(DZ)). Differential gene expression was determined by the ∆∆Ct method after normalization to 18S rRNA. Levels are expressed relative to DZ(DZ) construct (calibrator). Each condition was done in triplicate and the experiment was repeated 4 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates a significant difference in expression for the selected gene. FGF-1, -2, -18 = fibroblast growth factor 1, 2, 18; FGFr2c = fibroblast growth factor receptor 2c, and FGFr3c = fibroblast growth factor receptor 3c.
Figure A8: Differential gene expression of extracellular matrix constituents in chondrocytes co-cultured for 7 days in mineralization-inducing medium. DZC were either co-cultured with SZC (DZ(SZ)) or DZC (DZ(DZ)). Differential gene expression was determined by the ΔΔCt method after normalization to 18S rRNA. Levels are expressed relative to DZ(DZ) construct (calibrator). Each condition was done in triplicate and the experiment was repeated 4 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates a significant difference in expression for the selected gene. AGG = aggrecan; COL I = collagen Type I; COL II = collagen Type II; SZP = superficial zone protein.
**Figure A9:** Inorganic polyphosphate accumulation within *in vitro*-formed cartilage. DAPI staining was used to demonstrate inorganic polyphosphate distribution within *in vitro*-formed DZ cartilage grown in mineralization-inducing medium for 7 days (A) DZ cultured without β-GP, (B) DZ cultured in mineralization-inducing medium with β-GP, (C and D) SZ co-cultured with DZ, (E and F) MZ co-cultured with DZ, (G and H) DZ co-cultured with DZ. (A, B, D, E, H = 1×10^6 cells; C, E, G = 2×10^6 cells) Epifluorescence microscopy; DAPI staining. The arrow indicates mineral deposits. * Indicates the membrane insert. The scale bars indicate a distance of 100µm.
Figure A10: Time course of mineral deposition in deep zone chondrocytes co-cultured with either superficial zone or deep zone chondrocytes. Mineral deposition in DZC co-cultured with SZC and DZC on membrane inserts in mineralization-inducing medium at 2, 4, 6, and 8 days. Mineralization in DZC co-cultured with DZC begins much earlier than DZC with SZC. Mineralization in DZC co-cultured with SZC is suppressed at earlier time points and rate of mineral deposition is lower. The scale bars indicate a distance of 500µm.
Figure A11: Media conditioned by superficial zone chondrocytes cultured alone inhibits deep zone chondrocyte mineralization. (A) Calcium and phosphate contents and (B) calcium-to-phosphorus ratio of tissue formed by DZ cartilage in vitro on membrane inserts after 7 days in media conditioned by different cell densities of SZC or DZC cultured alone. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates significant difference.
**Figure A12:** Soluble factors released by superficial zone chondrocytes inhibit deep zone chondrocyte mineralization. Calcium-to-phosphorus ratio of tissue formed by DZ cartilage *in vitro* on membrane inserts after 7 days in ITS media conditioned by SZC or DZC subjected to heat treatment (H.T.) or trypsinization (Trypsin). Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * Indicates significant difference.
Figure A13: Effect of fibroblast growth factor signaling inhibitor on inhibition of mineralization in deep zone cartilage by superficial zone chondrocyte conditioned media. Calcium-to-phosphorus ratio of tissue formed by DZ cartilage in vitro on membrane inserts after 7 days in ITS media conditioned by SZC or DZC supplemented with PD173074. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean.
Figure A14: Proposed mechanism for the modulation of polyphosphate and cartilage mineralization in healthy mature articular cartilage.
Figure A15: Proposed mechanism for the modulation of polyphosphate and cartilage mineralization in osteoarthritic articular cartilage.
Figure A16: PAGE of Pi, PPI, and linear chain polyphosphate species. PAGE was used to separate and visualize Pi, PPI and polyphosphate with average chain lengths of 5, 45 and 65 phosphate units.
References:


