THE IN VITRO PRODUCTION OF ENDOCHONDRAL BONE FROM BOVINE PERIOSTEAL EXPLANTS

By

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Abstract

In the present study, periosteal tissue explants were explored as a substratum for the production of endochondral bone tissue *in vitro*. Endochondral bone is formed when mesenchymal stem cells (MSCs) proceed through the chondrogenic lineage to produce a transitory cartilage template which eventually is ossified. The periosteum is of interest as this tissue is found enveloping long bones and has been shown to contain a resident cellular population capable of generating endochondral bone. Endochondral ossification was induced in periosteal explants through the successive application of chondrogenic and hypertrophic/osteogenic media simulating the *in vivo* progression of the process. Different chondrogenic and osteogenic media types were utilized in order to assess the best method for producing osseous tissue. The results indicated that endochondral bone could be produced from periosteum tissue *in vitro*. It was determined that chondrogenic culture with transforming growth factor β1 (TGFβ1) led to the development of immature (resting or proliferative) cartilage tissue while chondrogenic culture with bone morphogenetic protein 2 (BMP2) produced mature (hypertrophic or calcified) cartilage and osseous tissue. Osteogenic media generally failed to improve ossification in cartilaginous explants but did affect their progression through the endochondral process. Cartilaginous periosteum explants responded differently to osteogenic media types based on the method of chondrogenic pre-induction. Immature cartilage formed under TGFβ1 induction underwent maturation in osteogenic media with triiodothyronine (T3). Mature cartilage formed under BMP2 continued to undergo maturation in the presence of osteogenic media with BMP2 or T3. Overall, these findings suggested that BMP2 is crucial in the development of endochondral bone from periosteal explants *in vitro* and that the osteogenic media is unnecessary in promoting this process.

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Chapter 1 Introduction

1.1 The Clinical Need for Bone Tissue

Advances in the field of orthopaedics have led to significant improvements in the prognosis of bone related surgeries; however, when normal healing is inadequate to achieve bone union, further augmentative procedures may be required. In these instances, bone grafts are often utilized in conjunction with surgical interventions (fixation) to enhance repair by serving as ancillary or replacement tissue. An estimated 2 million bone graft procedures are performed worldwide annually, with at least 500,000 of these undertaken in the United States alone [1], [2]. Bone grafts are employed in a wide variety of orthopaedic surgeries for joint immobilization (arthrodesis), defect correction, and void reconstruction in the treatment of congenital defects, degenerative diseases, cancers, and trauma [3]. Between 1992 and 2007, data collected on the usage of bone grafts and bone substitutes in the U.S. indicated over half of all procedures were attributable to spinal injury, with the remaining fraction due primarily to fracture non-union and prosthesis correction. The recipients of these grafts were seen to be increasingly older over the period of study [4]. The currently high clinical need for bone tissue should only continue to rise as the global population ages, representing an epidemiological shift towards a demographic at higher risk of bone injury and with poorer healing capacity due to associated changes in mechanical and biological tissue function [5]–[8]

The osteoconductive, osteoinductive, and/or osteogenic components of a graft facilitate bone tissue integration and regeneration at the defect site. Each of these components represents a particular aspect of the body's natural healing response and necessarily promotes bone repair. An osteoconductive matrix supports osteogenic cell invasion, osteoinductive cues regulate the recruitment and differentiation of osteogenic cells, and osteogenic cells mediate the formation of new bone tissue [9]. A bone graft may be considered beneficial if it provides any one or more of these properties at a defect site. Autologous bone tissue grafts are the current 'gold standard' of repair in orthopaedic surgery, as they are fully histocompatible and confer all three of these qualities [10]. Harvest of autologous grafts requires a secondary surgery in which bone is excised from a donor site, typically the iliac crest or the intramedullary canal of long bones [11]. Complications such as infection and chronic pain are commonly reported following graft harvest [9], [12]. Furthermore, the total amount of bone tissue available is naturally limited to the patient's own supply, therefore autografting may be unfeasible in the treatment of larger defects. Allogeneic tissue grafts are thus frequently utilized in lieu of autogenous grafts as they are readily available, non-exhaustive in supply and negate donor site morbidities [13]. Allogeneic tissue grafts are sourced from living or cadaveric donors and are available in many forms including demineralized bone matrix (DMB), cortical, cancellous, and cortico-cancellous chips or morsels, and

osteochondral or whole bone segments [14]. The regenerative capacity of allografts is inferior to that of autologous tissue due to post-collection processing which removes the osteogenic component of the graft and diminishes its osteoinductive properties [14]. Allogeneic grafts also carry the risk of disease transmission and immune rejection despite the stringent donor screening process and downstream processing [14]. Furthermore, donor tissue may not be capable of meeting demand as the need for grafts continues to rise [2]. Limitations in sourcing bone tissue along with the issues associated with graft use prompted the development of bone substitutes for orthopaedic applications.

1.2 Strategies in Bone Tissue Engineering

Tissue engineering features the use of scaffolds, growth factors, and cells – alone or in combination – to create reparative or replacement tissue constructs for medical purposes. Assembly of components occurs *in vitro* and is followed by either immediate *in vivo* implantation or a further culture period prior to use. Direct implementation enhances repair locally by providing the basic constituents required for tissue regeneration at the defect site. Further *in vitro* culturing is intended as a means to create mature tissues for later *in vivo* incorporation [15]. A variety of therapeutic treatments have been developed in the field of tissue engineering to reduce the need for autologous and allogeneic bone grafts. Osteoconductive biomaterials represent some of the first examples of bone tissue engineered products and have been widely used as scaffolding in particular orthopaedic applications [16]–[18]. More recently, growth factors and mesenchymal stem cells (MSCs) have been loaded into osteoconductive matrices to facilitate bone repair by supporting osteogenesis at the defect site [19]–[22]. Osseous tissue formation may also be stimulated *in vitro* through the process of intramembranous ossification wherein bone tissue is deposited by differentiated osteoblasts [23]. MSCs within an osteoconductive matrix are therefore prompted to undergo osteogenic differentiation and produce bone matrix through the application of particular inductive factors [24]. Although many of these constructs have proven to successfully promote bone repair following *in vivo* implantation, the *in vitro* method of their production requires streamlining and improved efficacy for ultimate clinical use [25].

A newly emerging strategy in bone tissue engineering focuses on a biomimetic rather than a modular approach in the development of bone tissue. In this technique, MSC populations are induced to form bone through the process of endochondral ossification [26]. The endochondral mechanism of bone formation features the differentiation of MSCs to a cartilaginous template which undergoes successive stages of maturation until eventual ossification [23]. Endochondral ossification is a major contributor to skeletal system formation and maintenance – it is the primary process associated with limb morphogenesis, longitudinal bone growth, and fracture repair [27]. This method of bone production has been suggested to be advantageous over intramembranous ossification not only based on its functionary

roles *in vivo* but also due to the benefits imparted during *in vitro* culture and subsequent implantation [28]. Cartilage is naturally avascular, with chondrogenic differentiation and tissue survival promoted in hypoxic environments, indicating that the constructs produced may be capable of surviving the low oxygen conditions accompanied by scale-up or implantation [29]–[31]. Furthermore, mesenchymal stem cells are predisposed to hypertrophy – the terminal state of differentiation in the endochondral route of ossification - following *in vitro* chondrogenesis [32], [33]. Lastly the cartilaginous template is a naturally osteoconductive and osteoinductive scaffold capable of supporting vascular invasion and the infiltration and differentiation of host osteogenic cells in a similar manner as at the fracture callus and ossification zone of the growth plate [34]. Following a 'developmental engineering' paradigm, researchers have attempted to form bone by first chondrogenically differentiated MSCs *in vitro* prior to *in vivo* application [35]. Thus far, this technique has been quite promising, capable of producing larger amounts of bone-like tissue *in vivo* in comparison to other techniques [36]–[39]

1.3 Cellular Sources for Endochondral Ossification

Mesenchymal stem cells have been sourced from multiple somatic tissues including bone marrow, adipose tissue, peripheral blood, synovium, and the periosteum [40]–[45]. Although multi-lineage potential is a hallmark of this cellular population, the efficacy of differentiation varies based on their origin; MSCs are more effective when differentiating down lineages of the tissue from which they were derived [46], [47]. Therefore, MSCs from tissues involved in bone forming processes should be utilized for endochondral bone tissue engineering. Bone marrow derived MSCs (BMSCs) been featured in a large number of these studies due to the superiority of their chondrogenic differentiation and their ultimate progression to hypertrophy [48], [49]. A source for MSCs that has been gaining traction in bone tissue engineering is the periosteum. The periosteum is a fibrous tissue that covers the outer surface of long bones and is involved in both endochondral and intramembranous processes such as fracture repair, osteophyte formation, and growth plate maintenance [50], [51]. Periosteum derived cells have been determined to mainly contribute to endochondral bone formation during fracture repair while bone marrow derived cells instead give rise to intramembranous bone [52], [53]. This may indicate that periosteum is the superior choice from which to launch the endochondral process *in vitro*. MSCs derived from periosteal tissue were shown to have both chondrogenic and osteogenic potential approximately on par with that of bone marrow [54]. Indeed, periosteal tissue grafts have been utilized successfully *in situ* for the regeneration of large segmental bone defects [55]–[57].

Currently, bone tissue engineering is focused on utilizing isolated cells to recapitulate the endochondral process *in vitro* [58], [59]. Adoption of this technique in a clinical setting may prove challenging as chondrogenic differentiation of isolated MSCs requires very large initial populations and

results in the production of small tissue constructs which are often pooled together to meet volume demand [60], [61]. Core necrosis has also been reported in chondrogenically differentiated cultures and remains one of the main challenges of scale-up in bone tissue engineering [34], [58]. Interestingly, periosteum explants have been reported to undergo chondrogenesis *in vitro* [62], [63]. Although recapitulation of endochondral ossification has not been attempted in periosteal explants, it could feasibly negate the issues associated with isolation and culture of the tissue's stem cell fraction and allow for construct scale-up.

1.4 Research Objectives

The goal of this research was to determine whether endochondral bone could be induced from periosteal explants *in vitro*. While periosteal cells have shown osteochondral potential, the use of periosteal tissue for endochondral ossification *in vitro* has not yet been attempted. In the present experimentation, periosteum was therefore utilized as a substratum for the production of bone tissue through the endochondral route of ossification. The method of inducing the endochondral process in periosteal explants was further explored. Initial chondrogenic differentiation was followed by a hypertrophic/osteogenic culture period to simulate the *in vivo* process of endochondral ossification. Several chondrogenic media types and hypertrophic/osteogenic media types were utilized such that the best method of ultimately producing osseous tissue could be determined.

Chapter 2 Literature Review

2.1 Bone Biology

2.1.1 Structure and Function

Bone is a highly variable, dynamic tissue responsible for a multitude of physical and biological functions within the body including support, protection, movement, cellular production and mineral homeostasis. The tissue is comprised of an extracellular matrix with both organic and inorganic phases, along with cellular constituents that are responsible for tissue remodelling and maintenance through matrix resorption and deposition. Osseous tissue may be classified as either trabecular (cancellous, spongey) bone or cortical (dense, compact) bone (Figure 2-1). These bone types are localized within skeletal elements and confer different functional properties [27], [64], [65].

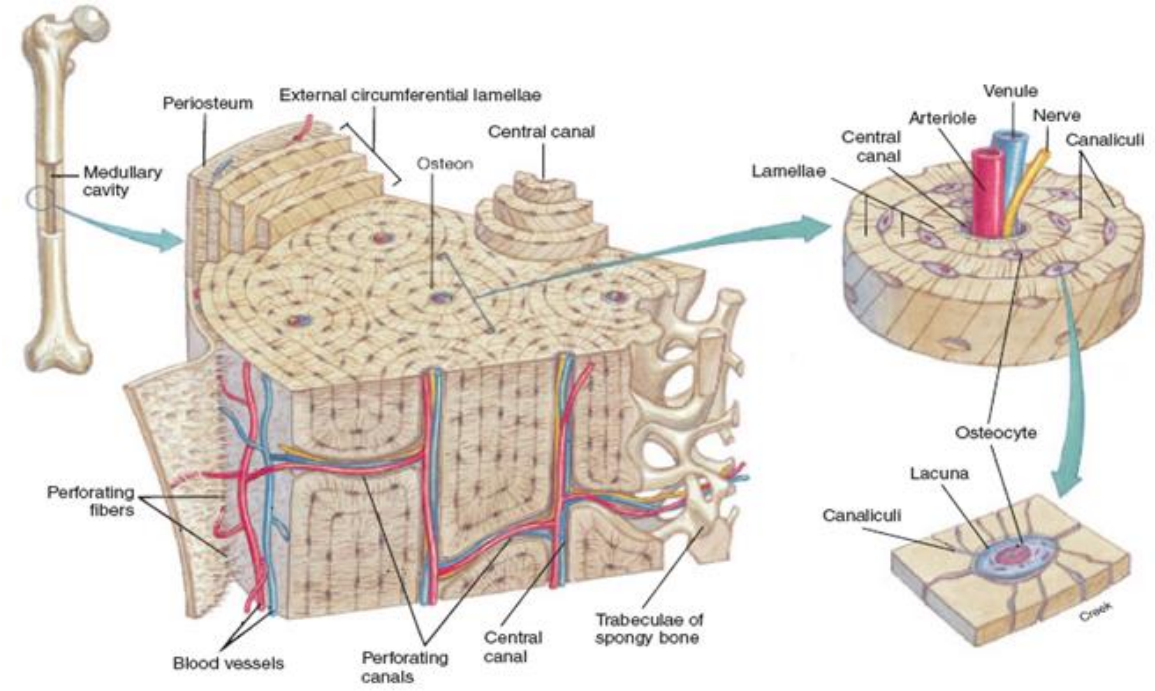

Figure 2-1: Bone tissue structure

Cortical bone is dense, strong, and flexible, forming the outer cortex of all bones. The functional unit of cortical bone is the osteon. Osteons consist of a central Haversian canal which runs longitudinally along the bone axis containing blood vessels and nerves, circumferentially enclosed within layers of bony tissue termed lamellae. Osteocytes are localized between the radiating lamellae within lacunar spaces which are connected through canaliculi. The canaliculi link resident bone cells with each other and with the vasculature thus creating a branching network for cellular communication, mechanical stress sensation, nutrient supply, and waste removal. The outer surface of bone is covered by the periosteum, a

fibrous tissue which houses the vasculature that supplies blood to cortical bone. Volkmann's canals perforate the bone surface and run between osteons, connecting vessels within the cortical bone with each other and with the external vasculature [27], [64], [65].

Trabecular bone is highly porous and forms the mesh-like bone marrow compartment in the interior of long bones. Trabeculae, the functional subunit of trabecular bone, are structured similarly as osteons, however they lack the centrally enclosed neurovasculature. Trabecular bone is unique in its capabilities as its high surface area to volume ratio enables calcium ion exchange and its vascularity provides the appropriate microenvironment for hematopoiesis and the housing of red bone marrow [27], [64], [65].

Bone may be considered either woven or lamellar, representing immature and mature bone respectively. Woven bone is characterized by the random orientation of the collagen fibers in its matrix, high cellularity, and a generally unorganized cellular population. Woven bone is most commonly found in areas where new bone is developing such as at the metaphysis of long bones in growing adolescents and at the fracture callus. In contrast, lamellar bone is highly regular with stress oriented collagen fibers that result in anisotropic mechanical properties. Lamellar bone results from the remodelling of woven bone and is found throughout the mature skeleton. The anisotropy of lamellar bone results in its general strength over that of woven bone [27], [64], [65]

2.1.2 Cellular Constituents

2.1.2.1 Osteoblasts and Osteocytes

Osteoblasts are derived from the osteogenic differentiation of mesenchymal stem cells (MSC) resident within bone. These cells are responsible for the production of bone osteoid and involved in the regulation of mineralization. Osteoblasts may be found lining bone surfaces in areas where new bone synthesis or remodelling is occurring. They are cuboidal in shape with a high amount of mitochondria and rough endoplasmic reticulum and a large Golgi apparatus, which is indicative of the high metabolic and secretory functioning of these cells. Markers of osteoblastic activity include alkaline phosphatase (ALP) and collagen I, along with several non-collagenous proteins of the osteoid matrix including osteocalcin, osteonectin, osteopontin, and bone sialoprotein [66], [67]. Depending on the stage of differentiation, osteoblasts temporally and spatially regulate the deposition of the non-collagenous constituents of the osteoid matrix. Osteoblastic lineage commitment is modulated by the expression of two main transcription factors: runt-related transcription factor 2 (*Runx*2) and osterix (*Osx*) [68], [69]. Differentiation of MSCs to osteoblasts is thought to occur by a two stage process; *Runx*2 mediates the transition of MSCs to a preosteoblastic state while downstream activation of *Osx* ultimately leads to the mature osteoblastic phenotype [68]–[70]. Preosteoblasts maintain bipotency indicating *Osx* is essential in

osteoblast commitment (Figure 2-2). Indeed, *Runx*2-positive, *Osx*-null progenitor cells were incapable of osteoblastic differentiation and instead retained a chondrogenic phenotype [71]. Preosteoblastic cells produce collagen I and ALP, while markers for mature osteoblasts also include osteocalcin and bone sialoprotein [70]. The direct deposition of bone by osteoblasts following MSC differentiation is known as intramembranous ossification [68]. Osteoblasts are highly involved in the regulation of osteoclastogenesis through the activity of surface membrane bound receptor activator nuclear factor κB ligand (RANKL) and the expression of the RANK decoy receptor osteoprotegerin, where the binding of RANKL to RANK expressed by osteoclast precursors triggers induction of osteoclast formation [72].

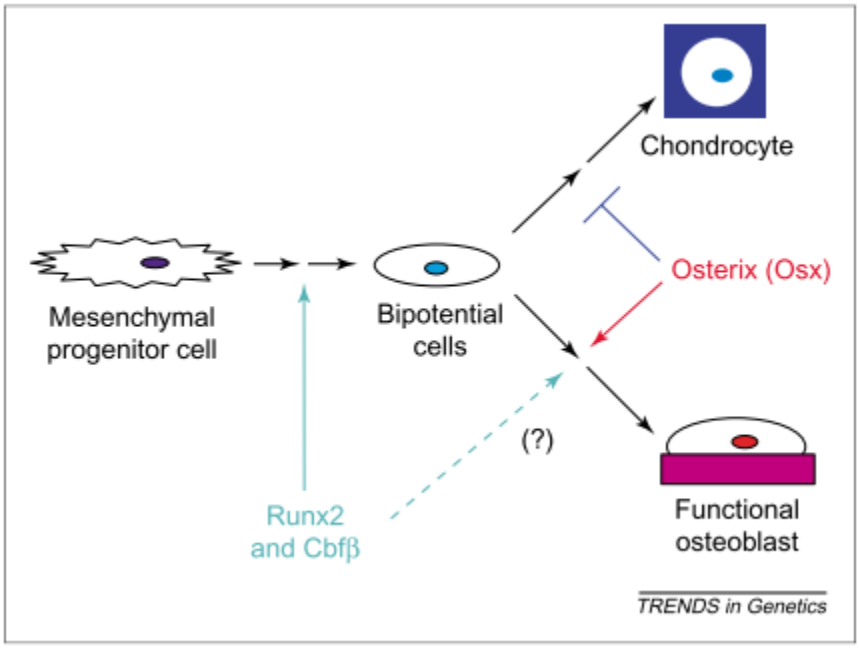

Figure 2-2: Bipotency of osteoprogenitors [69]

Osteocytes are the mature form of osteoblasts which have become encapsulated within the secreted bone matrix. They are found dispersed in an organized manner throughout the tissue, forming a network for cellular communication and mechanosensing. Cytoplasmic extensions occupy the canaliculi between cellular lacunae such that osteocytes may exchange nutrients, waste, and signalling factors through adjacent gap junctions. These cells thus participate in the maintenance of bone tissue and regulation of mineral homeostasis through recruitment of osteoblasts and osteoclasts, and through independent mechanisms as well [67], [68].

2.1.2.2 Osteoclasts

Osteoclasts are responsible for bone turnover and initiation of the remodelling process through resorptive action. Characteristically large and multi-nucleated, these cells are derived from the

hematopoietic cell lineage rather than the mesenchymal lineage and are thus related to macrophages rather than osteoblasts [27], [67], [68]. Their formation is the result of cytoplasmic fusion of precursor monocytes/macrophages in response to various cytokine cues and, more specifically, concomitant activation by stromal cell macrophage colony-stimulating factor (M-CSF) and osteoblastic RANKL binding. Osteoblastic expression of the competitive RANK ligand, osteoprotegerin, regulates the differentiation of osteoclasts and is therefore essential in modulating resorption within bone [72], [73]. Osteoclasts can be recruited to sites where bone resorption is required or can be induced towards osteoclastogenesis once at the site. Where resorption has already been initiated, they are found within degradative pits known as Howships lacunae. Along with their ability to resorb bone, osteoclasts are identified from macrophages through the specific production of tartarate resistant acid phosphatase (TRAP), cathepesin K, and calcitonin receptor. Morphologically, osteoclasts have the ability to polarize and thus have a characteristic ruffled border which is utilized to isolate a resorptive area [27], [67], [68]

2.1.2.3 Mesenchymal Stem Cells

The cellular precursors responsible for the development and maintenance of skeletal system tissues are mesenchymal stem cells (MSCs). MSCs are a population of non-hematopoietic stromal cells with multilineage differentiation potential that reside in connective tissues including bone marrow, cartilage, bone, adipose, periosteum, ligament, and synovium (among many others) [45], [74]. These cells were first identified in bone marrow, where attempts were being made to determine the cellular origin of the tissue's osteogenic capabilities [75]. They were distinguished from the hematopoietic fraction of bone marrow cellular isolate by their adherence to tissue culture plastic and the fibroblastic morphology of the cells upon *in vitro* expansion [75], [76]. Further *in vitro* analyses demonstrated clonality of this cellular population, as limiting seeding density resulted in the formation of colonies originating from single cells (colony forming unit – fibroblastic [CFU-f]) [77]. Differentiation of these cells to osteogenic, chondrogenic, adipogenic, myogenic, and tenogenic lineages established their multipotency [74], [78].

Initially termed 'bone marrow stromal cells' or 'osteogenic stem cells', widespread usage of 'mesenchymal stem cells' in reference to this cell type became popularized by Caplan et al. in the 1990s and has since been the subject of some contention [79]. While the term is pervasive in the realm of cellular research, some consider it a misnomer as both 'mesenchymal' and 'stem cell' are misleading, previously generating confusion when attempts were being made to develop a unifying description for the non-HSC cell resident in stroma [80]. The use of 'mesenchymal' in describing this cell type, along with the almost concurrent establishment of embryonic stem cells, indicated a post-natal stem cell type with a broad differentiation capacity potentially synonymous with the mesoderm of fetal tissue which has hematopoetic capacity [80]. 'Stem cell' meanwhile indicates unlimited self-renewal potential, which,

until recently, had not been truly tested *in vivo* – rather, previous assertions came to refer only to the ability of MSCs to sustain themselves long term in passage with the ability to differentiate upon subsequent passages [80], [81].

The current recommended name is 'multipotent mesenchymal stromal cell', although 'mesenchymal stem cell' is still synonymously used. However these issues of nomenclature, bring to the forefront the issue of defining the constitution of a true MSC. Until the consensus on the definition of an 'MSC', there was no unifying standards with which to categorize this cellular fraction [82]. Subsequently, a minimum criteria which these cells must satisfy in order to claim to be true 'MSCs' was established: Adherence to plastic, specific antigen surface markers, and multilineage differentiation potential [83] These minimum criteria does not discount a body of literature which may utilize several but not all of these methods as these were specifically designated in order to unify and standardize human MSCs in order to pave the way for downstream clinical usages [83]. MSCs are now more frequently being described as a heterogeneous population of cells – the exact nature of which are still under investigation.

Plastic adherence is a standard characteristic of MSCs, yet not unique to many cell types *in vitro*. This trait is used as an initial screen in isolation against most hematopoietic cells, however, as MSCs are increasingly obtained from tissues with adherent cell fractions, it does not provide very stringent exclusion of unwanted cell types [75], [77]. Extended passaging allows not only for expansion of the MSC pool, but can also help eliminate the small fraction of slow growing, adherent hematopoietic cells, which will be unable to viably compete *in vitro* under the culture conditions utilized [84]. Due to the low frequency of this cell type within tissues, expansion is indeed required in order to obtain working populations [77]. The expansion of MSCs is limited by the senescent changes they undergo in culture [85], [86]. Telomere length decreases *in vitro* due to culture conditions and repeat cellular division, resulting in irreversible negative changes in their differentiation capacity [85], [87], [88]. Therefore, *in vitro* expansion is suggested to be limited to the lowest useable passage [85].

A panel of surface antigens have been identified which are routinely utilized in the identification of MSCs. No single surface marker has been found yet which unequivocally targets the true MSC, and thus a variety of markers are employed towards these ends. As stated by the IPSC minimum criteria, a true MSC should be CD105+, CD73+, CD90+, and CD45-,CD34, CD14- or CD11b-, CD79a or CD19- and HLA-DR- as determined by flow cytometry [83]. Again, these criteria were outlined for human MSCs, with species based differences making the identification of these cells difficult and general heterogeneity of MSC-like cells further complicating the issue.

Multipotency remains the most definitive feature of MSCs and is often affirmed by differentiation assays. Osteogenic, chondrogenic, and adipogenic capacity of the cellular population is often tested in parallel to confirm tri-lineage potential [45]. These assays are well described and have become standard

practice among cellular researchers and tissue engineers. Osteogenic differentiation features the culture of MSCs in monolayer culture in the presence of β-glycerophosphate (βGP), ascorbic acid, and dexamethasone for two to three weeks. Bone morphogenetic proteins (BMPs) have also been added to the culture media to enhance osteogenic differentiation [89], [90]. Differentiation is typically confirmed by staining for calcium deposits and collagen I, although expression of the other non-collagenous bone matrix molecules (osteocalcin, bone sialoprotein) can be used to further confirm osteoblastogenesis [45], [91]. Adipogenic differentiation is promoted in MSC monolayer culture through the addition of dexamethasone, insulin, isobutyl methyl xanthine, and indomethacin. Lipids accumulate within intracellular vacuoles thus differentiation is assessed by staining for fatty oils [45], [92]. The standard practice of chondrogenic differentiation was described by Johnstone et al. who placed MSCs in high density culture in the presence of chondrogenic factors, attempting to mimic the environment which triggers cartilage formation during limb morphogenesis. Chondrogenic media typically includes dexamethasone, ascorbic acid, and a transforming growth factor (TGFβ) isoform (commonly TGFβ1 or TGFβ3) [93], [94]. BMPs have also been utilized as chondrogenic growth factors for MSC differentiation [95], [96]. Chondrogenic induction features the production of proteoglycans and collagen II, the two main extracellular constituents of cartilage, by chondrocytes, the cellular component of cartilage [29][45].

The distinguishing factors used to identify MSCs are assessed upon *in vitro* culture and thus the *in vivo* occupation of these cells is still poorly understood. The localization of MSCs *in vivo* has been challenging due to the lack of a universal MSC immunophenotype – the surface markers utilized for the *in vitro* identification of putative MSCs change with passage and isolation technique indicating they do not necessarily reflect the true status of these cells *in vivo* [97]. MSCs have been proposed to occupy a perivascular and an endosteal niche within bone marrow as demonstrated by the ability to heterotopically induce bone and marrow formation [97], [98]. Within the environment of the niche, MSCs may retain their stem-like qualities [99]. Periosteum has similarly also been considered niche for stem cells as periosteal elevation, growth factor administration, and injury all result in the local formation of bone [100]–[102]The MSC population within the periosteum lies bone adjacent, although the presence of blood vessels does not rule out the possibility of a contribution from a perivascular niche as well [50].

2.1.3 Extracellular Matrix

2.1.3.1 Organic and Inorganic Components

The extracellular matrix of bone is comprised of three principal phases: an inorganic phase, an organic phase, and a water phase, accounting for 60-70%, 20-40%, and 5-8% of the tissue respectively. Calcium phosphate salts in the form of hydroxyapatite (HA) crystals (Ca_{10} (PO_4)$_6$ (OH)$_2$) or analogous variations constitute a majority of the inorganic phase. Apatite crystallizes as a lattice form plate like

structures that are approximately 20-80 nm in length and 2-5 nm thick. Typical variations hydroxyapatite involve the substitution of atomic elements leading to the incorporation of magnesium, carbonate, acid phosphate, citrate, and fluoride into the crystal structure. These alterations result in changes in the structural properties of the matrix such as by decreasing solubility of the HA crystals which allows for matrix resorption as is the case with the carbonate analog [103].

Type I collagen is the principal constituent of the organic phase of the osteoid representing approximately 90% of the mass while the remaining percentage is composed of other collagen types, non-collagenous proteins, lipids, and various macromolecules. Molecular collagens of the bone matrix are organized into parallel, quarter staggered arrays to form fibrils which are further structured by aggregation into collagenous fibers. The arrangement of collagen into this fibrillar structure leads to resultant gaps between the parallel and consecutively aligned molecular collagens wherein hydroxyapatite crystals are localized, thus implicating a role of these pores in the mineralization of HA. While hydroxyapatite mineralization in bone is a poorly understood process, the presence of noncollagenous proteins within these pores is thought to precipitate hydroxyapatite from the extracellular fluid by providing nucleations sites for mineralization to commence. The osteoinductive properties of bone arise from the entrapment of cytokines, hormones, and growth factors within the osteoid matrix due to the excretions of native bone cells and foreign cells alike. These factors affect various processes within the bone including proliferations and differentiation of osteoprogenitor cells, cellular migration, and matrix resorption and remodelling [103]. Non-collagenous proteins constitute a small proportion of the organic bone matrix but are pivotal in matrix mineralization, turnover, and structure. Principal non-collagenous proteins include osteocalcin, osteopontin, and bone sialoprotein which are often considered markers of bone formation [104]–[106]

2.1.3.2 Mineralization

The mineralization process of bone may be considered as two events, consisting of initial formation of discrete mineral deposits, followed by crystal growth within the osteoid. While still under investigation, two main mechanisms have been proposed for the initiation process: seed crystals of hydroxyapatite are directly formed at nucleation sites within the osteoid or seed crystals are formed within matrix vesicles followed by subsequent deposition within the organic matrix. These seed crystals serve as heterogeneous nucleators for secondary nucleation in the stage of crystal propagation. Amorphous calcium phosphate has been considered a precursor nucleator with solid phase transition from this metastable form to crystalline hydroxyapatite under defined conditions for the secondary stage of crystal growth [107]. Matrix vesicles have been suggested to take part in initial mineralization through both regulation of extracellular ion concentrations by enzymatic action and by provision of nucleators

such as phospho-proteins. Enzymatic cleavage of extracellular pyrophosphate (PPi) by membranous ALP of matrix vesicles simultaneously reduces the content of this mineralization inhibitor and provides phosphate (Pi) for extracellular hydroxyapatite crystal formation. Local increases in calcium and phosphate ion concentrations at nucleation sites within the organic matrix is thought to result in direct initiation of hydroxyapatite crystal formation. Alternatively, increased concentrations of calcium and phosphate ions within matrix vesicles due to the action of ALP along with the presence of intravesicular nucleators may result in the formation of hydroxyapatite seed crystals which may then be deposited onto associated collagen fibrils [108]. Crystal propagation occurs mainly through secondary nucleation where HA crystals grow from the pre-existing mineral nuclei and fuse to form larger crystals. HA crystals align along their c-axes in the channels formed by gaps of the collagen fibrillar array that run parallel to the stress acting on the bone thus reflecting the anisotropic properties of the bone matrix [107].

2.1.3.3 Resorption and Remodelling

The process of bone resorption and remodelling features the recruitment, proliferation, and differentiation of osteoclast precursors with subsequent degradative action upon bone matrix and new bone deposition. In accordance with Wolff's law, bone is continuously remodelled in order to adapt to changing applied loads even after cessation of growth and therefore the resorptive process is highly necessary for the health of bone tissue [109]. Hematopoetic monocytic precursors are recruited to the site of bone resorption from the bone marrow and blood stream upon retraction of bone lining cells. The precursors proliferate to form a respective population and develop in maturity subsequent to M-CSF and RANKL binding, forming multinucleated polykaryons which are commited to osteoclastic differentiation and termed resting osteoclasts. Octeoclastic resorption is stimulated through the interaction of these cells with the surrounding matrix as well as by induction by local cytokines and hormones. Osteoclasts attach to the bone matrix through integrin action and form a sealed extracellular microenvironment in which dissolution of the mineralized osteoid occurs. Adhesion to the extracellular matrix triggers rearrangement of the osteoclast cytoskeleton that results in a polarized cell structure. The cell membrane adjacent to the bone becomes ruffled in morphology with increases surface area for resorptive purposes and the opposing side remains smooth as a secretory domain. Fusion of lysosomal vacuoles to the plasma membrane mediates this morphological change at the ruffled border, potentially through binding of the αvβ3 integrin, which serves to increase the content of surface bound proton pumps for acidification and which also releases degradative enzymes into the sealed zone. The sealed zone of the osteoclast is a circular structure that encases the ruffled membrane and is comprised mainly of filamentous actin (F-actin). F-actin of the sealing zone forms punctate complexes in the plasma membrane known as podosomes which associate with integrins and are analogous to focal adhesion points. Integrins of the podosome complex

interact with exposed RGD motifs of the osteoid matrix due to the presence of proteins including collagen I, fibronectin, osteopontin, and bone sialoprotein [73].

Matrix resorption begins with the dissolution of the inorganic phase of bone by acidification within this microenvironment. Carbonic anhydrase II (CAII) initially generates protons through the reaction of water and carbon dioxide intracellularly. Vacuolar H+ATPases embedded in the ruffled membrane pump protons against the concentration gradient thereby establishing and maintaining the pH in the extracellular compartment. The energy independent exchange of bicarbonate ions (produced from the enzymatic action of CAII) for chloride ions at the osteoclast secretory domain results in a regulation of internal pH. Chloride ions are passed through channels on the ruffled border generating hydrochloric acid in the microenvironment thus dropping the pH to a range of 4-4.5 and dissolving the mineralized hydroxyapatite. This concerted exchange of bicarbonate and chloride ions maintains the electroneutrality of the osteoclast. The demineralized osteoid is then available for degradation through the action of enzymes such a cathespin K, matrix metalloproteinases, and TRAP. Cathespin K is a lysosomal cysteine protease capable of functioning optimally at low pH and thus is utilized within the acidic microenvironment to degrade collagenous proteins of the matrix. TRAP is a membrane bound enzyme that hydrolyzes phosphoproteins, ATP, and other triphosphates and is thus involved in the breakdown of many non-collagenous proteins of the extracellular matrix. Matrix metalloproteinases (MMPs) are zinc activated zymogens involved in the osteoclastic degradation of the osteoid matrix with action on a variety of collagens and proteoglycans. Degradative products of the resorption process are endocytosed by the osteoclasts and released into the extracellular fluid through the secretory domain. The action of osteoclast arrestment is not well understood, however arguments have been made for cellular sensing of calcium concentration within the microenvironment while still others suggest apoptosis of osteoclasts through the action of estrogen mediated through TGF-beta. Remodelling occurs as osteoblasts invade the resorbed pit upon removal of osteoclasts and lay down a new osteoid layer for mineralization [73].

2.2 Mechanisms of Bone Formation

The process of bone development occurs through two mechanistic pathways: intramembranous and endochondral ossification. Intramembranous ossification leads to the direct deposition of bone matrix by osteoblasts whereas bone formation proceeds via mesenchymal stem cell differentiation through an intermediate cartilage template prior to chondrocyte hypertrophy and matrix mineralization in endochondral ossification. Although the two processes are not mutually exclusive and often are employed in conjunction as with fracture repair, endochondral ossification is primarily associated with long bone development and longitudinal growth, while intramembranous ossification is associated with flat bone formation and bone remodelling.

2.2.1 Development and Growth

2.2.1.1 Limb Morphogenesis

Limb development is initiated in the embryo with condensation of mesenchymal stem cells which form an anlage that serves as a template for the transitory cartilage stage prior to bone formation (Figure 2-3 A) [110]. Cell-cell adhesion proteins, namely N-cadherin and neural cell adhesion molecule (N-CAM) mediate the aggregation and proliferation of MSCs in the condensation. Interaction of MSCs with the primordial matrix of fibronectin and proteoglycan further facilitates this process. Chondrogenesis commences upon activation of the transcription factor *Sox*9 by paracrine signalling factors which regulates the phenotypic conversion of MSCs to chondroprogenitors termed chondroblasts (Figure 2-3 B). *Sox*9 coordinates chondrogenic differentiation through its transcriptional control over key cartilaginous genes including *Sox*5, *Sox*6, and collagen II. The two other members of the *Sox* family are necessary for the production of aggrecan. The synergistic action of the *Sox* trio promotes the chondrocyte phenotype, causing cellular proliferation and deposition of a cartilaginous extracellular matrix, and resulting in interstitial growth of the anlage [111], [112].

Delineation of an early periosteum (termed periochondrium at this time) occurs during chondrogenesis of the limb anlage, as mesenchyme condense at the periphery of the structure. The perichondrium encloses the newly forming bone, extending over the shaft and ending at the joint cavity. As vascular invasion of the perichondrium occurs, progenitor cells of the deepest layer undergo osteogenic differentiation to form an osseous shell surrounding the inner cartilaginous mass (Figure 2-3 C). The delimited structure is now termed the periosteum at this stage and henceforth contributes to appositional bone deposition by supplying newly differentiated osteoblasts to the bony collar. Although considered different tissue types, periosteum tissue is a derivative of perichondrium tissue; after epiphyseal plate closure, perichondrium transforms to periosteum. It is thought that perichondrium will differentiate to periosteum accordingly with the status of differentiation of the underlying bone tissue [113], [114].

Chondrocytes of the anlage eventually cease proliferation and become hypertrophic, entering the terminal stages of endochondral ossification. Chondrocytes which exit the cell cycle undergo hypertrophic onset arbitrated through the transcription factor *Runx*2 [115]. At this stage, chondrocytes increase their cellular volume and secrete a distinct matrix which promotes extracellular matrix mineralization. Hypertrophic chondrocytes are fated to eventually undergo apoptosis although recently transdifferentiation of these cells to the osteoblastic lineage has been confirmed [116]. As the hypertrophic chondrocytes die, the transverse septa created by the surrounding matrix are broken down, thus allowing the entry of blood vessels which supply osteoblast and osteoclast progenitors to the

ossification front. Local and extrinsic factors act to induce differentiation of these progenitors and thus allow the ossification process to proceed, as osteoclasts resorb most of the cartilaginous matrix while osteoblasts deposit osteoid onto the residual mineralized matrix fragments. This process initially occurs at the center of the anlage, which is termed the primary center of ossification (POC; Figure 2-3 D). The ossification front advances longitudinally along the bone axis, forming the diaphysis (bone shaft) and metaphysis (conical shaft ends), while the adjacent cartilaginous regions continue the process of interstitial growth. Secondary ossification centers (SOC) later form at proximal and distal ends of the anlage (epiphyses) with an area of cartilage retentive between the two fronts (Figure 2-3 E). This cartilage does not ossify neonatally but rather forms the growth plate [117], [118]. The morphological and regulatory mechanisms of endochondral ossification are thus described in the context of the growth plate (Figure 2-3 F).

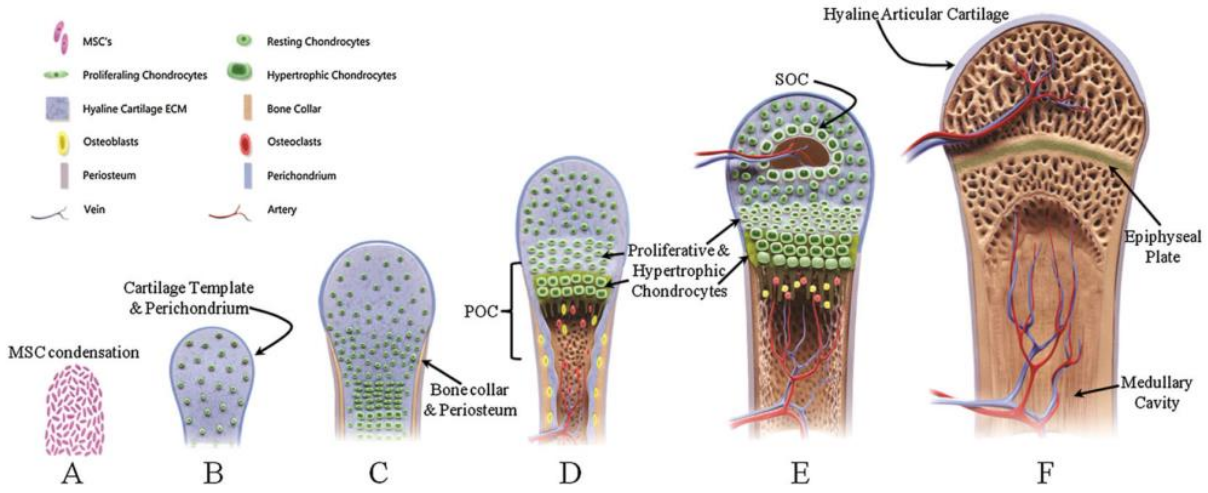

Figure 2-3: Endochondral ossification mediates the process of long bone formation [35]

2.2.1.2 The Growth Plate

The growth plate is a layer of cartilaginous tissue that lies between the epiphysis and metaphysis of long bones and functions in sustaining longitudinal bone growth through the intercalation of continuously ossifying chondrogenic tissue as per the endochondral route of bone production [119]. The growth plate persists throughout childhood, remaining open but progressively thinning until the end of puberty, after which growth is arrested as full fusion of the plate occurs [120]. The growth plate has a highly organized structure, comprising distinctive zones that contain phenotypically unique chondrogenic cells, arranged in a manner that allows for the regulated progression of cartilage to bone. There are four main zones: the resting (reserve) zone, the proliferative zone, the hypertrophic zone, and the ossification zone (Figure 2-4) [119].

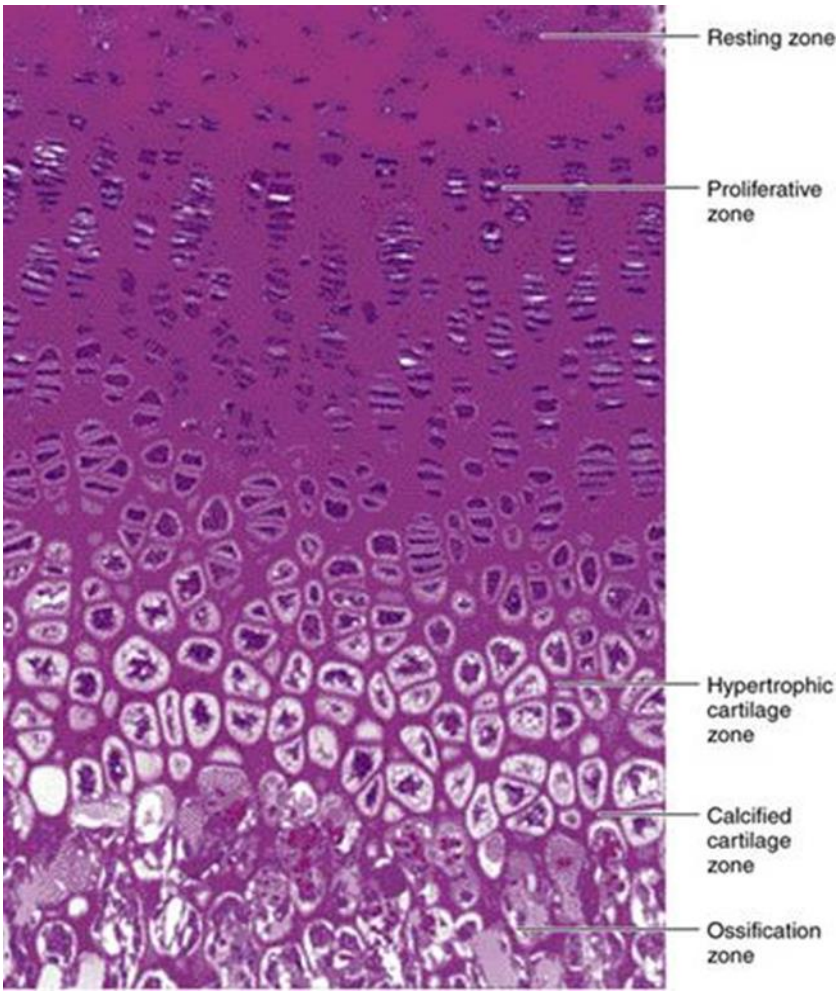

Figure 2-4: The epiphyseal growth plate [119]

The resting zone of cartilage lies adjacent to epiphyseal bone and furthest from the ossification front. Within this zone are reserve chondrocytes: small, spherical to ovoid shaped cells dispersed randomly throughout a large volume of matrix, occupying lacunae singly or as paired units [119], [121]. Resting zone chondrocytes secrete a cartilaginous matrix (collagen II, proteoglycans) under the action of *Sox9* [122]. The ECM of this zone contains aggregated proteoglycans, which inhibit matrix mineralization, and an abundance of collagen II maintained as an unorganized network of fibrils. Some studies have suggested that chondrocytes of the reserve zone have a stem-like quality, where division of these cells may result in the generation of two daughter cells – a proliferative clone whose subsequent divisions establish a new proliferative column and a clone that retains its immature stem-like state [123]. Growth plate senescence associated with age has thus been thought to be caused by depletion of the reserve zone due to finite doubling capacity of this cell pool [124], [125]. They have also been thought to produce a factor which orients cells of the underlying proliferative zone and maintains unidirectional bone growth [123], [126].

Transition from the resting zone to the proliferative zone is marked by an increase in cellular organization along with changes in mitotic activity, chondrocyte morphology, and matrix structure. While cells of the resting zone are generally quiescent, the proliferative zone houses – as the name suggests – highly active, proliferating cells. This zone is the most cellular with a high cell/matrix volume ratio. Cells of this zone are discoid in shape (flattened longitudinally) and stacked in a columnar fashion parallel to the bone axis [121]. Studies have indicated that each proliferative column is born from the expansions of a singular cell with subsequent divisions of progeny causing lengthening of the column [127]. As cells proliferate they orient perpendicular to the bone axis, then subsequently rearrange their position to lie atop each other. The family of cells within a column and the surrounding pericellular and territorial matrix is termed a chondron [128]. The structure of the proliferative zone cartilage can thus be discretized as a series of chondrons running in parallel, separated by interterritorial matrix. Matrix composition of the chondron differs from the surrounding interterritorial matrix. The composition of the interterritorial matrix within this region differs from the overlying resting zone, with increased production of collagens and orientation of fibrils in a longitudinal arrangement [119]. Regulation within this zone ensures chondrocyte proliferation is modulated and hypertrophic onset is not premature. An essential signalling mechanism inherent to the growth plate is the parathyroid hormone related peptide (PTHrP) - indian hedgehog (ihh) negative feedback loop [129]. PTHrP is expressed in resting zone chondrocytes where it prevents the transition of proliferative chondrocytes to hypertrophy [130]. Indian hedgehog is produced by hypertrophic chondrocytes where it diffuses to the resting zone to stimulate the production of PTHrP. An imbalance in either zonal population will thus concomitantly effect the other [129].

The hypertrophic zone of cartilage is distinguished by the phenotypic changes in its cellular population. Hypertrophy occurs as proliferative cells exit the cell cycle, having exhausted their expansion potential. The most drastic observable change is the increased volume and sphericity of these cells [121], [131]. Enlargement of hypertrophic cells is the main contributor to bone elongation, although cellular division and matrix synthesis within the proliferative zone are factors as well [132][133]. The general tissue structure is maintained from the proliferative to hypertrophic zone, but with compression of the interterritorial matrix [134]. Apart from the changes in cellular morphology, the hypertrophic zone is characterized by extensive remodelling of the extracellular matrix. Synthesis of novel matrix constituents including collagen X, matrix metalloproteinases (MMPs), ALP and vascular endothelial growth factor (VEGF) is concomitant with degradation of collagen II and aggrecan. Alteration of the extracellular matrix occurs in preparation for mineralization and blood vessel invasion. MMPs degrade mineralization inhibiting proteoglycans and collagen II, ALP regulates extracellular phosphate levels, Collagen X facilitates calcification, and VEGF stimulates vascular invasion [135], [136]. Hypertrophic cells also secrete matrix vesicles which are distributed throughout the interterritorial matrix (longitudinal septa) and

act as nucleation sites for mineralization [137], [138]. Membrane bound ALP converts the mineralization inhibitor pyrophosphate to phosphate (Pi) and thus promotes crystallization in a calcium rich environment [108].

The zone of ossification constitutes the final stage of endochondral ossification wherein the calcified cartilage matrix is replaced by bone. Secretion of VEGF by hypertrophic chondrocytes and cleavage of antiangiogenic factors stimulates capillary invasion of the calcified matrix. Apoptosis of terminal hypertrophic chondrocytes upon vascularization provides routes of tissue entry to invading cells [136]. Apoptosis may be trigger in cells by disruption to mitochondria during vascularization, an event known to initiate the caspase cascade and which may result in release of mitochondrial calcium stores, thus further promoting mineralization of longitudinal septa [139]. A variety of cells infiltrate these channels including chondroclasts, osteoclasts, osteoblasts, endothelial cells and pericytes. Bone deposition occurs as invading cells resorb the calcified cartilaginous matrix and deposit osteoid on the remnant struts [136].

2.2.2 Repair

Bone has a naturally robust repair process which features the synthesis of an osteoconductive matrix that induces recruitment of cells with osteogenic potential for recapitulation of endochondral and intramembranous ossification at the wound site. Unlike other tissues, bone is able to repair itself without the formation of a scar and with almost complete integration into the bordering bone tissue. The series of events in fracture repair progresses through stages of activation, inflammation, soft callus formation, hard callus formation, and remodelling. A hematoma is initially formed as disruption of the vasculature leads to activation of the blood clotting cascade. This fibrinous matrix of the hematoma functions in attracting and temporarily housing inflammatory cells such as macrophages and monocytes at the wound site. Macrophages facilitate hematoma removal and phagocytose cellular debris from injury, while monocyte-derived osteoclasts resorb necrotic bone at the ends of the fracture gap. Secretion of potent chemotactic factors by these inflammatory cells recruits fibroblasts and MSCs to the area from their local niches in the periosteum, endosteum, and vasculature. Continued expression of these cytokines promotes proliferation, differentiation, and matrix production by fibroblasts and MSCs. Granulation tissue replaces the hematoma as fibroblasts synthesize a collagenous extracellular network which hosts the proliferating MSCs and supports neovascular ingrowth [140].

Hypoxia within the fracture gap - the result of arteriolar disturbance during injury – encourages the chondrogenic differentiation of MSCs. Chondrogenesis begins centrally but eventually extends throughout the granulation tissue to connect the fracture ends. The fibrocartilaginous plug forms the soft callus which provides some mechanical stability to the fracture and which, through continuation of the

endochondral process, eventually becomes bone. Chondrocytes eventually hypertrophy, apoptose, and mineralize their surrounding environment much like at the growth plate. Concurrently, intramembranous ossification at the fracture ends repairs damage to the bone cortex as resident osteoblasts and osteogenic MSCs of the periosteum and endosteum directly deposit bone osteoid. The process begins at an area removed from the fracture site but progresses inwards where it eventually interfaces with hypertrophic cells of the soft callus. Osteoblastic cells and blood vessels thus invade the cartilaginous scaffolding to deposit bone and mediate the formation of hard callus in a spatially regulated pattern which proceeds from structure periphery to the centre. The hard callus improves mechanical stability of the fracture and consequently the bone reaches a status where it is capable of supporting loading. The structural organization of bone tissue is re-established upon tissue remodelling in response to application of physiological stresses [140].

2.2.3 Pathology

Although not fully understood, several theories have been proposed to account for the pathology of osteoarthritis (OA), and specifically, reactivation of the transitory cartilage route to eventual bone formation has been hypothesized. Cartilage may be classified as either permanent or transitory, where the tissue phenotypes differ only in their developmental fate. Transitory cartilage eventually progresses through the cartilaginous phenotype to form bone through endochondral ossification (as previously described for the growth plate) in contrast to permanent cartilage where this default route is arrested and thus stable cartilage is formed which is capable of lasting for decades in humans. Aberrations in the stable cartilaginous phenotype leads to the progression of osteoarthritis (OA) which occurs with the onset of age and results in breakdown and mineralization of the cartilaginous matrix [141]. The coinciding events of matrix degradation and progression of tidemarks indicating bone formation in OA patients led some to investigate the potential of chondrocyte hypertrophy and terminal differentiation as the mechanistic pathway through which OA develops [141], [142]. The upregulation of specific hypertrophic markers such as collagen X, MMP-13, *Runx2*, VEGF, and ALP and downregulation of *Sox*9 have been identified in OA cartilage and thus have indicated that in some cases, OA may be due in part to chondrocyte hypertrophy and the advancement endochondral ossification within the permanent cartilage [141], [142].

The process of endochondral ossification is further observed in the development of osteophytes, a pathological phenomenon often associated with progression of osteoarthritis [143]. This is characterized by the formation of bony projections at the margins of joint capsules in diarthrodial joints. The process leading to osteophyte onset is not fully understood mechanistically, but two theories have arisen that

suggest their propagation is due to either mechanical instability or a change in the molecular milieu within the joint space, both of which result in the activation of a subset of cells primed for undergoing an endochondral-like route of cartilage formation and ossification [143]. These induction methods may not be mutually exclusive either, as mechanical injury may lead to subsequent biochemical changes within the joint capsule and trigger osteophyte formation. In either case, the periosteum is a tissue highly implicated in their development. Periosteum is found within the joint capsule covering the bone and bordering the articular cartilage. The cambium layer of the periosteum lies adjacent to bone and contains a resident cell population comprised of a heterogeneous mixture of cells, including mesenchymal progenitors, which are capable of recapitulating endochondral bone formation. In models of osteoarthritis, osteophyte formation begins with activation of these cells in the periosteum at the cartilage-bone junction, leading to their proliferation, chondrogenesis and subsequent ossification. Several specific factors - members of the TGF-beta superfamily known for their role in skeletogenesis - are thought to initiate this event. In several experiments, it was seen that intra-articular injections of TGF-β and BMPs or viral overexpression of TGF-beta isotypes mediated the formation of osteophytes in vivo which were colocalized to regions of periosteum [143]–[146]. Conversely, inhibition of exogenous TGF-beta reduced osteophyte formation and cartilage repair in collagenase induced experimental OA [147]. An inflammatory response from macrophages of the synovial lining has been suggested to simulate this process in vivo, where degradative products from OA or mechanical insult may elicit the production of these factors or inductive co-factors locally [147], [148].

2.3 Periosteum

2.3.1 Structure and Function

Periosteum is a dense, irregular connective tissue forming a membranous sheath that covers the outer surface of most bones, excluding areas of articulation, sesamoid bones, and tendon attachment sites. It is comprised of two distinct layers which provide its functional properties (Figure 2-5). The outer layer consists of fibroblasts residing within a fibrous network of collagens and elastins. This layer is highly vascularized and innervated, providing the underlying bone tissue and the overlying muscle with a blood and nerve supply. Blood vessels of the periosteum traverse the bone perpendicularly through Volkmann channels and feed directly to Haversian canals, thus ensuring nutrient delivery and waste removal within the cortical bone. Sharpey's fibers, bundles of mainly type III collagen, anchor the periosteum to the bone, transecting both layers and penetrating the lamellae of the bone cortex. Sharpey's fibers are found to vary in density along the length of bone, dependent on the tensions experienced at a particular surface. At the diaphysis, periosteum is thicker but easily separate from the adjacent bone, while at the epiphysis and metaphysis, the junction between the tissues is tightly fused. The inner layer of periosteum, termed

the cambium, is highly cellular containing a variety of cell populations including mesenchymal stem cells (MSCs), osteoprogenitor cells, fibroblasts and osteoblasts. Due to the presence of a vascular network within both layers of periosteum, endothelial pericytes capable of osteogenic induction are also resident within the tissue [50], [51].

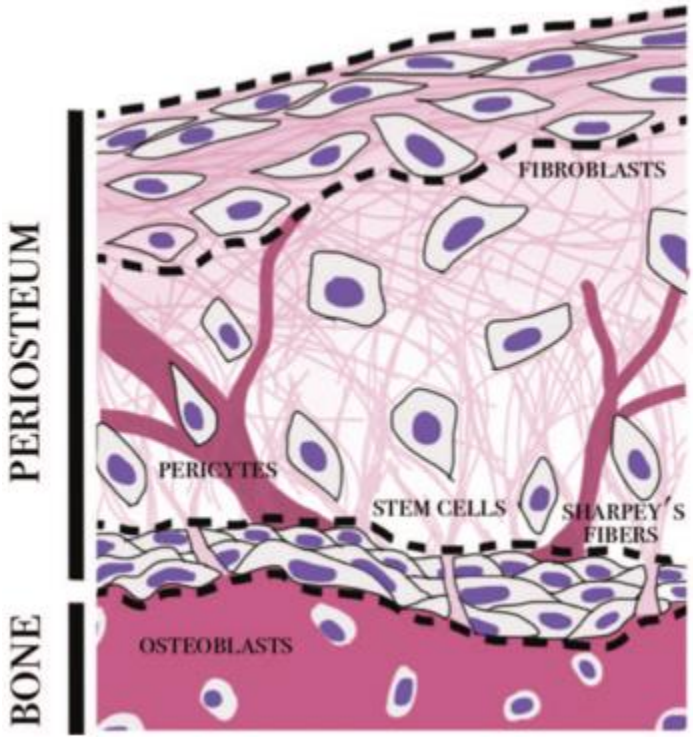

Figure 2-5: Periosteum tissue structure [50]

Periosteal tissue is continuous over the diaphysis, showing general homogeneity in organization and form. Its extension upwards towards the epiphysis is marked by more distinguishing structural particularities such as the ring of Lacroix and the groove of Ranvier. The groove is proximal to the joint space relative to the ring of Lacroix [51]. It may be described histologically as a wedging of the perichondrium into the growth plate, where cells of the perichondrial tissue visually appear to flow into the resting zone. Cells supplied to the growth plate at the groove of Ranvier allow for latitudinal expansion of the growth plate [149]. Just below the groove of Ranvier is the ring of Lacroix, a fibrous band that circles the epiphyseal cartilage, supplying mechanical properties to the relatively weak growth plate and confining its development [150]. The transition between perichondrium and periosteum occurs at the ring of Lacroix which is contiguous with both tissues, despite appearance of single tissue continuity [51].

2.3.2 Role in Skeletal System Formation and Maintenance

Periosteal cells contribute to a variety of activity along the bone surface including appositional bone growth and bone tissue repair however, the potential of this layer to recapitulate these processes varies with age and location. While contributing vastly to appositional bone deposition in the fetus and during early childhood, this ability dwindles with age. Progression into adulthood is accompanied by decreased cellularity of the cambium, thinning of the overlying fibrous layer, and recession of the microvasculature[151]. Despite tissue atrophy, the periosteum retains the ability to be stimulated from quiescence to an active state during fracture repair. The contribution of periosteal cells to the healing potential of bone is substantial; an initial proliferation stage increases the number of periosteal stem cells at the fracture site which then undergo endochondral or intramembranous ossification within the hematoma to bridge the defect [152], [153].

Differences in bone forming capacity are observable between periosteum tissue taken from distinct skeletal locations. It follows that areas at higher risk of fracture or injury require highly regenerative periosteal tissue; load bearing bones like the tibia have periosteum with greater osteogenic potential as compared to flat bones like calvaria [154]. Even along the length of a bone, the ability of periosteum tissue to affect an osteogenic response is variable, with significant differences between the diaphyseal and metaphyseal regions of femur and tibia [155], [156]. In this respect, it has been suggested that osteogenic potential in the periosteum improves with proximity to the epiphyseal plate. Cellularity appears to be a main determinant of periosteal potential where a positive correlation exists between cambium cell count and chondrogenic tissue deposition in samples taken from various locations [157]. The coincidence of decreased cellularity along with decreased osteogenic potential agrees with the observed age-related changes in periosteum as well [151].

2.4 Techniques in the Surgical Repair of Bone

Surgical intervention in the treatment of high energy trauma, tumour resection, and deformity correction is often accompanied by substantial bone loss which results in the formation of critically sized defects. Spontaneous healing is unlikely to occur in skeletal defects greater than 2 cm with stabilization alone, therefore fixation is utilized in conjunction with bone stimulating techniques or grafts [158].

2.4.1 Fixation

Fixation is the principal technique for the stabilization of bone following fracture. Fracture fixation aims to support union through the immobilization and apposition of the segmental bone ends. Although fixation can be achieved through non-operative methods (casting or traction), surgical fixation

is often required in the instances of open reduction where debridement of necrotic tissue at the fracture edges and removal of bone fragments leads to large skeletal voids. Fixation maintains the alignment and displacement between adjacent fracture pieces following the reduction procedure. Internal fixation devices include intramedullary nails or rods, plates and screw, and pins and wires [159]. The intramedullary nail is inserted through the medullary canal in the diaphyseal shaft of long bones and function as an internal splint. Screws at the end of the nail lock the device in place until the fracture has completely healed. Plates are affixed to the external cortical bone and secured by screws where they prevent displacement of the fracture segments or span a fracture gap. Pins and wires are utilized to set bone fragments prior to rigid fixation or for the small bones (such as in the hand or wrist) which cannot be fixed with screws. External fixation devices have been developed which feature trans-osseous pins and wiring connected to scaffolding [160].

2.4.2 Distraction Osteogenesis

Distraction and transformational osteogenesis are two techniques that do not require supportive osteogenic procedures for surgical encouragement of bone formation. In distraction osteogenesis, bone is willfully fractured and the segments progressively distanced such that the osseous tissue is continuously formed within the defect gap. Correspondingly, transformational osteogenesis involves the application of compressive forces at bony interfaces to allow for tissue necrosis followed by revascularization with subsequent distraction for bone growth [161]. The founding principles of these processes can be attributed to the work of Ilarizov, who initially described these methods for bone transport using external fixation in the ‘Ilarizov technique’ [162]. The Ilarizov technique makes concerted use of distraction and transformational osteogenesis for the repair of large defects in the diaphysis of long bones by bone transport. This procedure results in bone lengthening and consolidation of defects as a metaphyseal bone segment is transported across a large intercalated defect by distracting the segment at the proximal bony surface and compressing the leading surface upon meeting the distal bone. In later years these osteogenic techniques, specifically distraction osteogenesis, have been adapted use with internal rather than external fixators [161]. Although initially described for orthopaedic uses, these techniques have also been applied in other surgical areas such as cranial, maxillofacial, and orofacial surgeries [163].

2.4.3 Electric Stimulation

Pulsed electromagnetic fields (PEMF) and low-intensity pulsed ultrasound (LIPUS) are adjunctive treatments for bone growth stimulation in fracture and non-union repair. In PEMF therapy, electromagnetic fields are applied through bone tissue to stimulate healing. The therapy has been proven

to affect a beneficial healing response in fracture non-unions, however the mechanism of action is poorly understood. PEMF therapy is typically applied subsequent to non-union or delayed union formation with few pre-emptive or early uses in fracture healing [164]. Ultrasound is a mechanical energy transmitted as auditory pressure waves that are outside the boundary of human hearing. Typically used for diagnostic purposes at low intensity and therapeutic purposes at high intensity, ultrasound has recently been seen to have beneficial therapeutic effects for fracture repair at low intensity. Low intensity pulsed ultrasound (LIPUS) exerts micromechanical forces at the fracture which trigger cellular and molecular mechanisms that culminate in improved tissue healing. The use of this technique for stimulating fracture healing is controversial, as there are conflicting results based on several randomized studies [165].

2.4.4 Bone Grafting

Proper bone healing is promoted by the development of an osteoconductive matrix that supports the invasion and growth of blood vessels and cells, an osteoinductive environment where the presence of growth factors and cytokines promotes proliferation and differentiation of osteoprogenitor cells, and a source of cells with osteogenic potential. Bone grafts are considered the best choice in defect repair, as they are capable of imparting all the necessary stimuli for proper bone regeneration while simultaneously providing a mass of bone ready for integrative purposes. Grafts can be classified as autologous, allogeneic and xenologous, however for clinical purposes, they are typically autologous and allogeneic [3].

2.4.4.1 Autologous Grafting

Autologous grafting refers to the transfer of bone from its native site to an ectopic region within the body for augmentative healing purposes. Autografts are the most desirable for skeletal defect repair, as they are non-immunogenic, histocompatible, and pose low risk for infection transfer. Autografts can be taken directly from debridement of bone at the surgical site or from foreign regions within the body. Differing types of bone may be resected depending on the grafting purpose as they confer varying osteoconductive, osteoinductive, osteogenic, and mechanical capabilities [166].

Cancellous bone grafts are distinctly advantageous for grafting as their porous structure provides an osteoconductive matrix for blood vessel invasion while supplying an initial source of osteoprogenitor cells resident within the marrow stroma. The proliferation of transferred osteoprogenitor cells at the new site and induction to osteogenic differentiation results in osteoblastic formation of substantial amounts of bone. Incorporation of the cancellous graft occurs as new bone is deposited over the dead struts of the trabeculae. Remodelling occurs up to a year after graft integration where the trabecular struts are oriented in relation to mechanical stresses. These grafts are non-vascular and therefore must be harvested fresh in order to maintain their cellular population. Although cancellous grafts provide poor mechanical support

initially, their relatively quick integration into the recipient site allows for recovery of structural integrity such that their strength is similar to that of cortical grafts within 6-12 months of surgery. Cancellous bone grafts are often harvested from the anterior or posterior iliac crest of the pelvis although they may be harvested from the distal ends of the radius and tibia. The lacking mechanical strength provided by these grafts infers their use for defects that do not require structural support such as non-unions [166], [167].

Cortical grafts provide surgeons with a mechanically strong osteoconductive substrate that is able to confer some osteogenic potential for bone segmental defect repair. Non-vascularized cortical grafts are integrated with the surrounding tissue through creeping substitution where bone is gradually resorbed then replaced by newly viable vascularized tissue. In contrast, vascularized cortical grafts heal quickly at the bone-graft interface where blood vessel anastomosis negates the need for resorption and revascularization and prevents tissue necrosis. Non-vascularized cortical grafts weaken for 6 weeks following the grafting procedure due to the resorption and revascularization process, however they regain strength similar to that of the vascularized grafts subsequent to this initial time period. Cortical grafts are commonly harvested from the fibula, ribs, and iliac crest and used for defects that require structural support [166], [167].

Bone marrow represents another source of autologous material available for utilization in grafting. Rich in stem cells, growth factors, and cytokines, bone marrow has both osteogenic and osteoinductive potential but lacks a structurally sound matrix. Bone marrow is harvested from the posterior iliac wing in volumes of 100-150 mL for injection at fracture sites or non-unions to promote healing. The aspirate may be centrifuged to concentrate the stromal osteogenic cell population when used in smaller defects. The osteogenic progenitor population is also capable of expansion *in vitro* without loss of plasticity and therefore, larger cell populations may be maintained for later usage. The use of bone marrow for bone regenerative purposes is beneficial because of the low risk of donor site morbidity and infection which can be attributed to the percutaneous method of its harvest. Issues remain with the use of bone marrow as the aspirate has a tendency to wash away from the recipient site. Various methods of applications for marrow have been tested with successes when used in combination with a carrier such as demineralized bone matrix for injection at the site of non-unions and delayed unions [3], [168].

The potential of autologous grafting is limited by the amount of bone which can be taken safely from a secondary region which prevents the possibility of using autologous grafting for large or multiple defects. Furthermore, morbidities associated with donor site harvesting include pain, risk of infection, potential fracture and tissue necrosis which may further compromise a patient's health. Although autografts are typically considered good options in terms of bone regeneration, the osteogenic potential of any graft varies among patients and therefore, autologous grafting may not always provide the optimal outcome. In a review of patients who had undergone autologous bone grafts, it was found that 8.6% of

patients had experienced a major complication while 20.6% of patients had experience a minor complication. Furthermore, a higher complication rate was found if the graft was harvested from within the same incision as the surgery was conducted. This illuminates the potential for serious issues to incur with autologous grafting despite the good results which are often associated with their usage [12].

2.4.4.2 Allogeneic Grafting

Allografts refer to the transplantation of bone harvested from a human donor or cadaver to a recipient patient. The limitations of autologous bone harvesting can be partially overcome with the use of allogeneic grafts, although several of the beneficial aspects of autografts are forfeit as other limitations are introduced. The use of allografts is considered advantageous in terms of ease of use and procurement as they are more readily available in several distinct forms and do not require the host to undergo the secondary operative harvesting procedures that may result in tissue morbidities. The drawbacks associated with allogeneic grafts such as risk of disease transmission, tissue rejection and related problems of harvest and storage methods remain challenges to be overcome. Allografts are available in several formats including demineralized bone (DMB) and morselized or complete cancellous and cortical bone [3].

Demineralized bone matrix (DBM) is a commercially available osteoconductive and osteoinductive substrate material widely used in dental, craniofacial, orthopaedic, and spinal surgeries for non-structural support in bone regeneration. The process of bone demineralization results in a powder product that is comprised mainly of collagenous proteins (Collagen I, IV, X) and growth factors (BMPs) with some non-collagenous proteins and residual amounts of calcium phosphate Bone is obtained from tissue banks which obtain samples mainly from cadavers. The bone removed from the donor is cleaned of excess soft tissues, lipids, and blood vessels prior to initial sterilization in antibiotic. It is then morselized to a homogenous particle size where acid treatment extracts most of the inorganic mineral phase from the organic matrix. Subsequent to freeze-drying, the demineralized bone matrix powder may be further manipulated to form putties, pastes, injectable materials or strips by mixture with suitable carriers. These composite materials are appropriate for use to fill bone voids and extend bone grafts. DBM may also be mixed with constituents that transfer osteogenic potential to the recipient site, such as bone marrow or autologous bone grafts [169]. The efficacy of this product may be affected by the donor age and gender, storage, processing and sterilization techniques. Sterilization of these materials is usually achieved chemically or by radiation, as more traditional methods have the potential to inactivate the proteins and growth factors of the product. DBM is known to elicit a low immune response due to the process of demineralization which largely removes antigenic markers from the material surface. Furthermore, the risk of transferring an infectious disease is low due to the stringent testing administered by tissue banks

prior to accepting bone donors. The sterilization techniques utilized by manufacturers with this product are also capable of rendering some infectious diseases ineffectual [14].

Cancellous and cortical bone is available commercially as a whole allograft or as a product that has been morselized into granules or chips of varying sizes. The cancellous products offer an osteoconductive matrix for blood vessel invasion and supports osteogenic differentiation towards osteoblastic activity and bone formation. These allografts are incorporated into bone defects in a similar manner as autologous cancellous bone grafts. Morselized allografts can be used as bone void fillers, graft extenders, or composites and are commonly used when autologous bone supply is limited such as with impact grafting in revision total hip replacements. Cortical bone as a whole graft is utilized for structural reinforcement of existing bone and as a morselized product is used for osteoconductive purposes as bone void filler [168].

2.4.5 Periosteal Grafting

The osteochondral potential of periosteum has long been known and is routinely exploited in orthopaedic procedures principally focused on bone fracture repair and articular cartilage resurfacing. Periosteum was first utilized in the 19th century by Leopold Ollier who pioneered the use of periosteal grafts for the augmentation of fracture healing after he noted its contribution to osseous tissue regeneration [170]. Periosteum has since been determined to play a critical role in bone graft incorporation where its removal from autologous bone tissue transplants resulted in major decreases in bone healing and remodelling capacity. Removal of the cells from the bone marrow compartment of the graft had minimal effects indicating periosteum supplies the graft with crucial osteogenic and vascular components [171]. Indeed, vascularized periosteal bone grafts are routinely utilized in the treatment of atrophic nonunions and large segmental defects greater than 6 cm long [55], [172], [173]. Periosteum was seen to undergo chondrogenesis when placed in an inducing environment such as the synovium and thus have been applied for condylar resurfacing [174], [175]. Similarly, periosteal flaps are used in autologous chondrocyte transplantation (ACI). ACI utilizes a periosteal flap to contain injected chondrocytes within an excised chondral lesion. In this procedure, the periosteal flap functions as a sealant but is also implicated in the mechanism of repair, postulated to either act to stimulate the injected cells towards the hyaline cartilaginous phenotype or to contribute and recruit cells to the area and thus regulate the process [176]. Periosteum grafts for chondral lesion repair are frequently observed to undergo hypertrophy, thus indicating differentiation to a nonstable chondrogenic phenotype [177].

2.5 Endochondral Bone Tissue Engineering

Bone tissue engineering has typically focused on producing bone substitutes through traditional methods whereby a scaffold, cellular populations, and an osteoinductive milieu are combined to recreate the structure and organisation of native bone *in vitro* such that graft integration may be achieved *in vivo* [178]. This design convention focuses on reproducing the events of intramembranous ossification whereby bone cells or osteogenic stem cells are induced to form bone tissue within a scaffold. A recent shift in the design paradigm which focuses on a biomimetic rather than a modular approach has led to a rise in the number of bone tissue engineering constructs which utilize endochondral ossification. Although ideally endochondral ossification *in vitro* would require only the cellular component as during limb morphogenesis, currently scaffolding and inductive cues facilitate this process [25].

Distinctive culturing methods have been set in place to induce the process of endochondral ossification in cellular populations. These methods originate from those utilized to induce chondrogenic differentiation in stem cell populations (MSCs) or those typically employed for the purposes of cartilage tissue engineering and growth plate modelling; mainly pellet (micromass) and high density (with or without scaffold) culture [94], [179].

The technique to induce cartilage tissue formation was described for mesenchymal stem cell culture by Johnstone et al. who used a pellet culture system with accompanied (and now quite standard) growth factor stimulation and noted chondrogenic differentiation [93], [94]. Along with the cartilaginous phenotype, markers of hypertrophy were also observed, however in the context of cartilage tissue engineering this was considered an undesirable consequence and not until later was this technique considered for endochondral ossification. The variety of species and tissue sources in which this technique has been replicated show a rather homogeneous tendency among MSCs to undergo chondrogenesis upon cell-cell contact and proper growth factor stimuli [46], [93], [180], [181]. While most often applied to MSC cultures, these techniques have been translated for use with many cell types in which endochondral ossification is being attempted including chondrocytes and embryonic stem cells [182], [183]. To further the maturation of the cartilaginous tissue and induce mineralization of constructs, the media formulation is altered. This is the newest aspect of the culturing method and was adapted specifically for progressing the endochondral route of bone formation in chondrogenic constructs [184], [185].

Johnstone et al. described the high cell density pellet culture as analogous to the precartilaginous condensations which form during embryonic development and initiate the process of endochondral ossification. In this study, the importance of both cell-cell contact and the addition of specific bioactive factors for MSC chondrogenesis was noted. It is of particular relevance then, to consider that physical

parameters of the culture are as necessary to the induction of chondrogenesis as soluble factors.

While the growth factors required to trigger the endochondral route of tissue formation *in vitro* have become generally standardized, it is the physical methodology for inducing bone tissue formation which has seen a great deal of attention. Thus, in attempting to reproduce the events of endochondral ossification *in vitro*, researchers should be cognisant of the physical requirements needed in their culture methods – a three dimensional environment (affecting both cell shape and cell-ECM interactions) and a critical cell mass (affecting cell-cell interactions). Techniques to replicate these physical interactions in culture in order to optimize and up-scale the process of endochondral ossification have been studied by experimentation with cell seeding density, scaffold material, scaffold architecture, and construct size.

Cell seeding density has been known to affect the chondrogenic differentiation of MSCs, with higher densities yielding better cartilaginous tissues. Higher cell densities provide the appropriate cell-cell interactive cues and furthermore may provide a 3D environment, conducive to maintaining the rounded shape believed to be associated with the cartilaginous phenotype. This is most obvious when considering the inability of MSCs to undergo chondrogenesis when cultured in monolayer [93]. Disregarding any specific interactions between cells and their ECM, a three dimensional environment which sustains the rounded cell shape may be conducive to chondrogenesis. In several studies, cell shape was postulated to be involved in the commitment of MSCs to a certain lineage, where rounded versus flattened cells differentiated towards divergent cell types [186], [187]. This may be further supported by the fact that chondrogenesis has been observed for MSCs encapsulated in inert hydrogels such as alginate and agarose, where cell-cell contact is limited and specific cell-ECM binding interactions are less pronounced. The cell-cell interactions that are established in high density culture perhaps help maintain the three dimensionality of the structure while pre-cartilaginous matrix is lain subsequent to the onset of chondrogenesis, as is observed in mesenchymal condensations [111]. This precartilaginous matrix then serves to support and help regulate chondrogenesis through specific cell-ECM interactions such as those observed with fibronectin. An optimal cell density required for chondrogenesis has not been established for *in vitro* cultures of MSCs. It was determined that a critical cell density of 5000 cells/mm^2 was required for chondrogenesis of mouse limb bud mesenchymal stem cells, however in the development of tissue constructs, cell density as it relates to the development of chondrogenesis has only been considered in limited cases. Typically constructs are made from aggregates of hundreds to millions of cells. Whether there is an optimal cell seeding density may be contingent upon the system itself.

Endochondral bone tissue engineering has been attempted within ceramic and natural and synthetic polymer scaffolds. The choice to utilize these scaffold materials lies not only in that they provide structural reinforcement for the endochondral process but, in the case of ceramics and natural polymers, they are related to the natural *in vivo* environment for which this tissue development occurs.

For example, ceramics have a long history of use in bone tissue engineering applications but are most frequently paired with MSCs directed towards undergoing intramembranous ossification. The likeness of ceramic materials to the natural mineral component of bone makes them osteoinductive, biocompatible, resorbable and therefore very well suited to supporting new bone formation. Similarly, natural polymers such as Collagen I, Collagen II, and GAG have been considered owing to their roles as structural molecules of the ECM during either mesenchymal condensation or MSC chondrogenesis. Scaffolds will most likely be necessary in the scale-up of endochondral ossification, as cellular aggregates are quite vulnerable to the effects of mass transfer in tissue culture.

Following a more traditional approach, endochondral bone tissue formation has been attempted in ceramic scaffolds with promising results. As discussed previously, Jukes et al. induced endochondral ossification in ESC-laden ceramic scaffolds with success [182]. Although not much is discussed concerning the use of the ceramic scaffold, their method was adapted from a protocol by Yuan et al. who showed the osteoinductive potential of biphasic calcium phosphate (BCP) scaffolds *in vivo* [188]. Janicki et al. compared the osteogenic potential of two different ceramics, β-tricalcium phosphate (TCP) and a hydroxyapatite-TCP mixture, on the formation of ectopic bone by human MSCs through the endochondral route of differentiation [189]. Constructs were created as composites through the pelleting of MSCs together with ceramic granules of varying diameters, followed by resuspension with fibrinogen and clotting with thrombin. A 6 week *in vitro* chondrogenic induction period preceded subcutaneous implantation and 8 week *in vivo* culture in mouse. Indicative of the scaffold's potential to effect the extent of bone tissue formation, it was found that the use of granular particles in a composite produced significantly more new bone tissue as compared to ceramic blocks and furthermore, that the diameter of the granules also influenced the volume of bone deposited, where smaller particle size led to greater areas of bone formation. The importance of the fibrin glue on these results should not be overlooked. The conducive environment for vascular invasion provided by the fibrin clotting of constructs plays a major role in facilitating ossification, one which is not possible for ceramic blocks alone. The larger areas of bone tissue observed in constructs with smaller particle sizes was postulated to be due to the greater surface area provided which led to a higher release of osteoinductive and mineralizing factors. The use of βTCP was originally hypothesized as an alternative to HA/TCP granules (in which ectopic bone formation had previously been reported upon seeding with MSCs) due to the better resorbability of material. Ceramics are notoriously good at supporting bone tissue formation but poorly resorbed and thus presents considerable problem in bone tissue engineering applications. While this study attempted to gauge the extent of resorbed βTCP, it was not clearly determined. The chondrogenic differentiation of MSCs within a fibrin-ceramic composite was established *in vitro* and resulted in bone tissue formation *in vivo*, however unfortunately there was no comparison between bone formation for chondrogenically

primed constructs versus osteogenic constructs. Although not stated in the discussion either, it appears as though bone formation in unprimed constructs is occurring mainly at the borders of the ceramic as compared to a distribution of chondrogenic tissue throughout the pores of the constructs which is undergoing full ossification. Chondrogenic primed constructs also formed bone marrow foci as compared to osteogenic constructs and thus is more reminiscent of the full bone organ.

Natural scaffolds appear to be relatively prolific in the application of endochondral ossification for bone tissue engineering, while synthetic polymers appear to be used less frequently. Studies experimenting with the endochondral BTE technique have used collagen I, GAG, chitosan, and even full cartilaginous tissue to structurally support this process [28], [34], [58]. Collagen I and GAG are commonly employed in cartilage tissue engineering and thus have not been assessed for relevancy in the studies of endochondral ossification, as they have already been deemed capable of supporting cartilage growth. Chitosan scaffolds were proven to be capable of supporting *in vitro* chondrogenesis and subsequent *in vivo* mineralization based on sequential experimentation by Oliveira et al [190], [191]. As previously discussed in some detail, a cartilaginous tissue matrix seeded with MSCs was capable of ossification *in vivo*, where the scaffold itself showed evidence of mineralization indicating perhaps that it functioned both a support structure and a matrix capable of recapitulation endochondral ossification [183]. Poly(lactic-co-glycolic-acid) (PLGA) and poly(ε-caprolactone) are relatively common synthetic scaffolds used in bone tissue engineering and are the only synthetic polymers which have been studied thus far along with the process of endochondral ossification. Electrospun fibrous meshes were found to be appropriate substrates for *in vitro* chondrogenesis and subsequent bone formation *in vivo*, showing improved endochondral ossification as compared to what was developed in parallel pellet cultures [192]. Hydrogels have also been to be able to support chondrogenesis and endochondral ossification, however the application of a hydrogel for bone tissue engineering purposes is not usually considered due to the incompatible (incomparable) properties between the two materials.

Chapter 3 Materials and Methods

The reagents utilized in this study were purchased from Sigma Aldrich Ltd. (St. Louis, MO, USA) unless otherwise indicated.

3.1 Inducing Endochondral Ossification in Periosteal Tissue Explants

To determine the potential of periosteal explants to undergo endochondral ossification *in vitro*, tissue biopsies were initially induced to cartilaginous differentiation through the application of chondrogenic media. Cartilaginous periosteal explants were then prompted to mineralize by subsequent application of osteogenic media. The best method to facilitate the full process of endochondral ossification was further explored by assessing the differences between chondrogenic media types on cartilaginous differentiation and the differences between osteogenic media types on ossification. The timed application of sequential media types was hypothesized to result in a guided transition through endochondral ossification, from chondrogenic to hypertrophic to osseous tissue.

3.1.1 Periosteal Tissue Harvest and Explant Culture

Preliminary experiments (data not shown) indicated animal age and location of dissected periosteum affected the tissue response to inducing stimuli *in vitro*. Periosteum was harvested through sterile dissection from directly above the distal epiphyseal growth plate on calf (<18 months) metacarpal bones obtained from a local abattoir following slaughter (Agram Meats, Georgetown, ON, Canada). Strips of tissue (length=1 cm x width=2 cm; thickness not controlled) were detached from the underlying bone using a scalpel scraping technique and transferred to a petri dish containing 15 mL Dulbecco's Modified Eagle Medium (DMEM) supplemented with 25 mM HEPES (*N*-2-hydrocyethylpuperazine-*N'*-2-ethanesulfonic acid; Bioshop®, Burlington, ON, Canada) and 2% antibiotic solution (100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B). After two washes in media, the periosteal tissue was biopsied into samples 4 mm in diameter (thickness ~ 4 mm). Samples were then allotted into 24 well plates placed cambial side up and fed with chondrogenic media for up to 6 weeks followed by transfer to osteogenic media for an additional 2 weeks of culture. Each explant was given 1 mL of media with feed changes occurring every other day. The samples were housed in an incubator at 37°C with 5% CO_2 in 95% humidity for the entirety of their culture period, with harvest for analysis occurring at 3, 6, and 8-week time points (n=6 per group).

For the chondrogenic induction phase, three treatments groups were tested: 6 weeks culture in chondrogenic media (CM) containing TGFβ1 (CM-TGF), 6 weeks culture in chondrogenic media containing BMP2 (CM-BMP), or 3 weeks culture in CM-TGF followed by an additional 3 weeks of

culture in CM-BMP (CM-Switch; Figure 3-1). The chondrogenic media formulation consisted of DMEM with 25 mM HEPES, 10% FBS, 1% antibiotics, 100 nM dexamethasone, 50 µg/mL ascorbic acid-2-phosphate, 6.25 µg/mL insulin and either: (i) 10 ng/mL TGFβ1 (CM-TGF; PeproTech Inc., Rocky Hill, NJ, USA) or (ii) 50 ng/mL BMP2 (CM-BMP; PeproTech Inc., Rocky Hill, NJ, USA). Subsequent to the 6 week chondrogenic induction, samples were fed with different osteogenic media types for a further two weeks (osteogenic induction phase; Figure 3-1). Osteogenic media consisted of DMEM with 25 mM HEPES, 10% FBS, 1% antibiotics, 100 nM dexamethasone, 50 µM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate, 10 nM calcitriol and either: (i) 50ng/mL BMP2 (OM+BMP), (ii) 1 nM Triiodothyronine (T3) (OM+T3), or (iii) no additives (OM).

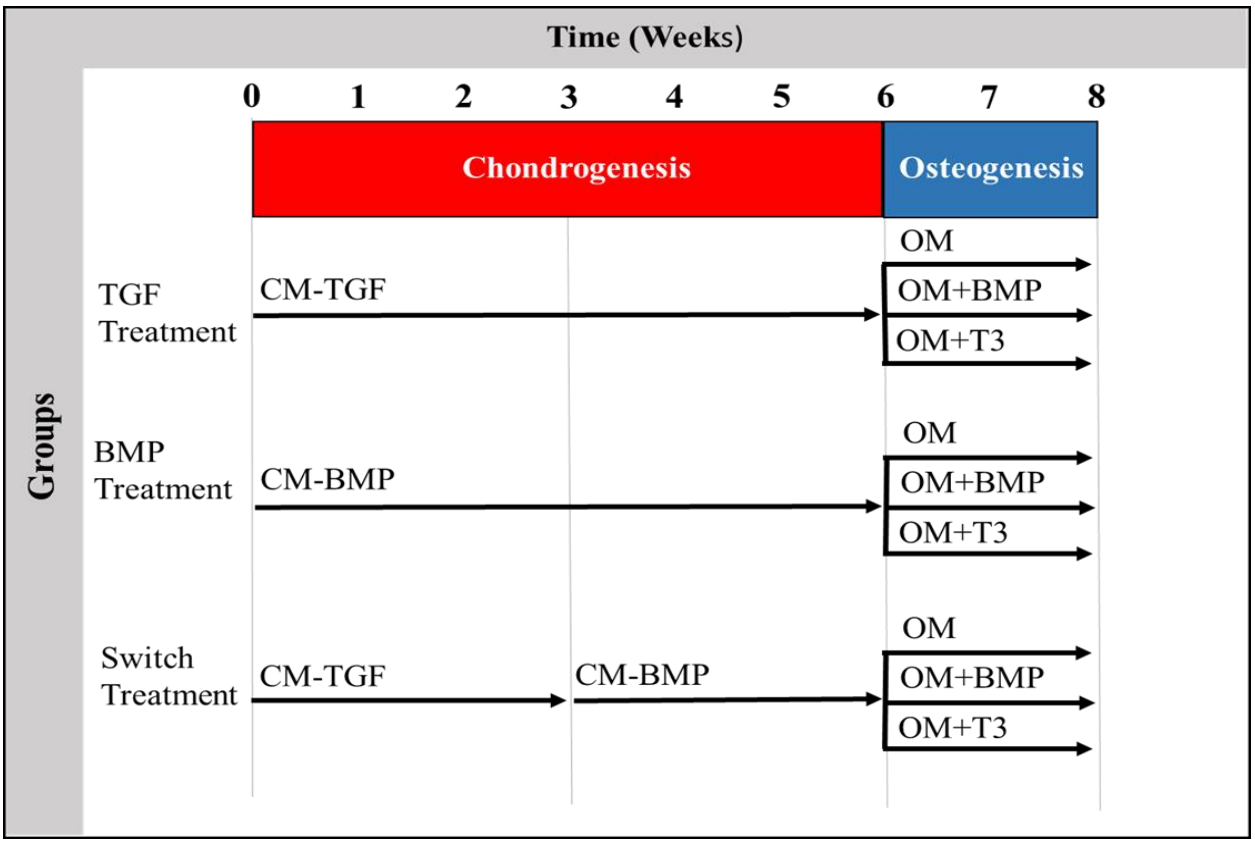

Figure 3-1: Periosteal explant media supplementation regime

3.1.2 Histological Evaluation

After the culture period, tissue samples (n=6) were harvested and fixed in 4% paraformaldehyde (in 0.1 M phosphate buffered saline, pH 7.2) for 24 hours and decalcified in a solution of 19% EDTA (in 0.1 M PBS, pH 7.4) for up to two weeks. Samples were then dehydrated in a graded ethanol series,

embedded in paraffin wax, and sectioned at 5 µm. In order to identify cartilaginous tissue (proteoglycans), sections were stained with safranin O (counterstained with fast green) following a protocol outlined by Tran et al. with some modifications [193]. Briefly, tissue sections were deparaffinised and rehydrated to water. Sections were then stained with a solution of Weigert's iron hematoxylin for 10 minutes. After a 10 minute rinse in tap water, a 0.05% fast green solution was applied to the sections for 5 minutes. Sections were differentiated in 1% acetic acid for 15 seconds, after which a solution of 0.05% Safranin O was administered for a further 5 minutes before subsequent dehydration and mounting with Permount™ resinous media (Fischer Scientific, Ottawa, ON, Canada).

3.1.3 Morphometric Analysis

Safranin O stained tissue sections were utilized to assess morphometric qualities of the developed tissue. Following this method, one section per sample (n=6) per group was analyzed microscopically using an Olympus BX50 microscope (Olympus America Inc., Melville, NY, USA). Based on the principles of endochondral ossification, the tissue produced was either chondrogenic (CH) or mineralized (M). These general categories were further expanded to include the phenotypic subdivisions of each, representing different stages of the endochondral process (Figure 3-2).

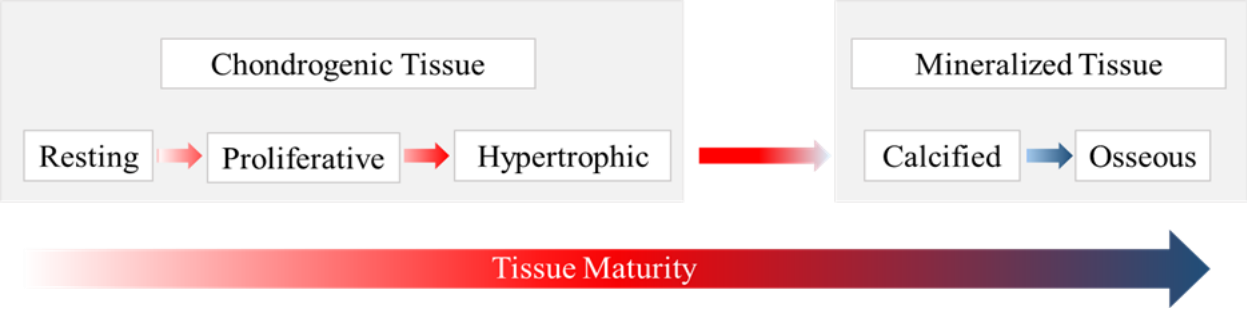

Figure 3-2: Stages of endochondral maturation

Chondrogenic tissue could be considered 'resting', 'proliferating', or 'hypertrophic'. Mineralized tissue was considered to be either 'calcified', or 'osseous'. A binary system was devised in which samples were considered either positive or negative for any of the tissue phenotypes (chondrogenic or mineralized). The categorization of the induced tissue as 'resting', 'proliferative', 'hypertrophic', 'calcified', and 'osseous' was based on well-defined histological characteristics of these phenotypes observed during *in vivo* processes of endochondral ossification. Table 3-1 indicates the histological hallmarks of each endochondral phenotype used in the binning of samples.

Tissue Phenotype		Hallmarks			
		Staining	**Cell Size**	**Cell Shape**	**Tissue organization**
Chondrogenic	Resting	Positive for safranin O (GAG)	Small (~10 μm)	Round/oval	1-2 cells/lacuna low cell-to-matrix volume ratio
	Proliferative	Positive for safranin O (GAG)	Small (~10 μm)	Oval/flattened	Multiple cells (>2)/lacuna High cell-to-matrix volume ratio Cells are stacked or clustered Discretization into chondron units and interterritorial matrix
	Hypertrophic	Positive for safranin O (GAG)	Large (>20 μm)	Round	Cells are stacked or clustered Discretization into chondron units and interterritorial matrix
Mineralized	Calcified	Positive for safranin O (GAG)	Cellular apoptosis	-	Apoptosis leads to cavities and branching tissue struts
	Osseous	Positive for fast green (collagens)	Small (~10 μm)	Round	Cells dispersed within branching tissue struts

Table 3-1: Histological characterization of tissue phenotypes [258]

This morphometric analysis allowed for the relative frequencies of induced tissue types under the various treatments to be determined. The number of samples which were chondrogenic (n_{CH}) or mineralized (n_M) were presented as a frequency (f_{CH}, $f_{M,}$ respectively) relative to the total number of samples per group (N_T=6). The number of samples with 'resting'(n_R), 'proliferating'(n_P), 'hypertrophic'(n_H), 'calcified'(n_{CA}), or 'osseous'(n_O) tissue phenotypes were also presented as a frequency (f_R, f_P, f_H, f_{CA}, f_O) relative to the total number of samples which went chondrogenic (N_{CH}), as this number represented the number of samples which produced any tissue at all. Thresholding using Image J software (National Institutes of Health, Bethesda, MD, USA) was employed in order to obtain area values for chondrogenic and mineralized tissue from the safranin O stained sections. Samples representative of group trends based on safranin O staining were further analyzed immunohistochemically.

3.1.4 Immunohistochemistry

Immunofluorescence was utilized to identify and localize collagen II (cartilage), collagen X (hypertrophy), and collagen I (bone) in tissue samples following a previously described methodology [194]. Tissue sections underwent enzymatic antigen retrieval to improve epitope-antibody binding through treatment with 0.25 units/mL chondroitinase ABC (in 40 mM tris-acetate buffer, pH 8.5) for 1 h at 37°C, followed by 0.25 units/mL keratinase (in 40 mM tris-acetate buffer, pH 8.5) for 30 minutes at 37°C. Endogenous peroxidase activity in the tissue sections was quenched with application of 1% hydrogen peroxide for 45 minutes at room temperature. Tissue samples were blocked with 1% bovine serum albumin (BSA) in a 1X phosphate buffer saline (PBS) for 30 minutes at room temperature to reduce nonspecific antibody binding. Sections were then incubated with mouse polyclonal anti-collagen II antibodies (1:100; Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-collagen X antibodies

(1:150; Abcam, Cambridge, MA, USA) or mouse polyclonal anti-collagen I antibodies (1:250; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), all diluted in 1% BSA (in 1X PBS), overnight at 4°C. Subsequent to primary antibody binding, sections were washed in 1X PBS and incubated with Texas Red (Collagen II, Collagen I) or FITC-labelled (Collagen X) anti-mouse or anti-rabbit secondary antibodies (1:200 dilution; Abcam, Cambridge, MA, USA) for 2 h at room temperature and in the dark. Slides were mounted with Vectashield mounting medium (Vector Laboratories Inc., Burlington, ON, Canada) containing a DAPI counterstain and imaged using an Olympus BX50 microscope with Cellsens™ software (Olympus America Inc., Melville, NY, USA). Nonspecific staining was assessed through the inclusion of a negative control in all immunofluorescent studies, which substituted the primary antibodies with a non-immune serum (BSA). No positive staining was observed in the negative controls (data not shown).

Chapter 4 Results

4.1 Effect of Chondrogenic Media Treatments on Cartilaginous Induction

Periosteal explants were cultured under different chondrogenic media in order to determine the best method, not only for inducing cartilaginous differentiation, but for priming constructs for eventual ossification. Samples were screened for cartilaginous differentiation through assessment of safranin O stained tissue sections. Morphological analysis was employed to further characterize the samples based on the tissue phenotypes (chondrogenic, mineralized) developed. The maturation of cartilaginous tissue was tracked over the chondrogenic induction phase from 3 to 6 weeks, prior to the osteogenic induction phase.

4.1.1 Induction of Chondrogenesis in Periosteal Explants

The percentage of periosteal explants which underwent chondrogenesis was >50% for all groups, but was much higher within each treatment when averaged over the three culture periods ($f_{CH,TGF}$=0.77, $f_{CH,BMP}$=0.73, $f_{CH,Switch}$=0.79; Figure 4-1). Chondrogenic induction (over the 6 week culture period) also appeared to be sustained through the osteogenic phase of culture, without a trending decrease in the number of explants that underwent chondrogenesis. No particular chondrogenic induction treatment (TGF, BMP, Switch) proved to be particularly better at inducing chondrogenesis in periosteum than any other according to treatment averages. Longer induction times (from 3 weeks to 6 weeks) also did not necessarily lead to an increase in the number of samples which underwent chondrogenesis.

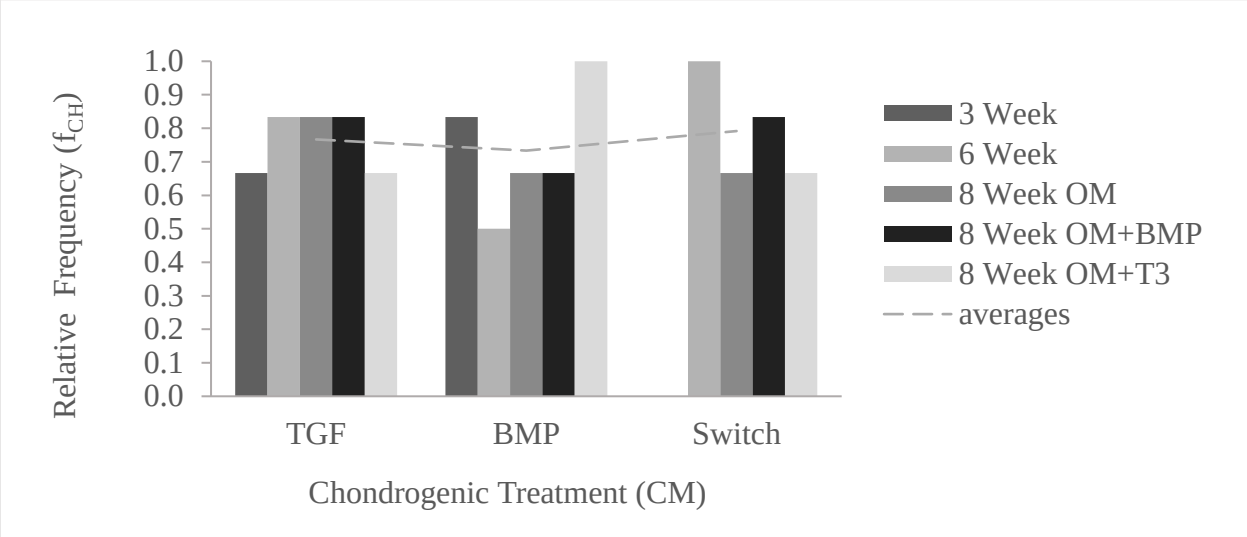

Figure 4-1: Frequency of chondrogenesis in periosteal explants

4.1.2 Effect of TGFβ1 Treatment on Induced Chondrogenic Tissue

After three weeks of chondrogenic induction by TGFβ1 (3W TGF), neotissue growth was identifiable off the cambial layer of periosteal explants. Positive staining for safranin O throughout the produced matrix indicated the induced tissue was cartilaginous (f_{CH}=0.67; Figure 4-2). The presence of collagen II within the GAG positive matrix further confirmed this observation (Figure 4-3 I).

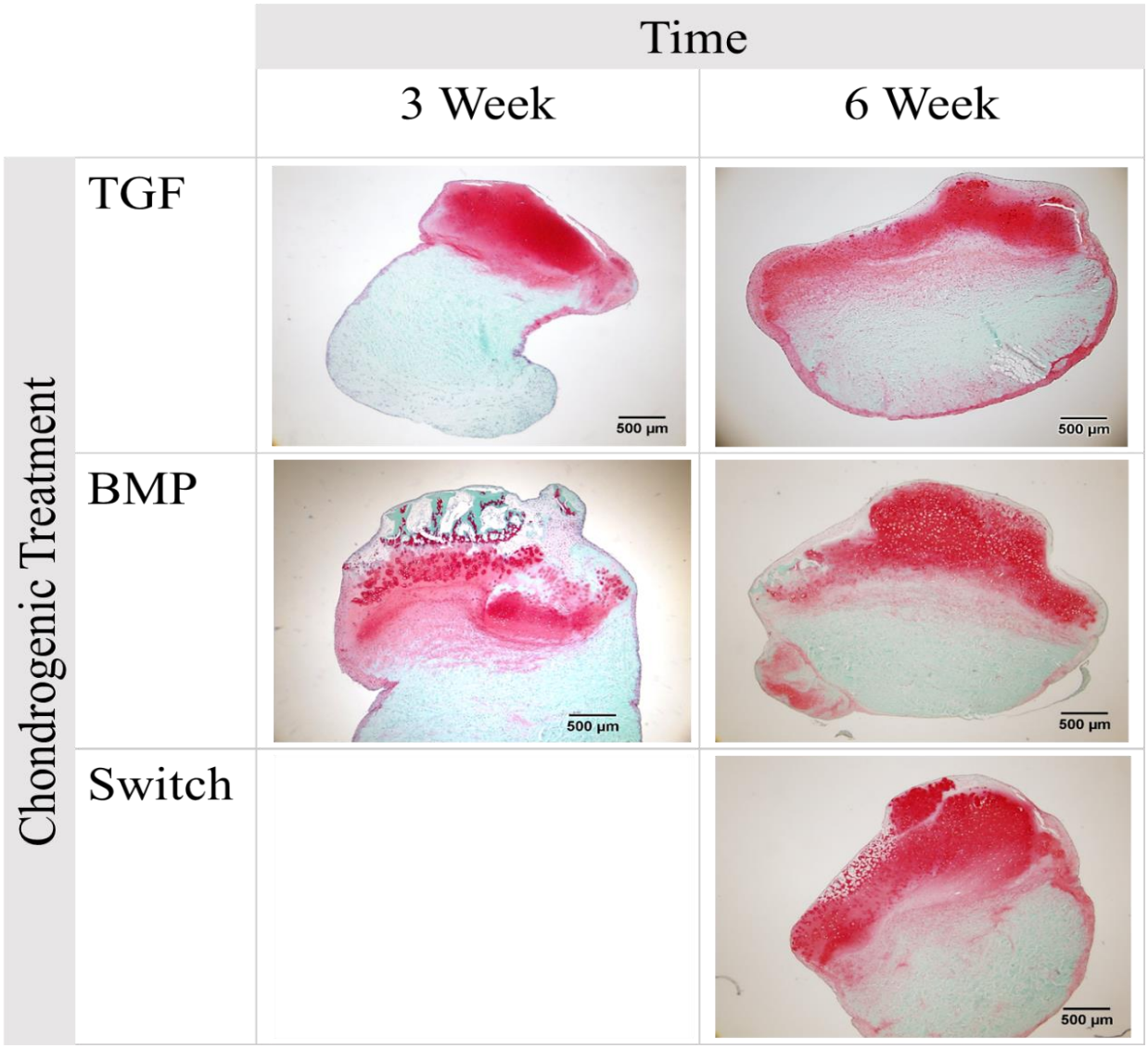

Figure 4-2: Chondrogenesis in periosteal explants as determined by safranin O staining

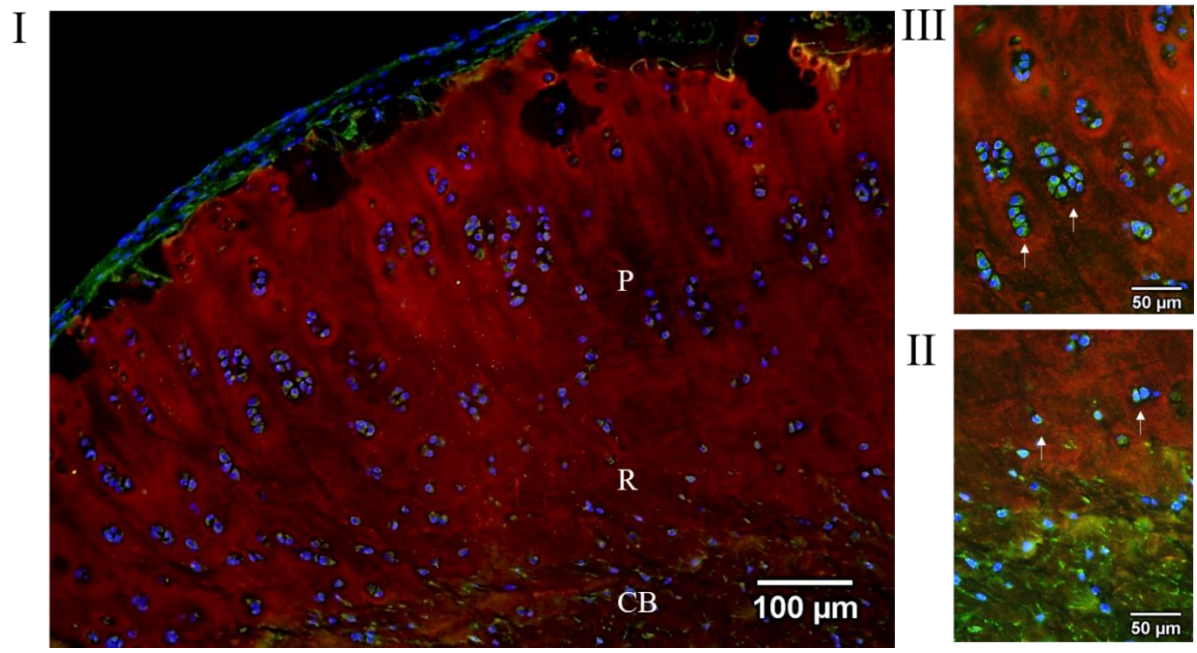

Figure 4-3: Immunohistochemical localization of collagen II/collagen X in cartilaginous tissue induced by 3 weeks culture in TGFβ1. I) Zonal arrangement of tissue proceeds from cambium (CB) through apparent resting (R) and proliferative (P) zones. II) Arrows indicate chondrocytes with resting zone phenotype. III) Arrows indicate chondrocytes with proliferative zone phenotype

The newly developed cartilage appeared morphologically heterogeneous in nature, with distinctive regions discernible throughout the tissue depth (Figure 4-4). At the base of the outgrowing cartilaginous structure (cambium adjacent; CB), cells were small, round to oval in shape, occurring individually or as doublets within lacunae and dispersed randomly throughout a large volume of matrix (Figure 4-4 I-II, Figure 4-3 II). A change in tissue organization occurred moving outwards towards the construct periphery marking a transition in cartilage phenotype. Cells resided mainly as multiples (>2) within lacunae, arranged side-by-side and atop each other as small stacks, some of which seemed to orient perpendicular to the periosteum surface (Figure 4-4 III-IV, Figure 4-3 III). These observations suggested that periosteal cells were capable of generating a growth plate-like structure featuring zones of 'reserve' (R) and 'proliferative' (P) cartilaginous tissue.

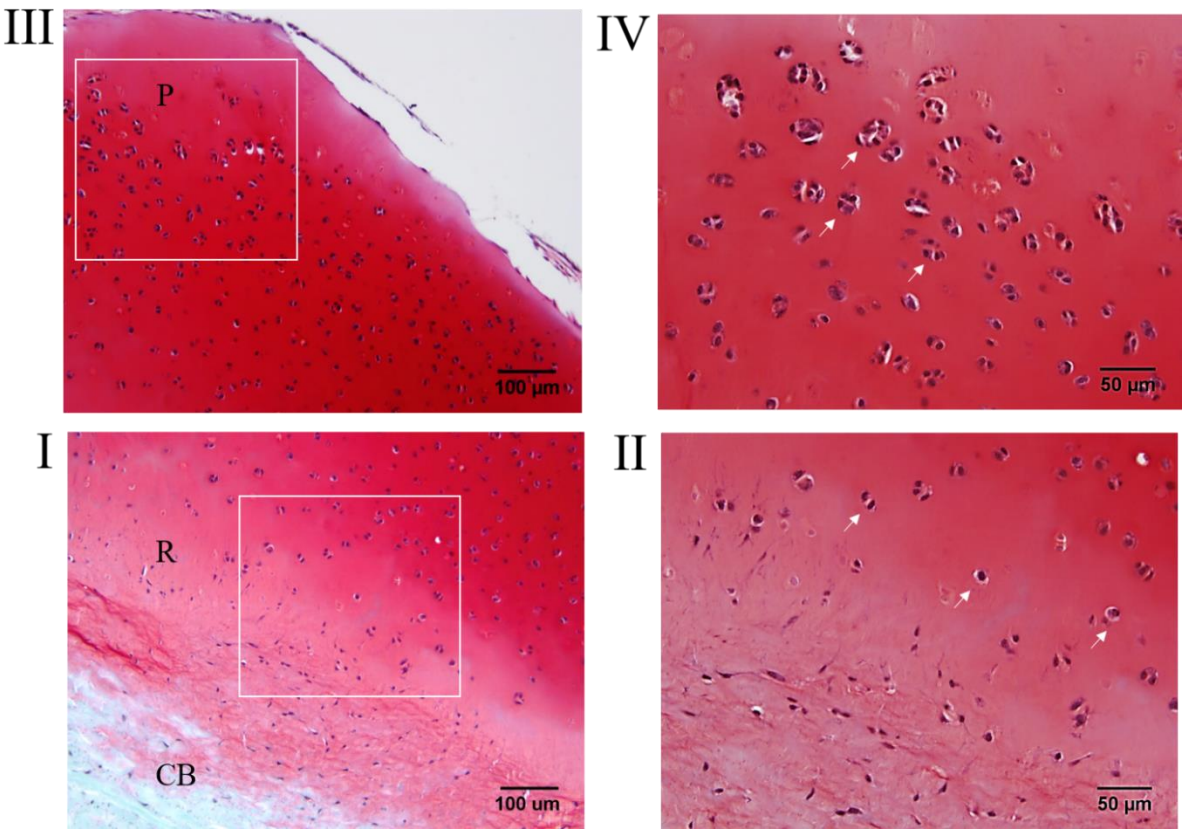

Figure 4-4: Zonal features of cartilage induced by 3 weeks culture in TGFβ1. I) Zonal arrangement of tissue proceeds from cambium (CB) through apparent resting (R) and proliferative (P) zones. II) Arrows indicate chondrocytes with resting zone phenotype. III) Arrows indicate chondrocytes with proliferative zone phenotype

Morphological assessment of 3W TGF samples indicated that a high number of the cartilaginous periosteal samples were positive for 'resting' and 'proliferative' chondrogenic phenotypes (f_R=1.0, f_P= 0.75) while a much smaller fraction of samples had developed characteristics of mature cartilage (hypertrophic) or mineralized (calcified, osseous) tissue (f_H=0.25, f_{CA}=0.25, f_O=0.25; Table 4-1)

			Groups				
			Chondrogenic Phase (TGF)		Osteogenic Phase		
			3 Week	6 Week	8 Week OM	8 Week OM+BMP	8 Week OM+T3
Tissue Phenotype	Chondrogenic (CH)	Resting (R)	1.00	0.80	1.00	1.00	0.50
		Proliferative (P)	0.75	0.80	0.60	0.60	0.75
		Hypertrophic (H)	0.25	0.60	0.40	0.60	0.75
	Mineralized (M)	Calcified (CA)	0.25	0.40	0.00	0.20	0.50
		Osseous (O)	0.25	0.40	0.40	0.40	0.50

Table 4-1: Frequencies of chondrogenic and mineralized tissue phenotypes in periosteal explants induced to chondrogenesis by TGFβ1

At the six week time point (6W TGF), similar regional differences were observed within the induced cartilage tissue (f_{CH}=0.83), but with a notable advancement in maturation of the chondrogenic phenotype (Figure 4-2). A similar 'reserve' zone appeared to give way to a 'proliferative' zone which, at its furthest extremity, appeared early 'hypertrophic' (H; Figure 4-5). Cells of the 'proliferative' area showed progression of differentiation from the previously described side-by-side and shortened stack arrangement of the early 'proliferative' zone (at three weeks), with a columnar organization of flattened cells that appeared to lengthen in a direction perpendicular to the periosteal surface (Figure 4-5 III). A 'hypertrophic' region was distinguishable at the edge of the tissue structures by the presence of enlarged cells within chondrons that were contiguous with the underlying 'proliferative' area (Figure 4-5 III). Collagen X was expressed extracellularly within this 'hypertrophic' region (Figure 4-6). The cartilage was negative for collagen I (Figure A-1).

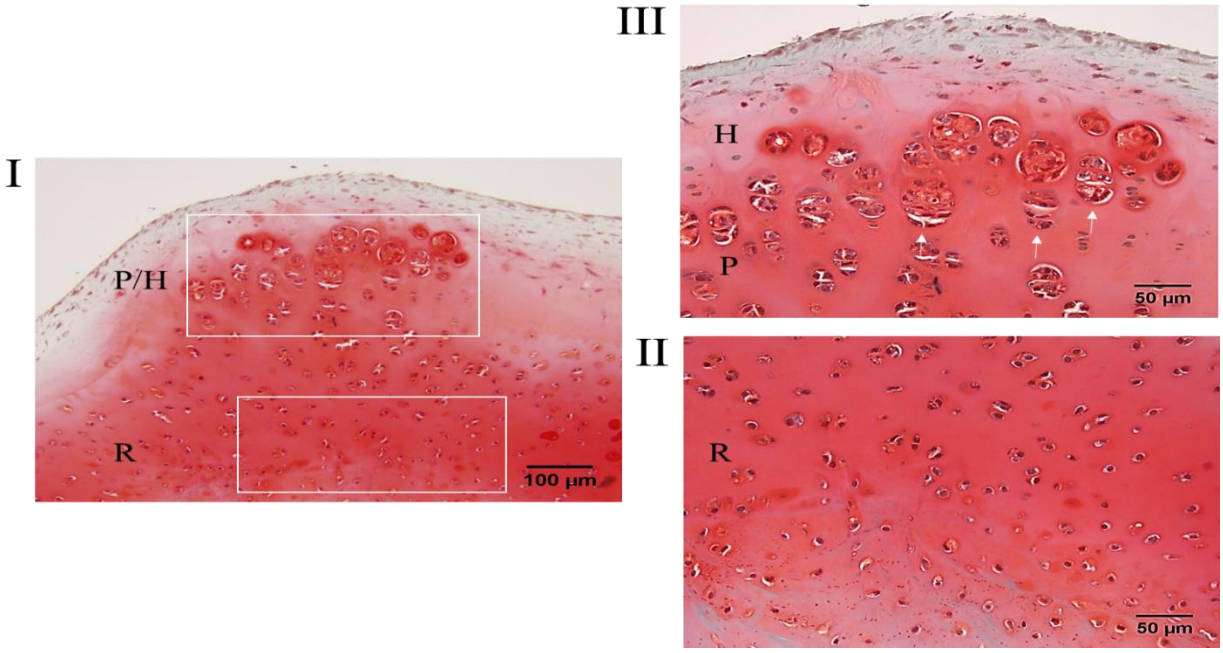

Figure 4-5: Zonal features of cartilage induced by 6 weeks culture in TGFβ1. I) Zonal arrangement of tissue proceeds from cambium (CB) through apparent resting (R), proliferative (P) zones to an early hypertrophic zone. II) resting zone-like tissue lies cambium adjacent. III) Arrows indicate chondrocytes with early hypertrophic phenotype

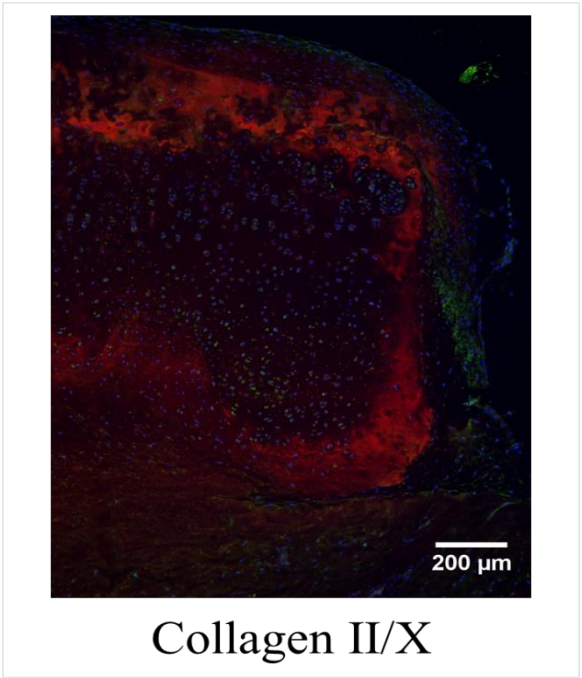

Figure 4-6: Immunohistochemical localization of collagen II/Collagen X in cartilaginous tissue induced by 6 weeks culture in TGFβ1.

Quantification of morphological hallmarks of tissue phenotypes indicated that a large number of explants had 'resting' and 'proliferative' cartilaginous tissue (f_R=0.80, f_P=0.80) similar to the samples cultured for three weeks under TGFβ1 (Figure 4-7). However, the number of samples with 'hypertrophic', 'calcified', and 'osseous' tissue appeared to increase by six weeks in culture (f_H=0.60, f_{CA}=0.40, f_O=0.40; Figure 4-7). Comparison between both time points (3 and 6 weeks) showed no major increase in area of induced chondrogenic tissue (Figure 4-8).

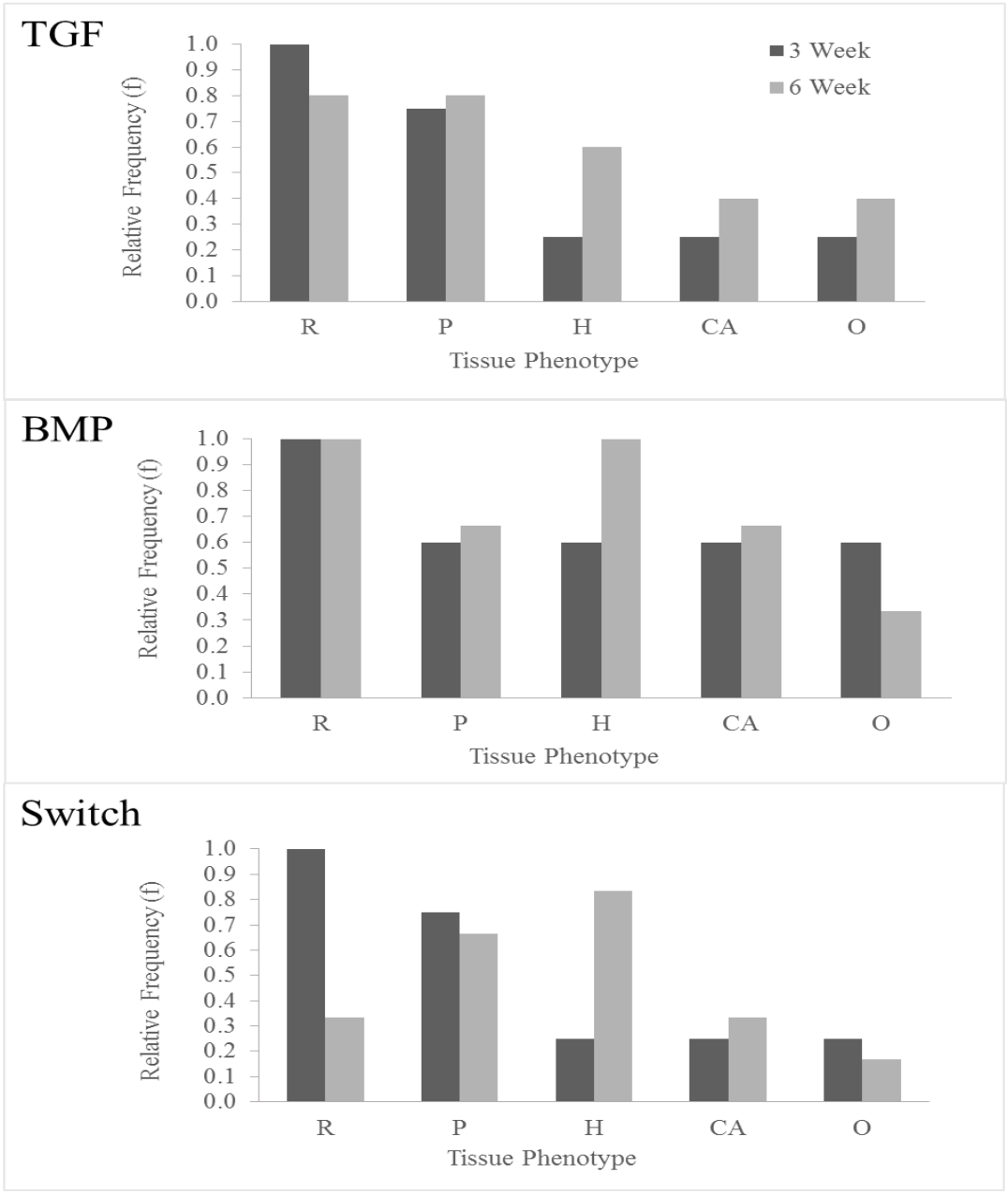

Figure 4-7: Comparative frequencies of tissue phenotypes for the various chondrogenic treatments between 3 and 6 week time points

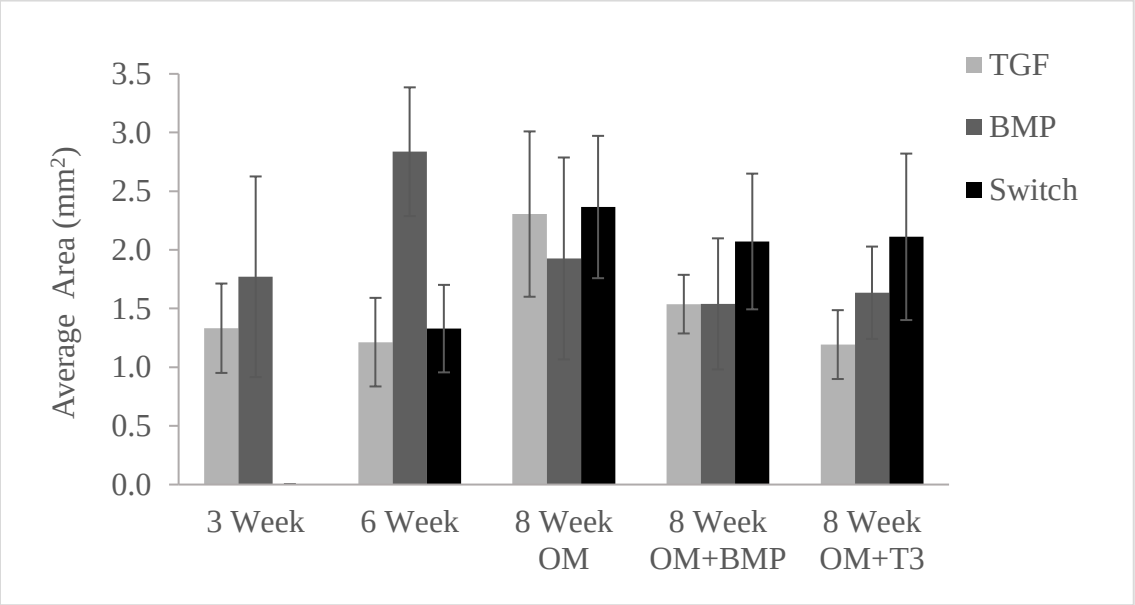

Figure 4-8: Cartilaginous tissue area produced by periosteal explants under the various chondrogenic treatments

4.1.3 Effect of BMP2 Treatment on Induced Chondrogenic Tissue

Under three week chondrogenic induction with BMP2 (3W BMP), a full recapitulation of the endochondral process had occurred in several explants (Figure 4-2). A high degree of tissue organization with 'reserve', 'proliferative', 'hypertrophic', 'calcified' and 'osseous' zones all observable within the newly developed tissue (Figure 4-9). Cells of the 'hypertrophic' area displayed columnar organization within chondrons running in parallel and separated by a collagen X positive interterritorial matrix (Figure 4-9 III-IV, Figure 4-10 II).

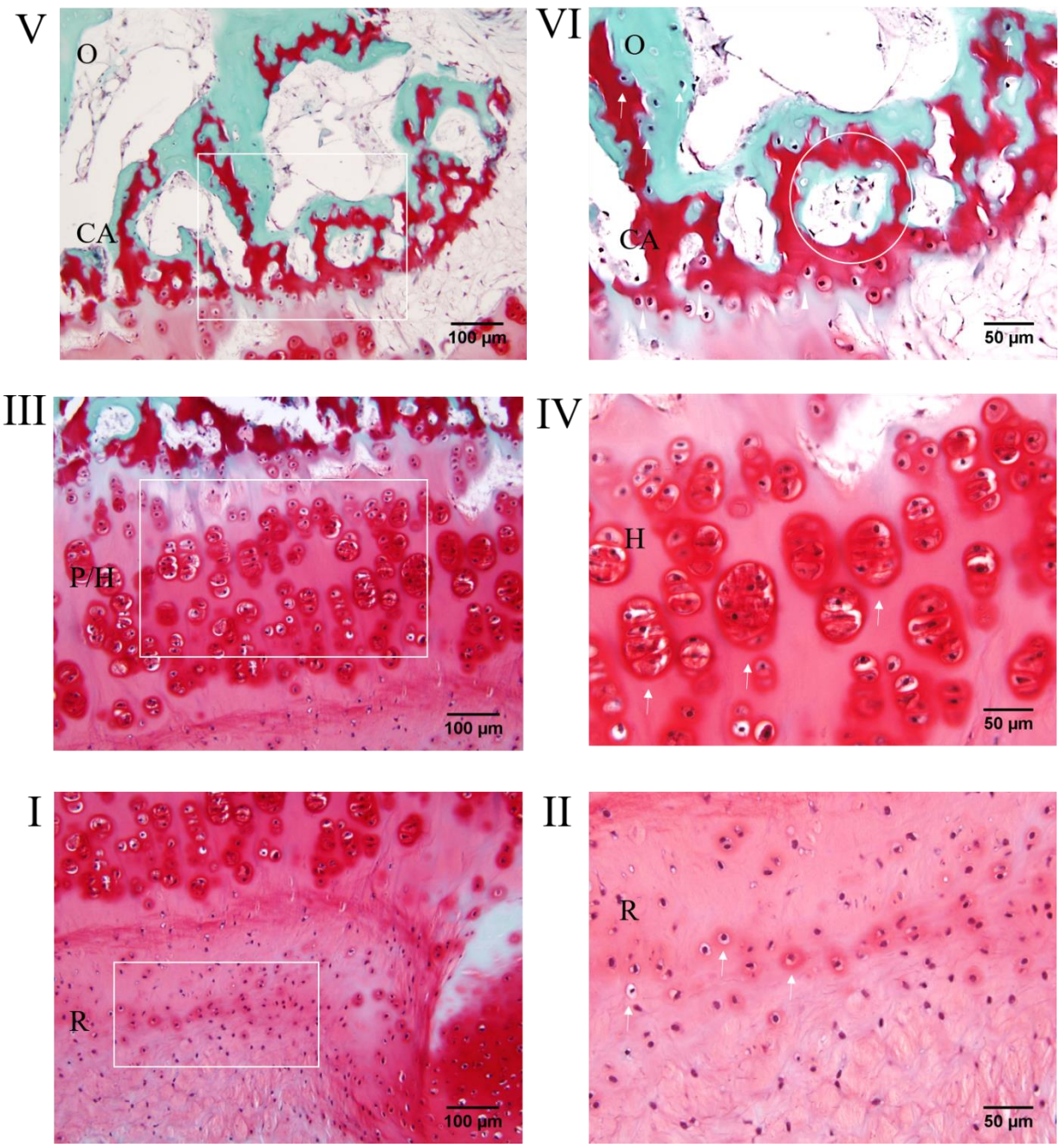

Figure 4-9: Zonal features of cartilage induced by 3 weeks culture in BMP2. I-II) Tissue proceeds from cambium (CB) through apparent resting (R) zone with arrows indicating chondrocytes with resting phenotype. III-IV) Proliferative/hypertrophic zone lies resting zone adjacent with arrows indicating chondrocytes with hypertrophic phenotype. V-VI) Calcified tissue is found atop the hypertrophic zone and is progressively lost to be replaced by overlying osseous tissue. Arrows indicate embedded osteogenic cells and a bone marrow compartment is circled.

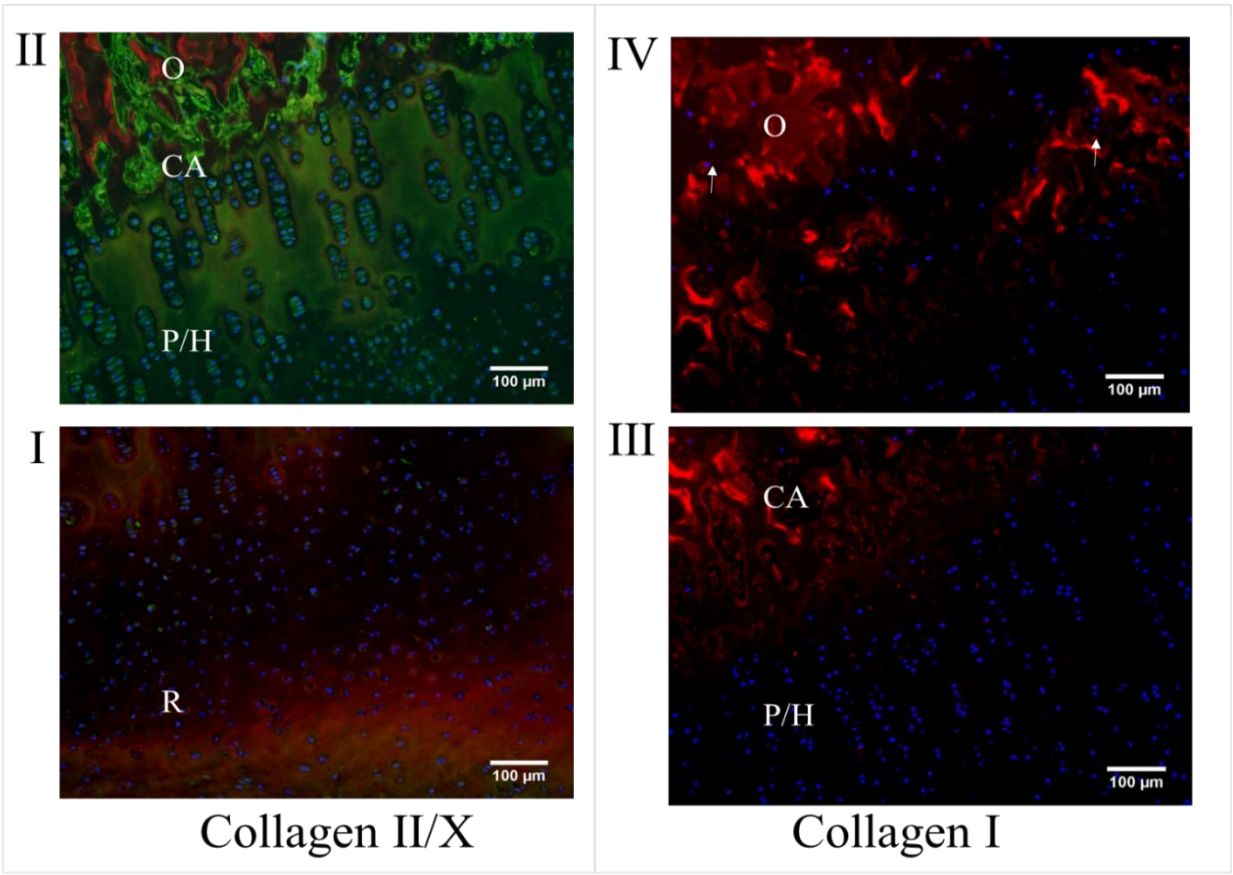

Figure 4-10: Immunohistochemical localization of collagen II/collagen X and collagen I in cartilaginous tissue induced after 3 weeks culture in BMP2. I) The apparent resting zone (R) is collagen II positive. II) Proliferative/hypertrophic (P/H) zone contains extracellular collagen X. III-IV) The ossified region is collagen I positive in certain areas and living cells are observed within this tissue.

Cells resident to the chondrons varied in shape from flattened to round and were visibly larger than those of the underlying regions. Of note was the overall order within the chondrons of this region, as cells were either maintained in single file or branched to form a separate column. Whilst GAG staining was diffuse throughout the cartilaginous neotissue, intense staining occurred in the pericellular and territorial matrix surrounding the chondrons. This intense GAG staining was co-localized with collagen II, which appeared only within this space within the 'hypertrophic' zone (Figure 4-10 I-II). Unidirectional lengthening of stacks extended to the 'calcified' (CA) zone. This area comprised of tissue which stained intensely for GAG and which appeared to be undergoing cavitation, potentially coincident with the observed loss of cellularity (Figure 4-9 V-VI, Figure 4-10 III). Remnants of GAG positive matrix ('calcified' tissue) were embedded within the overlying collagenous tissue; however, these regions were progressively lost moving towards the construct periphery (Figure 4-9 V-VI). The collagenous tissue (collagen I/II positive) had structural features characteristically similar to trabecular bone such as open cavities (marrow compartments) and branching struts (Figure 4-9 VI, Figure 4-10 II-IV). While the tissue of the 'osseous'

(O) zone was much less cellular than the cartilaginous regions, live cells did appear to be encased within the collagenous tissue (Figure 4-9 VI, Figure 4-10 IV).

Analysis of 3W BMP samples indicated that a high number of explants had not only 'resting' and 'proliferative' tissue (f_R=1.0, f_P=0.60), but also displayed 'hypertrophic', 'calcified', and 'osseous' tissue (f_H=0.60, f_{CA}=0.60, f_O=0.60; Table 4-2) The comparative frequency of the mature chondrogenic and mineralized tissue phenotypes (hypertrophic, calcified, and osseous) was greater for samples induced under BMP2 as compared to those induced under TGFβ1 at three weeks (Figure 4-11). Similarly, the average area of developed tissue was larger for the BMP2 group as compared to the TGFβ1 group under three weeks chondrogenic induction (Figure 4-8).

			Groups				
			Chondrogenic Phase (BMP)		Osteogenic Phase		
			3 Week	6 Week	8 Week OM	8 Week OM+BMP	8 Week OM+T3
Tissue Phenotype	Chondrogenic (CH)	Resting (R)	1.00	1.00	0.75	1.00	1.00
		Proliferative (P)	0.60	0.67	0.50	0.25	1.00
		Hypertrophic (H)	0.60	1.00	0.75	0.25	0.50
	Mineralized (M)	Calcified (CA)	0.60	0.67	0.25	0.25	0.33
		Osseous (O)	0.60	0.33	0.50	0.00	0.00

Table 4-2: Frequencies of chondrogenic and mineralized tissue phenotypes in periosteal explants induced to chondrogenesis by BMP2

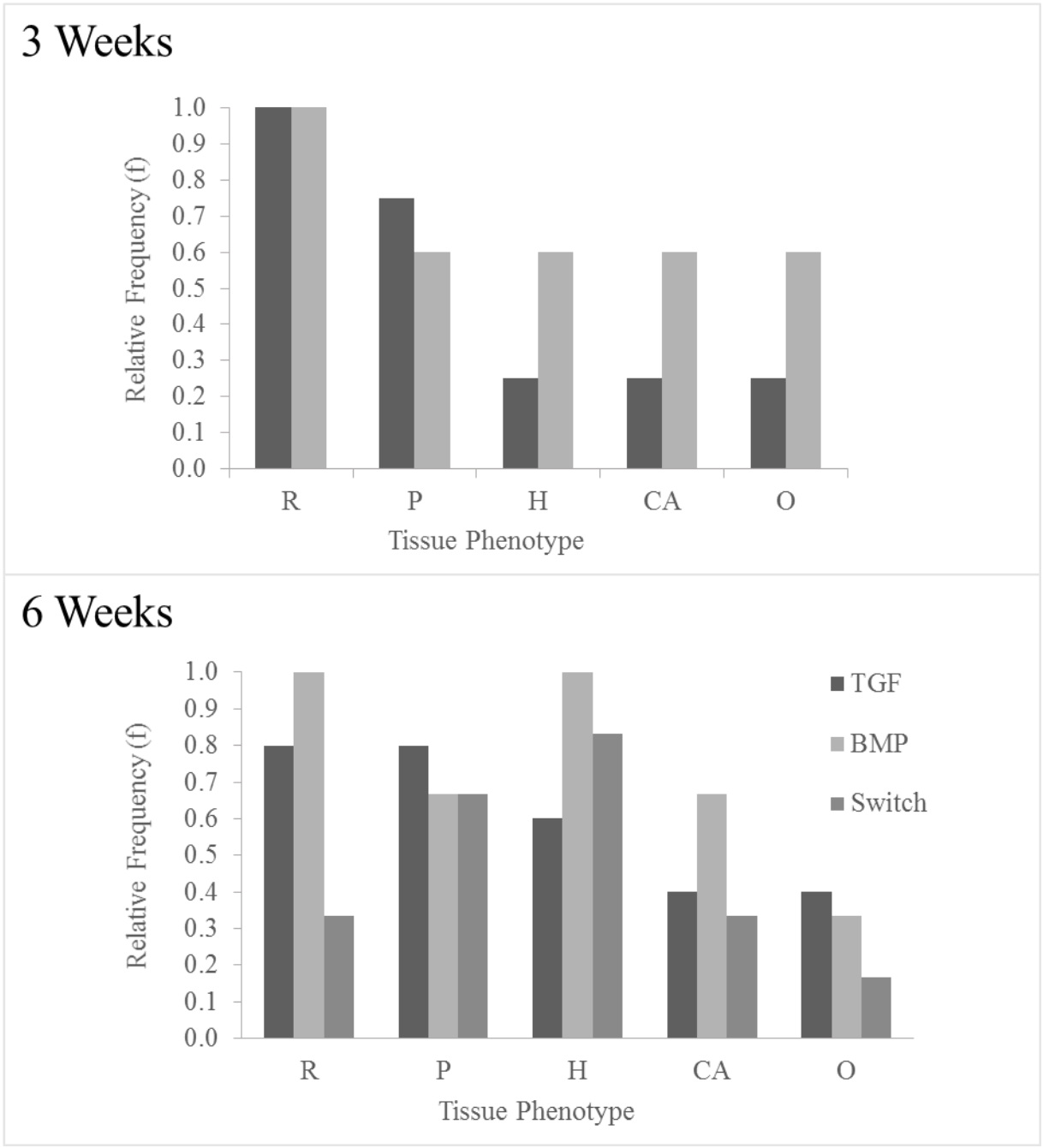

Figure 4-11: Comparative frequencies of tissue phenotypes between various chondrogenic treatments at 3 and 6 weeks.

Periosteum induced under the six week BMP2 supplementation (6W BMP) was comparatively similar to the three week BMP2 constructs (Figure 4-2). Again, the progression of the chondrogenic phenotype was observed (Figure 4-12); however, the 'mineralized' tissue appeared to be less developed than in the constructs cultured for a shorter time period. A 'calcifed' zone was observable at the end of the 'hypertrophic' zone as indicated by its predominant features: less cellular GAG positive matrix forming

into strut-like structures but with no branching collagenous trabeculae (Figure 4-12 III-IV). The tissue bulk was positive for collagen II and collagen X which appeared to be co-localized (Figure 4-13 I). The calcified matrix also appeared to contain less cells than the underlying tissue and stained slightly for collagen I (Figure 4-13 II).

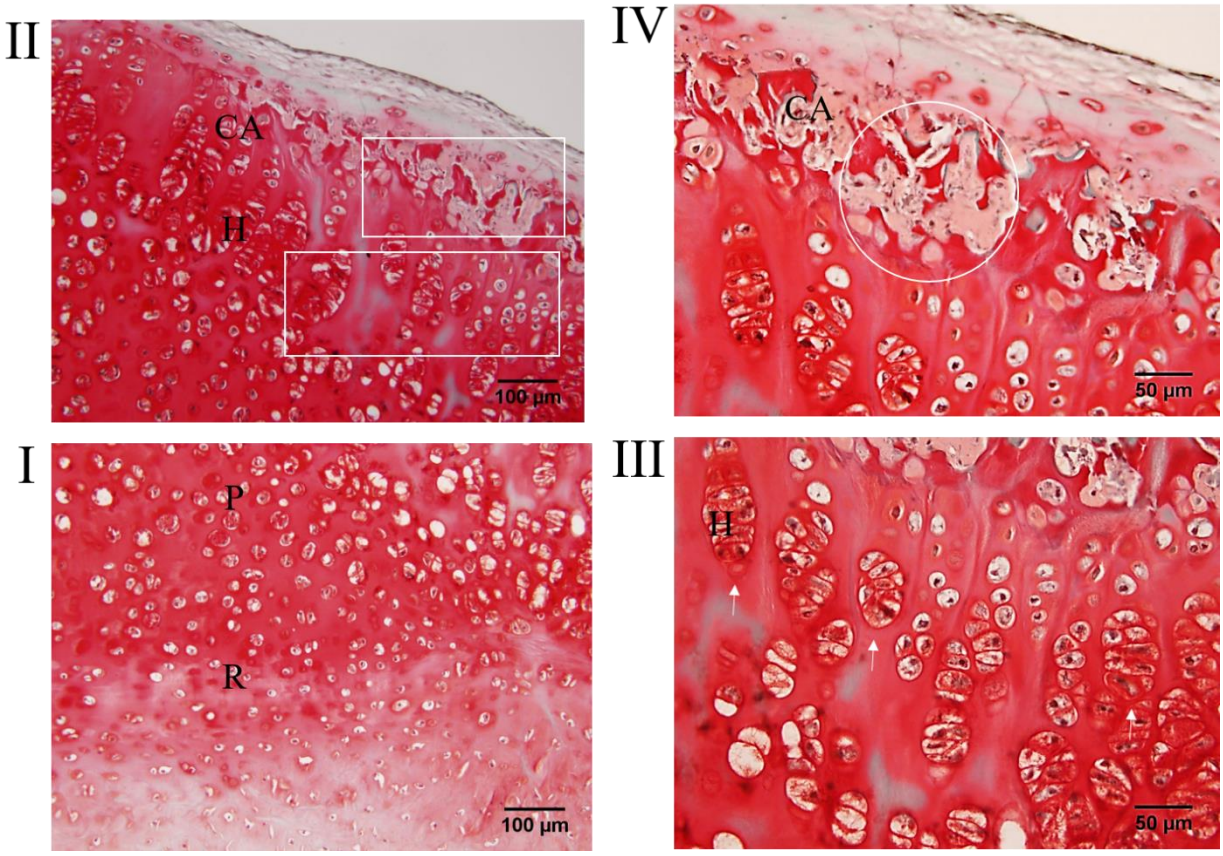

Figure 4-12: Zonal features in cartilage tissue induced by 6 weeks culture in BMP2. I-II) Endochondral process progressed through resting (R), proliferative (P), hypertrophic (H) zones to a calcified (CA) zone. III) Arrows indicate cells with hypertrophic phenotype. IV) Encircled is a bone marrow compartment.

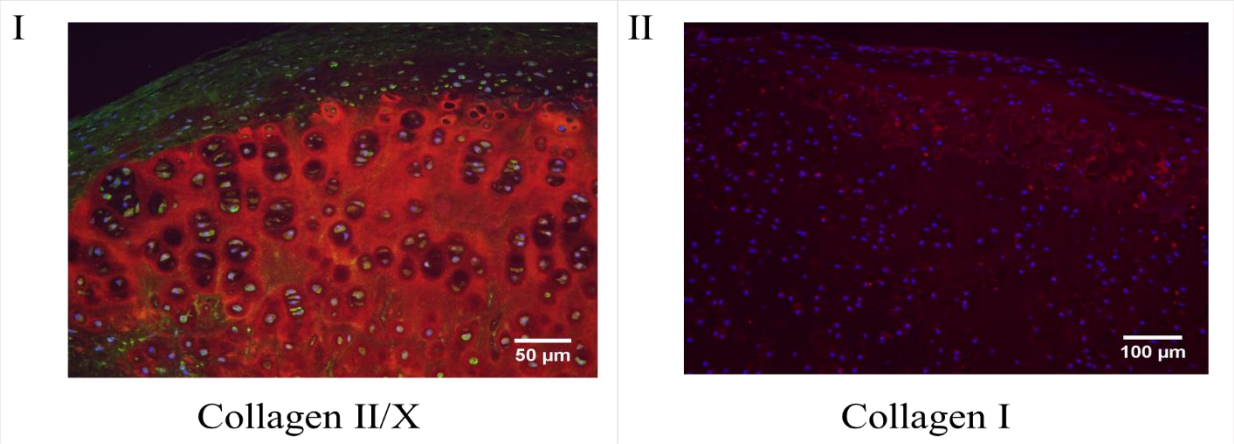

Figure 4-13: Immunohistochemical localization of collagen II/collagen X and collagen I in cartilaginous tissue induced after 6 weeks culture in BMP2

Samples cultured for six weeks under BMP showed a similar frequency of 'resting' and 'proliferative' tissue types compared to 3W BMP samples (f_R=1.0, f_P=0.67); however, with greater incidence of 'hypertrophic' and 'calcified' tissue (f_H=1.0, f_{CA}=0.67), and lower incidence of 'osseous' tissue (f_O=0.33) than at the earlier time point (Figure 4-7). BMP2 treated explants appeared to be more likely to develop mature chondrogenic and mineralized tissue ('hypertrophic', 'calcified') as compared to TGFβ1 treated explants at six weeks (Figure 4-11). The average area of developed tissue increased from three to six weeks under BMP2 induction, and was comparatively greater than that of 6W TGF samples (Figure 4-8).

4.1.4 Effect of Switch Treatment on Inducted Chondrogenic Tissue

At six weeks under the switch treatment (6W Switch), zones of the growth plate were recapitulated up to a 'calcified' or early 'osseous' phase (Figure 4-2). Several discrepancies were observed within the zones under this regime which did not appear to follow previous observations. The 'hypertrophic' zone displayed a lack of interterritorial collagen X staining, although there was formation of an overlying 'calcified' zone (Figure 4-14 I, Figure 4-15 I). The 'calcified' zone appeared to contain the standard hallmarks including loss of cellularity and formation of GAG positive matrix struts, some of which appeared to be undergoing remodeling with collagenous tissue lining their surface (Figure 4-14 II, Figure 4-15 II).

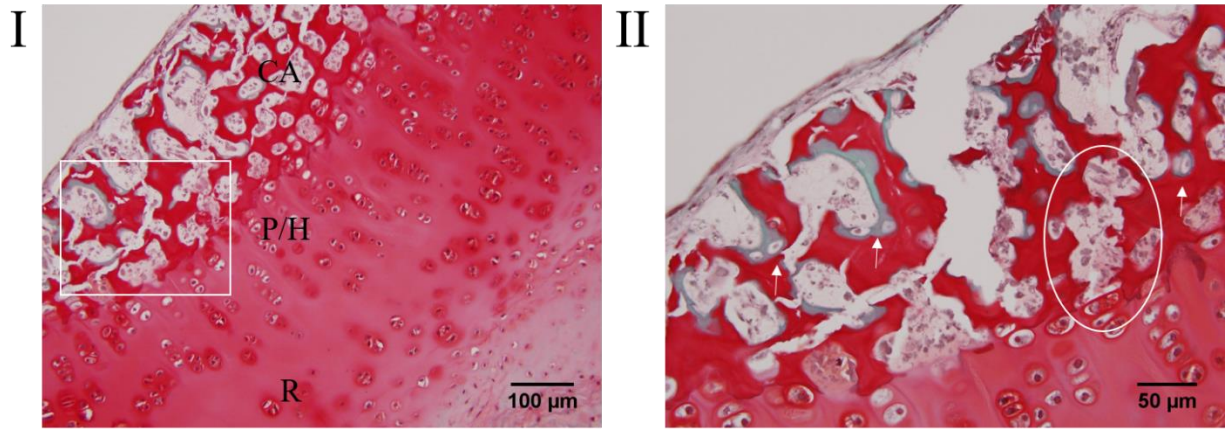

Figure 4-14: Zonal features in cartilage tissue induced by 6 weeks switch treatment. I) Recapitulation of endochondral process occurs to a calcified (CA) region. II) Arrows indicate embedded osteogenic cells and encircled is a bone marrow comparentment.

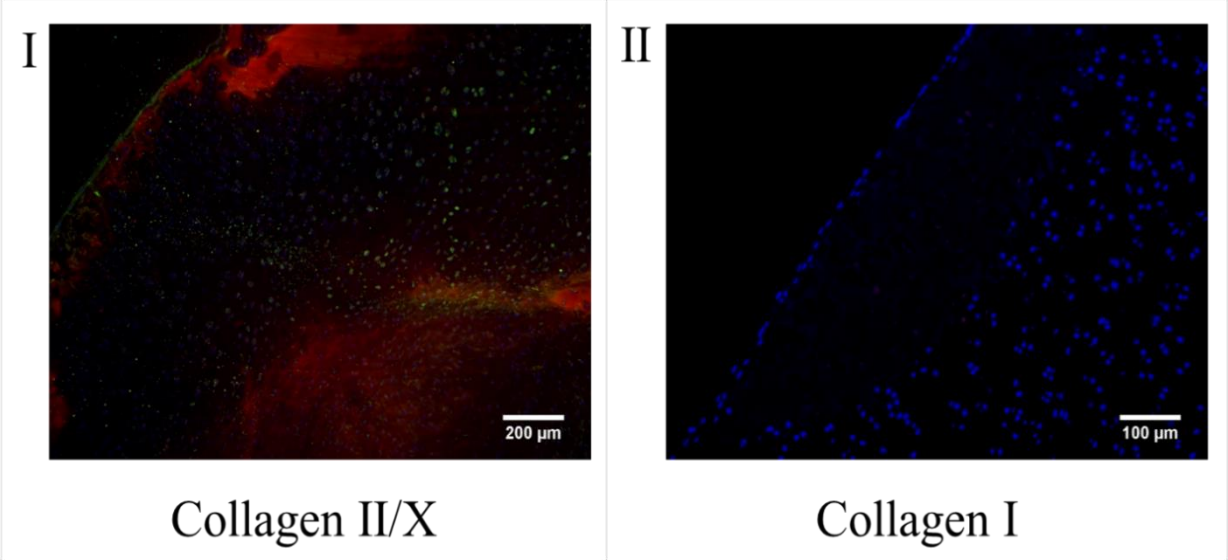

Figure 4-15: Immunohistochemical localization of collagen II/collagen X and collage I in cartilaginous tissue induced after 6 weeks Switch treatment. I) Collagen X does not appear extracellularly within the apparent hyperophic (H) zone. II) Loss of DAPI staining within the calcified region suggests apoptosis of hypertrophic chondrocytes is occurring.

Morphometric analysis on the switch treatment revealed a rather low incidence of samples with 'resting', 'calcified', and 'osseous' tissue phenotypes (f_R=0.33, f_{CA}=0.33,f_O=0.17) but a high incidence of the 'proliferative' and 'hypertrophic' cartilaginous phenotypes (f_P=0.67,f_H=0.83; Table 4-3). From three weeks (3W TGF) to six weeks, the switch condition increased in both 'hypertrophic' and 'calcified' tissue

types (Figure 4-7). In comparison with the other treatments after six weeks of culture, switch samples were less frequently 'proliferative' relative to TGFβ1 samples but more so relative to BMP2 samples (Figure 4-11). 'Hypertrophic' tissue was induced less frequently than in BMP2 samples but more often than in TGFβ1 samples (Figure 4-11). The area of tissue which developed under the switch treatment did not increase from the three week to six weeks and was less than what was produced under the action of BMP2 alone (Figure 4-8).

			Groups				
			Chondrogenic Phase (Switch)		Osteogenic Phase		
			3 Week	6 Week	8 Week OM	8 Week OM+BMP	8 Week OM+T3
Tissue Phenotype	Chondrogenic (CH)	Resting (R)	1.00	0.33	0.75	0.80	1.00
		Proliferative (P)	0.75	0.67	0.75	0.60	1.00
		Hypertrophic (H)	0.25	0.83	0.75	1.00	1.00
	Mineralized (M)	Calcified (CA)	0.25	0.33	0.00	0.40	0.50
		Osseous (O)	0.25	0.17	0.50	0.60	0.00

Table 4-3: Frequencies of chondrogenic and mineralized tissue phenotypes in periosteal explants induced to chondrogenesis by Switch treatment

4.2 Effect of Osteogenic Media on Endochondral Ossification in Periosteal Explants

Following six weeks of chondrogenic induction, periosteal explants were placed in different osteogenic media types for a further two weeks to assess their propensity to undergo ossification. Utilizing this stratagem, the chondrogenic induction technique and the osteogenic media type best suited to encourage endochondral bone formation was to be determined. Samples were stained for markers of hypertrophy (Collagen X) and bone tissue (collagen I), along with standard cartilaginous stains (GAG/Collagen II) for gross histological and morphometric analysis.

4.2.1 Induction of Osteogenesis in Cartilaginous Periosteal Explants

Approximately half of all cartilaginous periosteal explants underwent mineralization (Figure

4-16). The osteogenic phase of induction did not generally improve the tendency of explants to mineralize. Samples were equally, if not more prone to mineralize during the chondrogenic phase of induction (TGF, BMP; Figure 4-17). Only 8W BMP OM and 8W switch OM+BMP showed an average increase in frequency of mineralization (f_M=0.75 and 0.80, respectively) as compared to samples with the corresponding chondrogenic treatments at six weeks (Figure 4-16). All chondrogenic treatment groups (TGF, BMP, Switch) produced the most amount of mineralized tissue during the chondrogenic phase of treatment (between three and six weeks) (Figure 4-17). Samples under chondrogenic induction by BMP2 developed the largest amount of mineralized tissue while Switch treated samples led to the least amount of mineralized tissue (Figure 4-18).

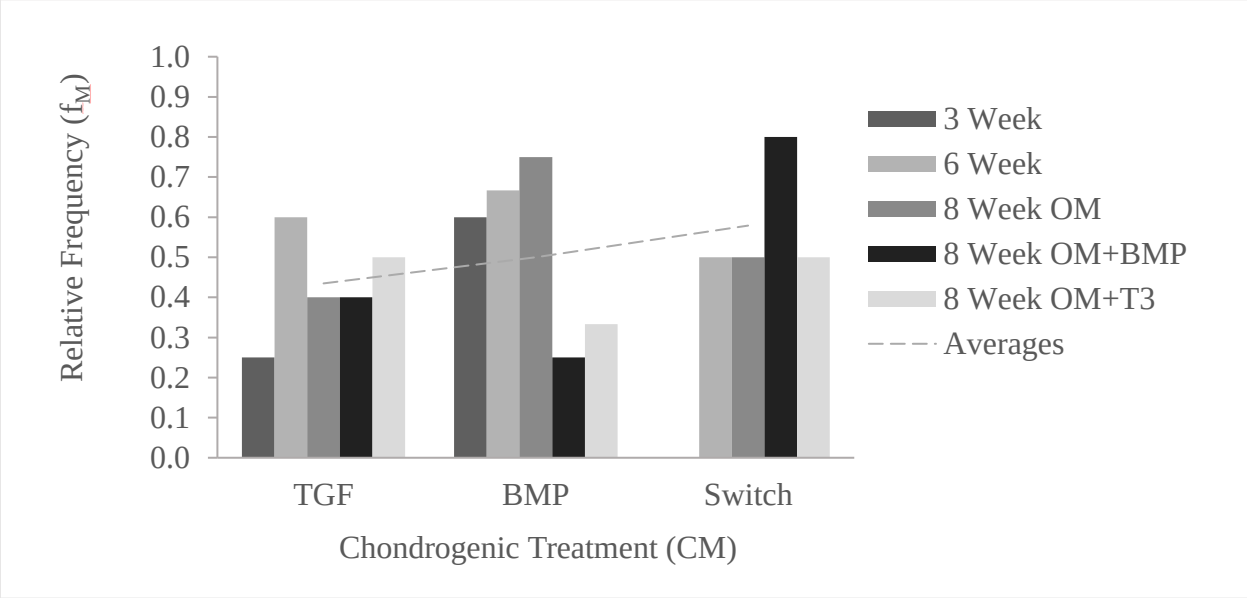

Figure 4-16: Frequency of mineralization in periosteal explants

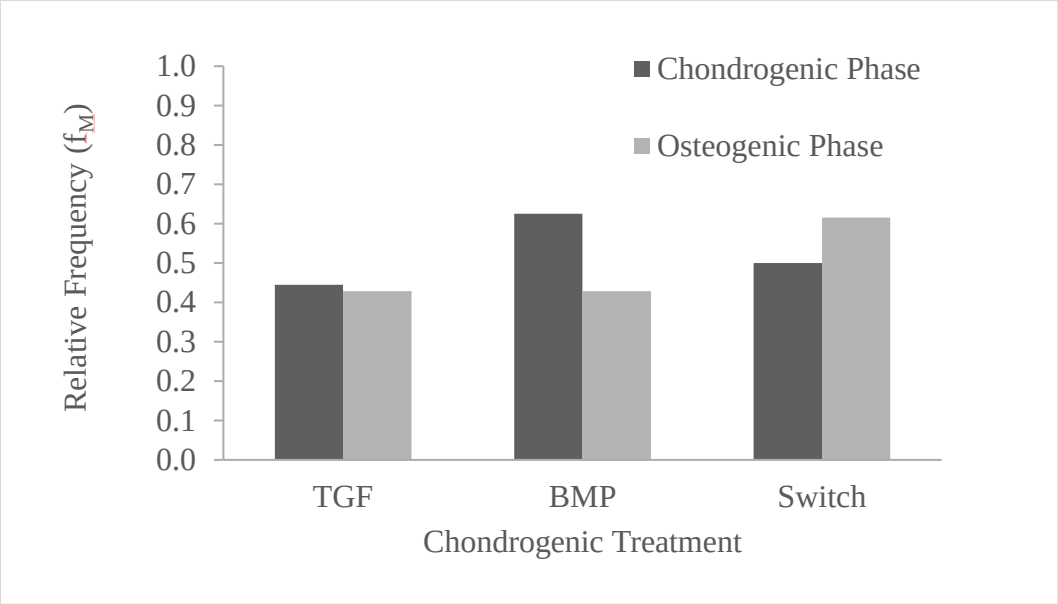

Figure 4-17: Average relative frequency of mineralization for both chondrogenic and osteogenic phases of induction.

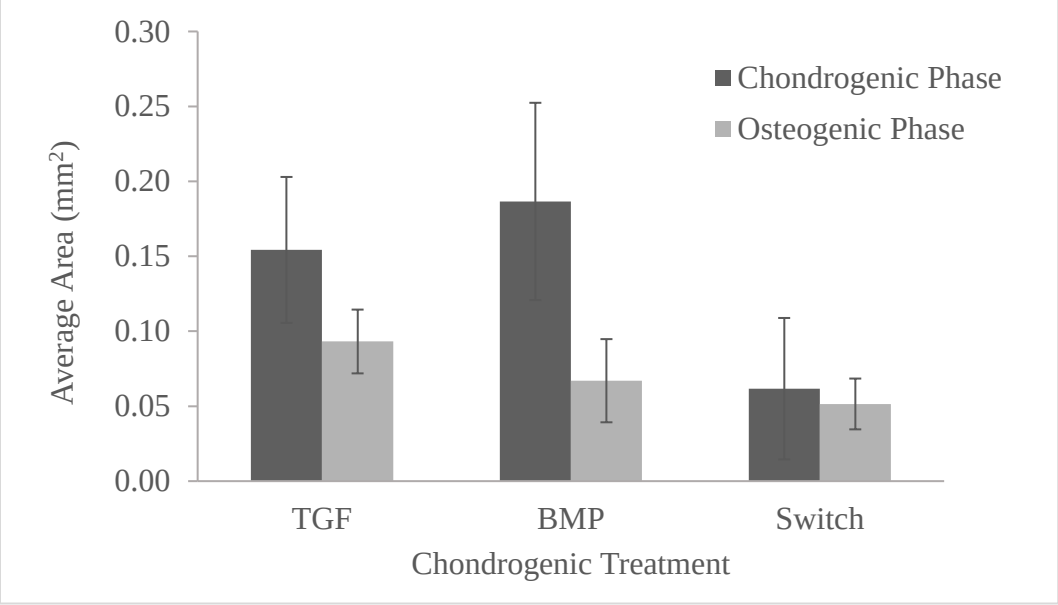

Figure 4-18: Average mineralized tissue areas for both chondrogenic and osteogenic phases of induction

4.2.2 TGFβ1 Treated Chondrogenic Periosteal Explants in Osteogenic Media Types

Subsequent to the osteogenic induction phase of culture, samples were found to be less prone to undergo mineralization as compared to at six weeks under TGFβ1 chondrogenic (6W TGF) induction

(Figure 4-16). Furthermore, the amount of mineralized tissue produced was similarly lower, with the largest amount produced by three and six week TGF induced samples (Figure 4-20). Samples given osteogenic media with T3 (OM+T3) were the most likely to mineralize (OM:f_M=0.4, OM+BMP:f_M=0.4, OM+T3:f_M=0.5). Only osteogenic media with T3 (8W TGF OM+T3) appeared to push samples further towards a mature cartilaginous or mineralized tissue phenotype (f_R=0.50, f_P=0.75, f_H=0.75, f_{CA}=0.50, f_O=0.50) as compared to samples that underwent only chondrogenic induction (6W TGF; Figure 4-20). Comparatively against osteogenic media alone (8W TGF OM), addition of BMP to the osteogenic media (8W TGF OM+BMP) resulted in increased frequency of observed hypertrophic and calcified tissue types, whilst addition of T3 resulted in a decreased frequency of the 'resting' phenotype, and increase in 'proliferative', 'hypertrophic', 'calcified', and 'osseous' tissue types (Figure 4-20). The addition of T3 appeared to follow a similar trend to the 6W switch samples, where a positive shift in tissue maturation was observed.

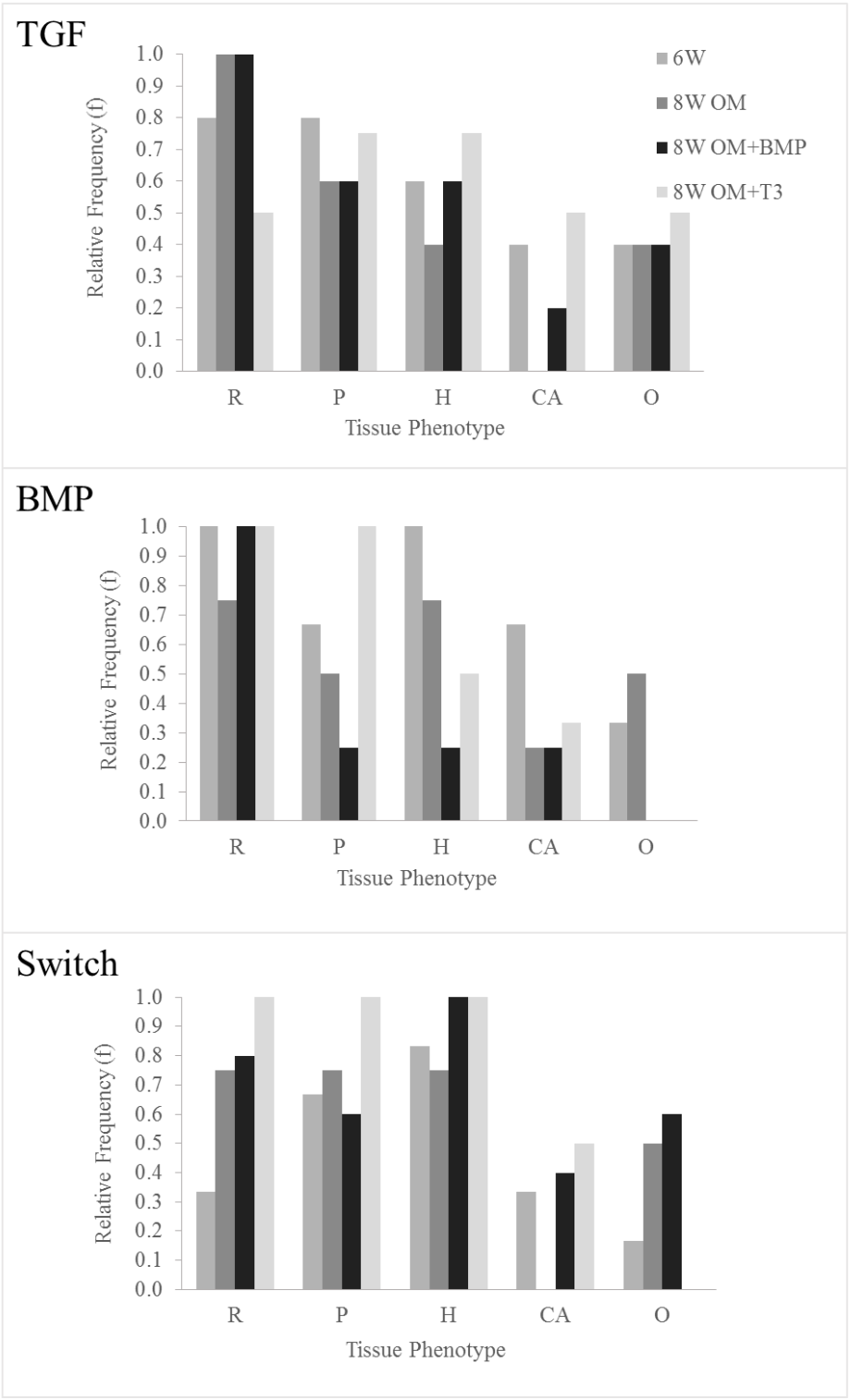

Figure 4-19: Comparative frequencies of tissue phenotypes between osteogenic media groups by the method of chondrogenic induction

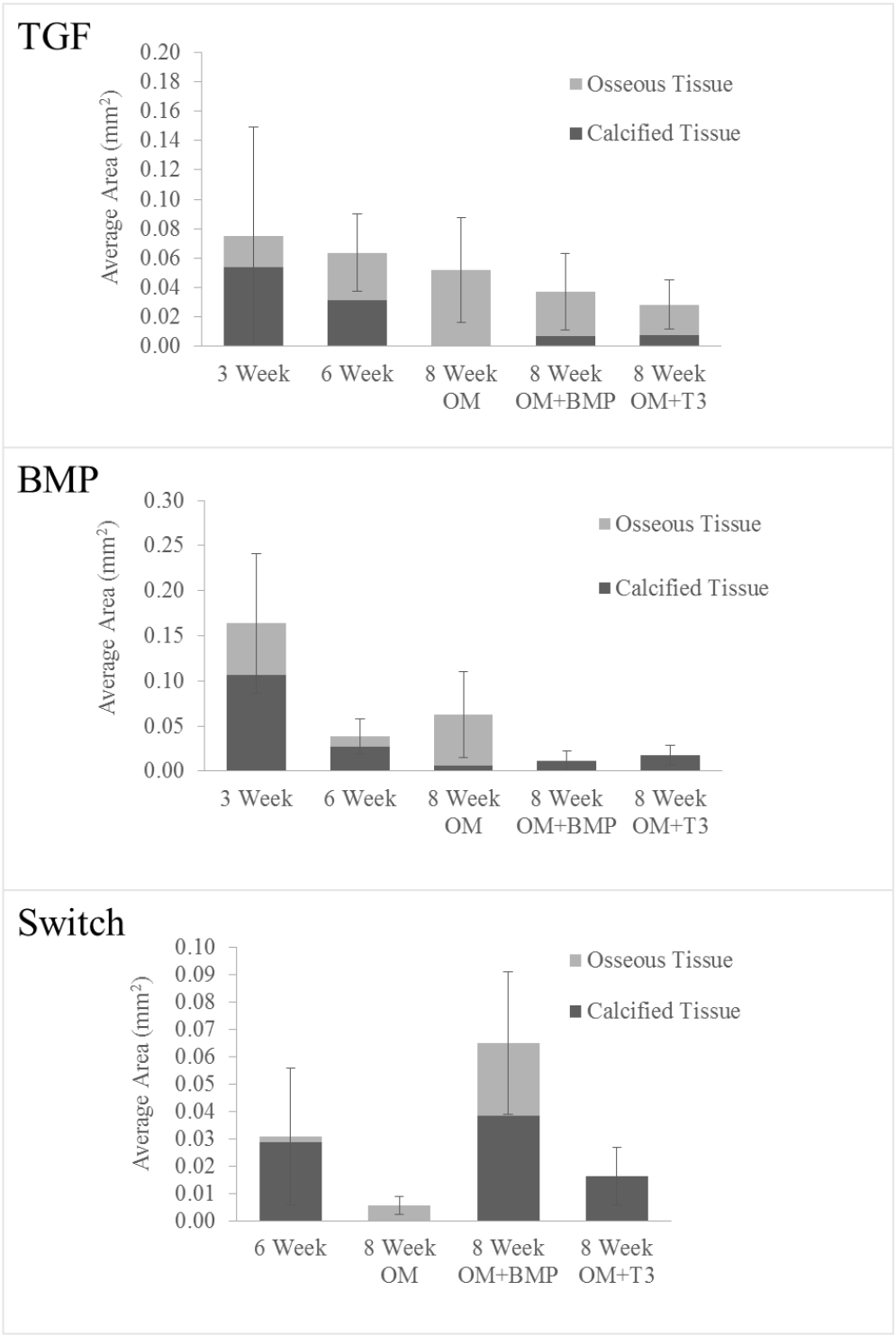

Figure 4-20: Mineralized tissue area during osteogenic induction phase by chondrogenic induction treatment

Observation of samples chondrogenically differentiated under TGFβ1 and cultured in osteogenic media (8W TGF OM), showed that while some bone formation had occurred in certain samples, the induced tissue was almost entirely cartilaginous and specifically appeared to be 'proliferative' (Figure 4-2, Figure 4-21 I). Although a thin 'resting' zone lay adjacent to the cambium (Figure 4-21 II) tissue was mostly highly cellular, with a large number of cells resident within singular chondrons (Figure 4-21 III). GAG staining was intense throughout the developed tissue and was present both within and without the cells. Expression of matrix constituents followed a similar pattern as seen previously under the action of TGFβ1 during the chondrogenic phase of induction. Collagen II was expressed in a small region at the base of the cartilaginous structure in what may be a 'reserve' zone and was then partially lost moving into the tissue bulk, before again staining intensely at the tissue periphery (Figure 4-22). Collagen X was found intracellularly within cells of the 'proliferative' zone (Figure 4-22). The developed matrix was negative for collagen I (Figure A-2).

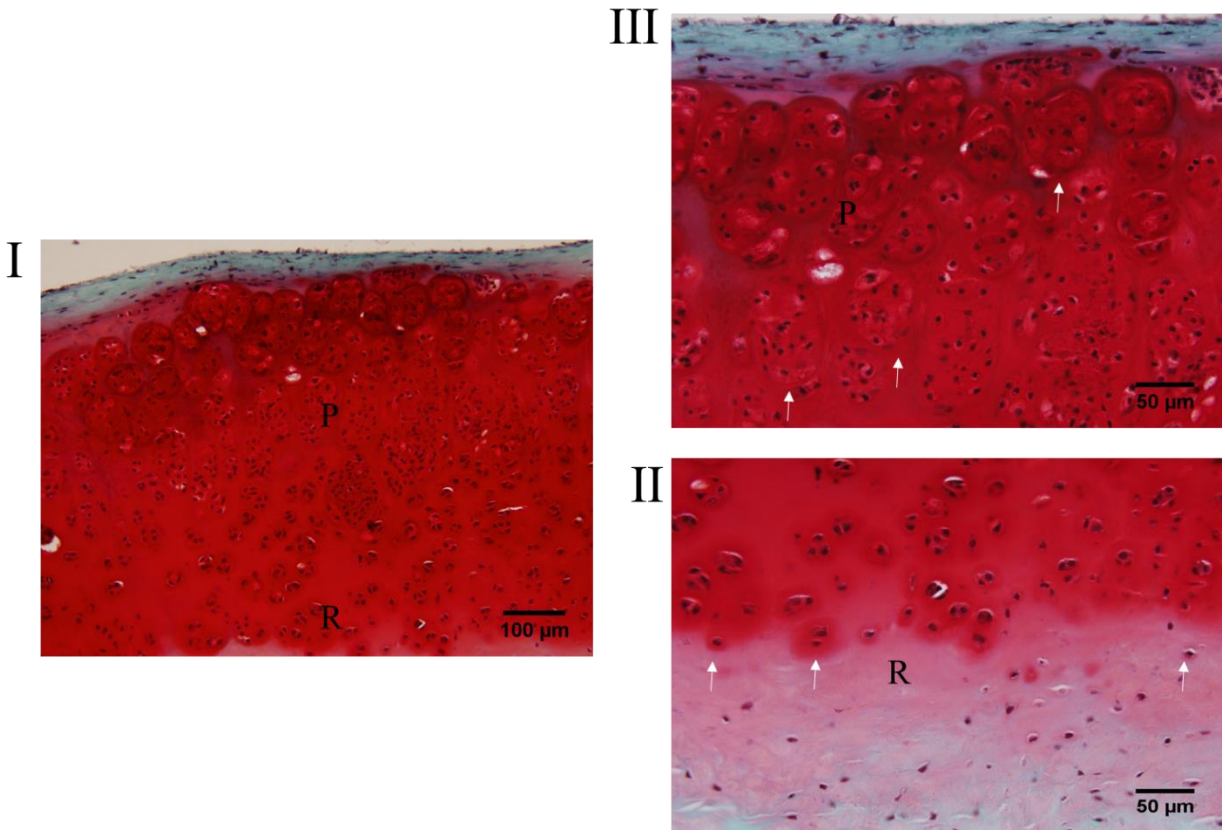

Figure 4-21: Zonal features in cartilage tissue induced by 6 weeks of TGFβ1 followed by osteogenic culture in OM. I) Tissue was seen to be mainly resting (R) and proliferative (P). II) Arrows indicate cells with resting chondrocyte phenotype. III) Arrows indicate highly proliferative chondrocytes.

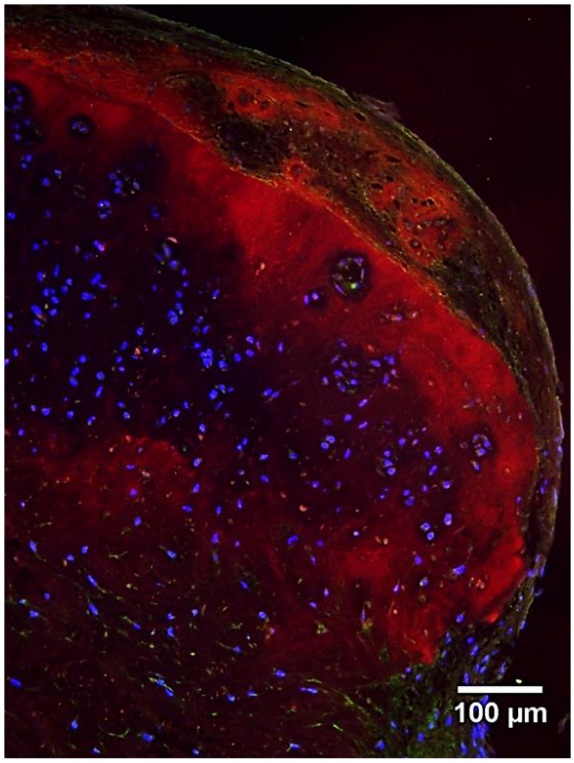

Figure 4-22: Immunohistochemical localization of collagen II/collagen X in cartilaginous tissue induced after 6 weeks of TGFβ1 followed by osteogenic culture in OM

Under two weeks of induction with osteogenic media and BMP2 (8W TGF OM+BMP), TGFβ1 treated samples showed evidence of ossification (Figure 4-2). Trabecular-like structures were visible close to the edge of the neotissue (Figure 4-23 III-IV). These structures were collagenous (Figure 4-24 II) and lay atop GAG matrix remnants (Figure 4-23 III-IV). Underlying the osseous structures was cartilage tissue containing features of 'reserve', 'proliferative', and 'hypertrophic' zones. Cellular alignment within the 'proliferative' zone was shifted with columns of cells forming diagonally or parallel to the periosteum surface (Figure 4-23 I-II). Immunohistochemical staining shows an even distribution of cells along fibrous bands of the periosteum, which, beginning around the 'reserve' zone', flow into the overlying cartilaginous tissue, forming 'proliferative' zone-like cell stacks (Figure 4-24 I). Despite the directional disparity, the 'proliferative' zones are phenotypically similar to those previously described, and give way to the 'hypertrophic' zone which culminates in the calcified and osseous tissue structures. The 'hypertrophic' zone is tentatively identified by the expression of extracellular collagen X and proximity to the 'osseous' zone (Figure 4-24 I). Cells within this zone appear to be contributing to the formation of

osseous tissue without undergoing apoptosis. Small, spherical cells are encased randomly within a GAG matrix and surrounded by collagenous tissue (Figure 4-23 IV).

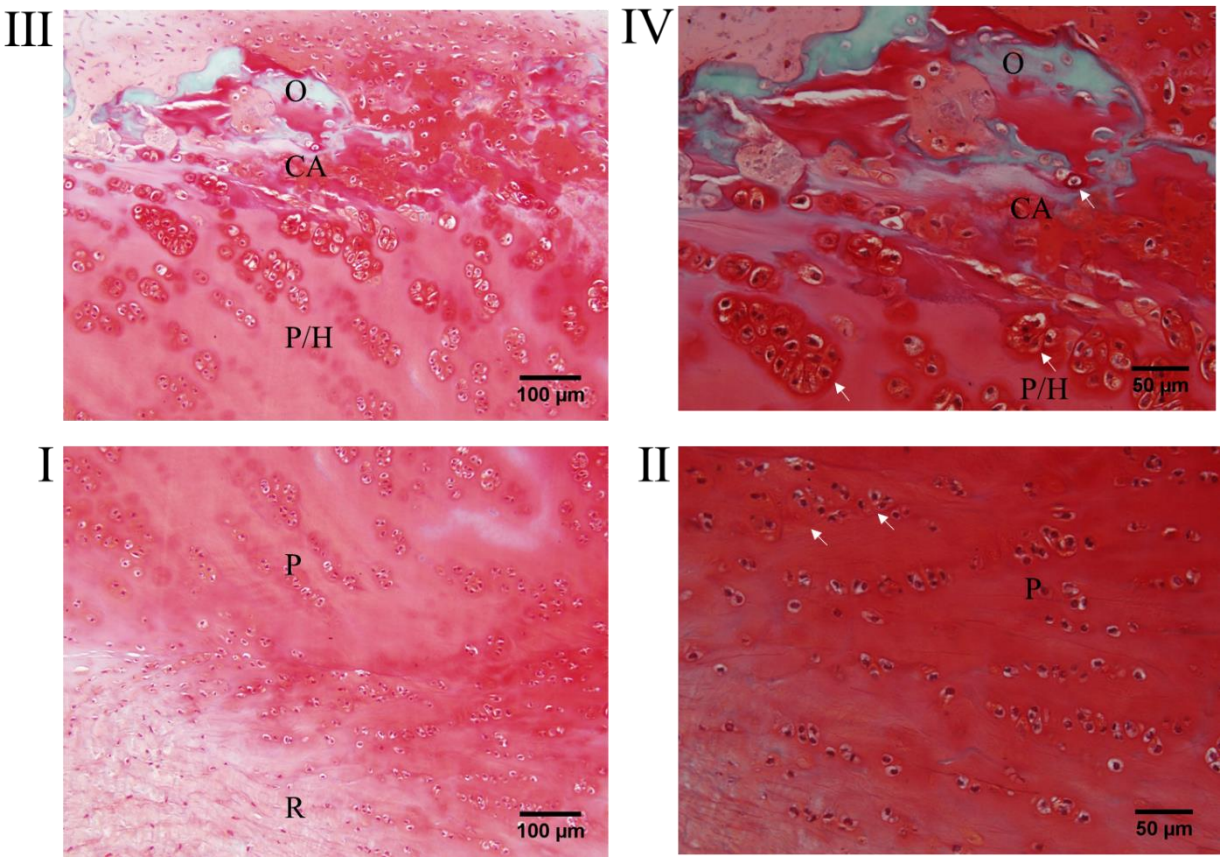

Figure 4-23: Zonal features in cartilage tissue induced by 6 weeks of TGFβ1 followed by osteogenic culture in OM+BMP. I,III) Progression of endochondral ossification occurred to osseous (O) tissue production. II) Arrows indicate proliferative cells. IV) Arrows indicate cells with hypertrophic phenotype.

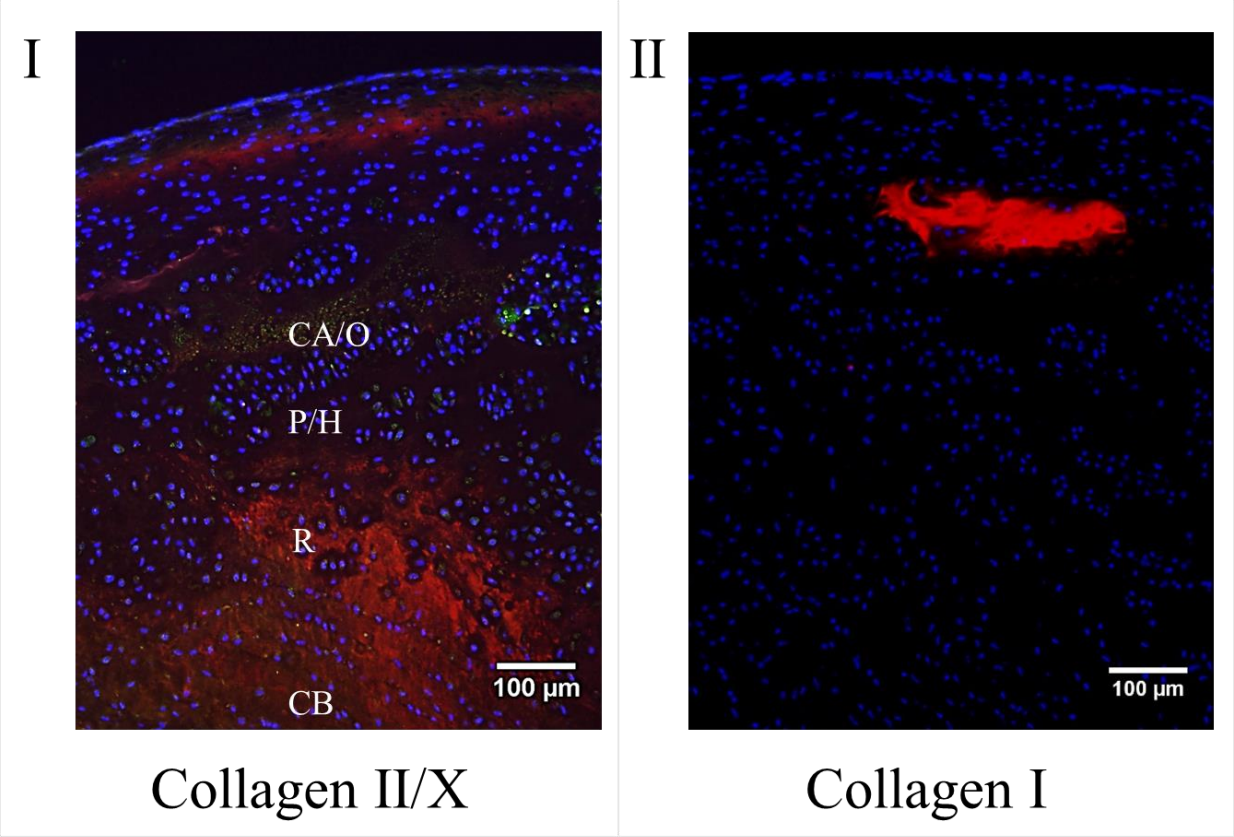

Figure 4-24: Immunohistochemical localization of collagen II/collagen X and collagen I in cartilaginous tissue induced after 6 weeks of TGFβ1 followed by osteogenic culture in OM+BMP.

Subsequent to two weeks of culture in osteogenic media and T3 (8W TGF OM+T3), TGFβ1 treated explants also showed evidence of osseous tissue formation (not in sample shown) along the typical endochondral route (Figure 4-2). 'Reserve', 'proliferative', and 'hypertrophic' zones were all identifiable within the neotissue (Figure 4-25). The 'hypertrophic' zone was positive for extracellular collagen X (Figure 4-26 I) although many of the other typical hallmarks of hypertrophy were lacking such as cellular enlargement (Figure 4-25 IV).

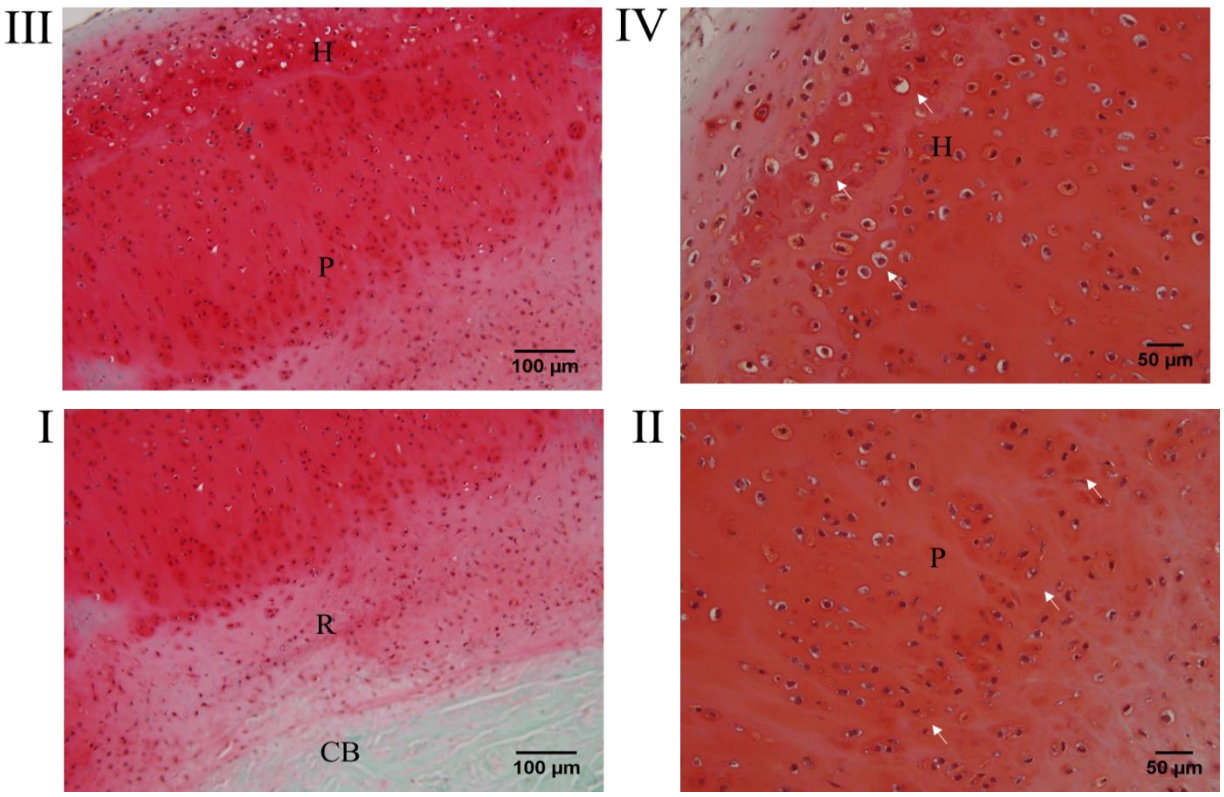

Figure 4-25: Zonal features in cartilage tissue induced by 6 weeks of TGFβ1 followed by osteogenic culture in OM+T3. I, III) The endochondral process continued to the hypertrophic stage of differentiation. II) Arrows indicate what appear to be proliferative columns of cells. IV) Arrows indicate cells with hypertrophic phenotype.

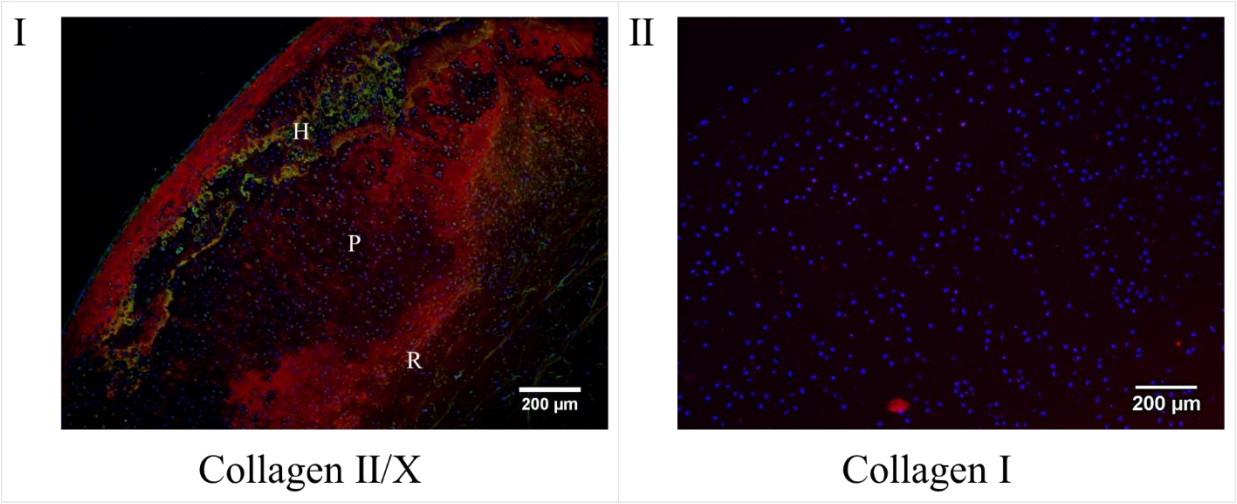

Figure 4-26: Immunohistochemical localization of collagen II/collagen X and collagen I in cartilaginous tissue induced after 6 weeks of TGFβ1 followed by osteogenic culture in OM+T3

4.2.3 BMP2 Treated Chondrogenic Periosteal Explants in Osteogenic Media Types

Culture in various osteogenic media types did not improve the overall tendency of samples to undergo mineralization as compared to culture in chondrogenic media alone (BMP2 induction; Figure 4-16). Samples treated with osteogenic media (8W BMP OM) were the only group that appeared to show an improved frequency of mineralization (f_M =0.75) as compared to samples only chondrogenically differentiated (3W BMP f_M=0.60, 6W BMP f_M=0.67; Figure 4-19). Along with the increased frequency of mineralized tissue in 8W BMP OM samples (from 6W BMP) there was a concomitant increase in the area of mineralized tissue (Figure 4-20). Despite this, 3W BMP samples appeared to be the best suited to undergo endochondral ossification. The osteogenic phase of induction appeared generally to slow or impede the progression of endochondral ossification. Among the different osteogenic media types, osteogenic media alone (OM) led to hypertrophic and osseous tissue the most often (Figure 4-19). Addition of BMP to the osteogenic media (OM+BMP) seemed to greatly inhibit tissue maturation, with samples displaying a high incidence of the resting cartilaginous tissue phenotype and low (or no) indications of others. Addition of T3 to osteogenic media (OM+T3) also appeared to similarly hinder the progression of tissue maturation, albeit to a lesser degree than BMP2, as many samples exhibited 'resting' and 'proliferative' phenotypes while fewer were found to have 'hypertrophic', 'calcified', or 'osseous' tissue.

Samples chondrogenically induced with BMP2 followed by culture in osteogenic media alone (8W BMP OM) appeared quite similar to the 6W BMP samples (Figure 4-2). A 'reserve' zone gave way to a region of tissue reminiscent of the aforementioned 'proliferative' zone, but with several dissimilarities (Figure 4-27). This region was GAG positive and collagen II negative, with cells containing intracellular collagen X (Figure 4-28). Cells of the region were dispersed randomly throughout their matrix, with no indication of directional alignment, instead forming clusters of >2 (Figure 4-27 II-III). Despite this lack of cellular organization, cells appeared to progress in phenotype moving towards the periphery of the structure. The GAG positive matrix is replaced by a collagenous matrix (collagen II; Figure 4-28), with cells resident to the clusters appearing in some areas to have become flattened and stacked (Figure 4-27 III). Collagen X appeared extracellularly in specific locations within this outer region (Figure 4-28). The matrix was negative for collagen I (Figure A-3).

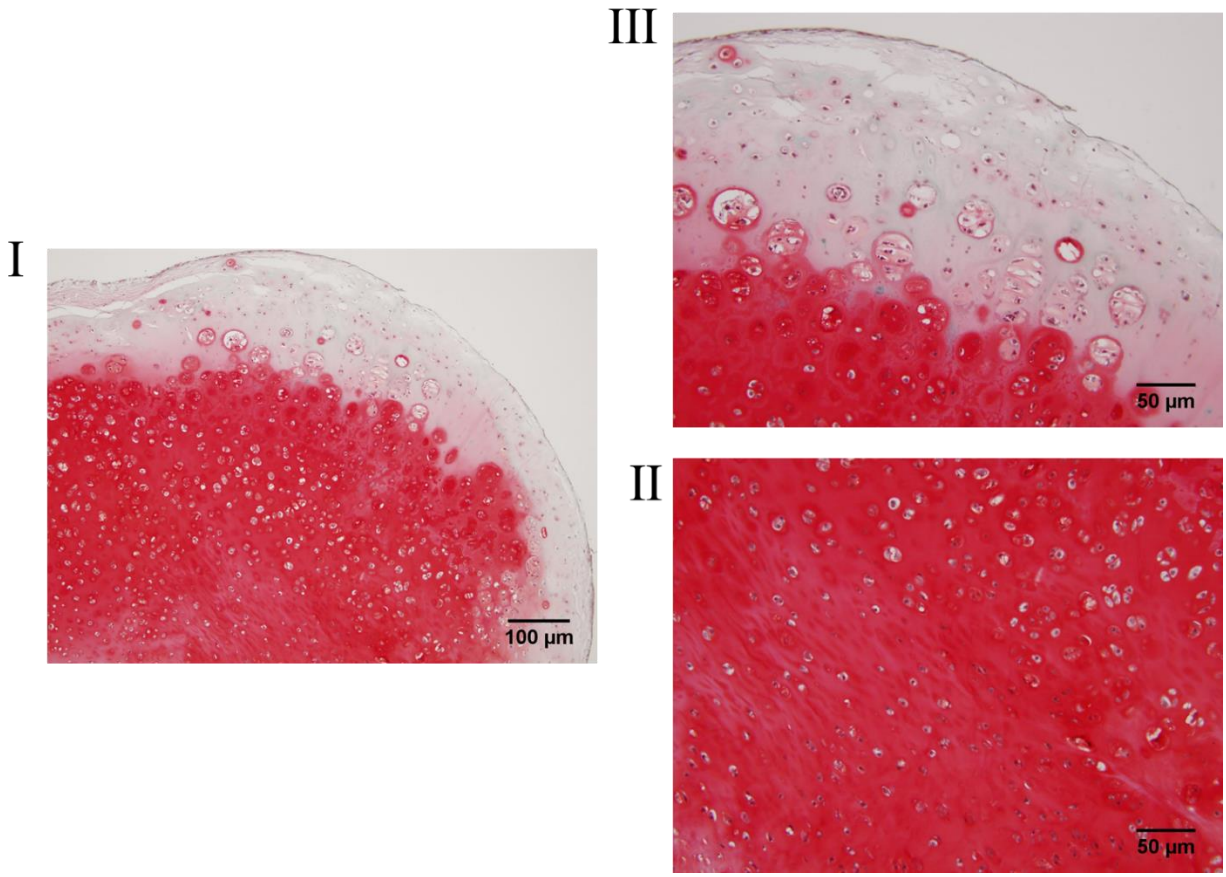

Figure 4-27: Zonal features in cartilage tissue induced by 6 weeks of BMP2 followed by osteogenic culture in OM

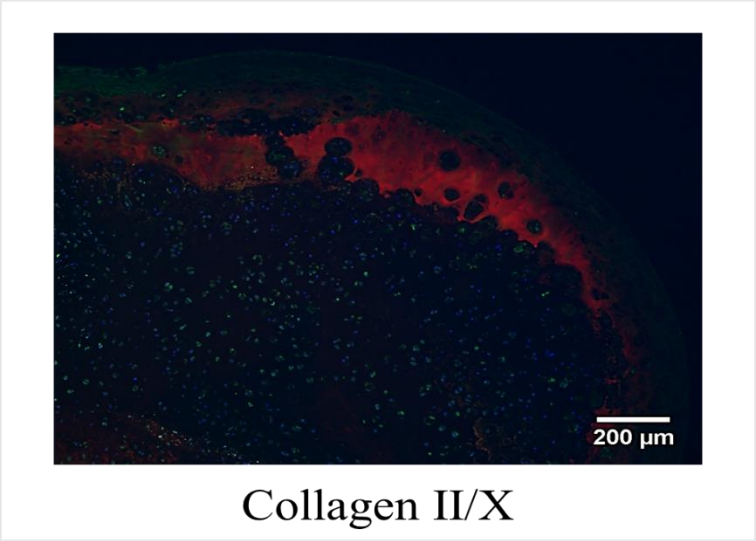

Figure 4-28: Immunohistochemical localization of collagen II/collagen X in cartilaginous tissue induced after 6 weeks of BMP2 followed by osteogenic culture in OM

After a two week induction in osteogenic media with BMP2 (8W BMP OM+BMP), BMP2 treated chondrogenic periosteal samples did not appear to ossify (Figure 4-2). These samples had a similar structure to that seen under osteogenic (OM) treatment only. Samples featured a 'reserve' zone which fed into a region of chondrocyte clusters containing intracellular collagen X, the surrounding matrix of which was GAG positive and collagen II negative (Figure 4-29, Figure 4-30). Extracellular collagen X was visible in the interterritorial matrix around the chondrons beginning at the tissue interior (Figure 4-30). Collagen X expression overlapped with that of collagen II moving towards the tissue extremity but eventually tapered, while collagen II expression remained intense at this outermost region (Figure 4-30). One small area showed indications of progression towards osseous tissue with the formation of cell negative GAG tissue struts (Figure 4-29 III). The matrix was negative for collagen I (Figure A-4).

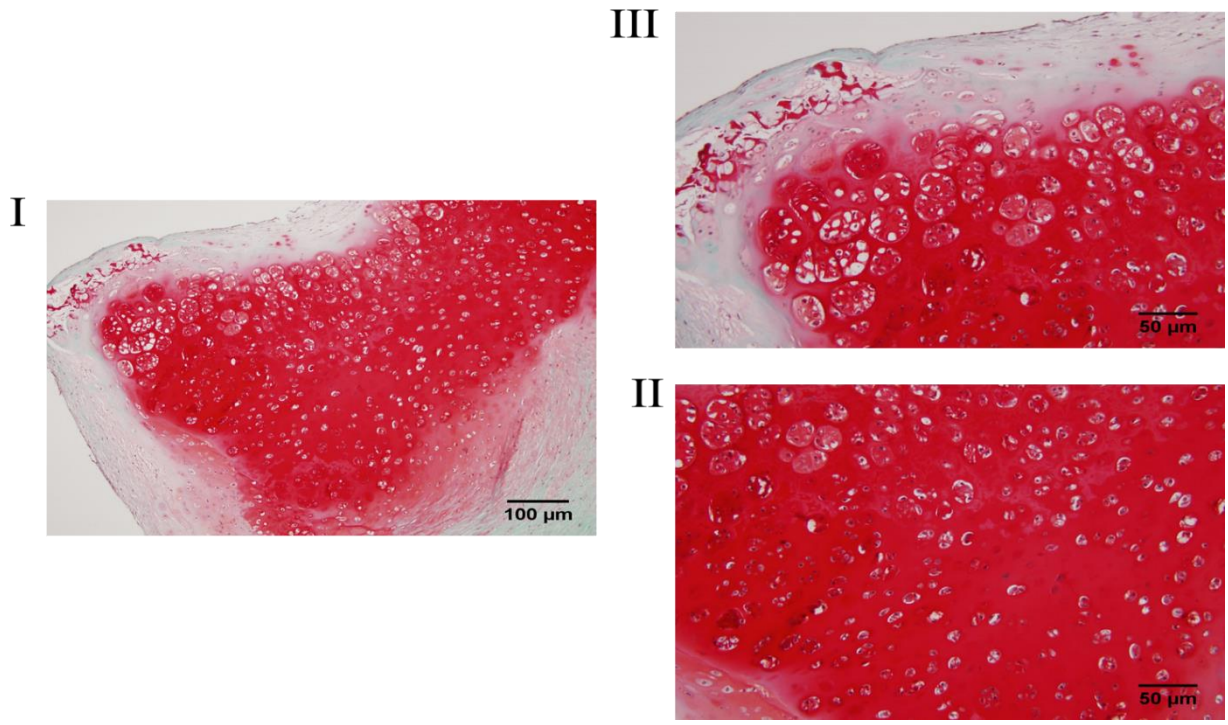

Figure 4-29: Zonal features in cartilage tissue induced by 6 weeks of BMP2 followed by osteogenic culture in OM+BMP

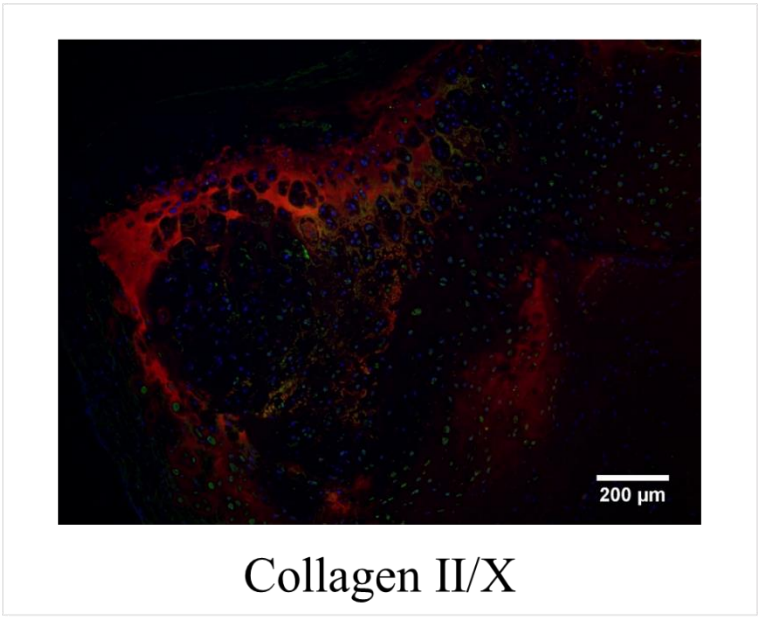

Figure 4-30: Immunohistochemical localization of collagen II/collagen X in cartilaginous tissue induced after 6 weeks of BMP2 followed by osteogenic culture in OM+BMP

BMP2 treated chondrogenic periosteum explants cultured in osteogenic media with T3 (8W BMP OM+T3) also did not appear to undergo ossification (Figure 4-2). The cartilaginous tissue produced was distinctly unorganized. There was little to no 'reserve' zone, with the bulk of the tissue comprised of cell clusters and small 'proliferative' chondron units (Figure 4-31). Cells of these 'proliferative' chondrons were stacked and flattened, while clustering cells were generally rounded and grouped within their territorial matrices (Figure 4-31 II). 'Proliferative' chondrons were found to orient in differing directions, both parallel and perpendicular to the periosteum surface. The matrix of this region was GAG positive and contained a large region of collagen II surrounding 'proliferative' chondrons closer to the base of the neotissue (Figure 4-32). Most cells of the neotissue contained intracellular collagen X. Moving towards the periphery, the collagen II was lost and collagen X was expressed extracellularly. At the furthest extremity of the tissue, cells were small, rounded and dispersed in a GAG negative, collagen II positive matrix (Figure 4-31 III, Figure 4-32). Collagen I was not observed within the developed matrix (Figure A-5).

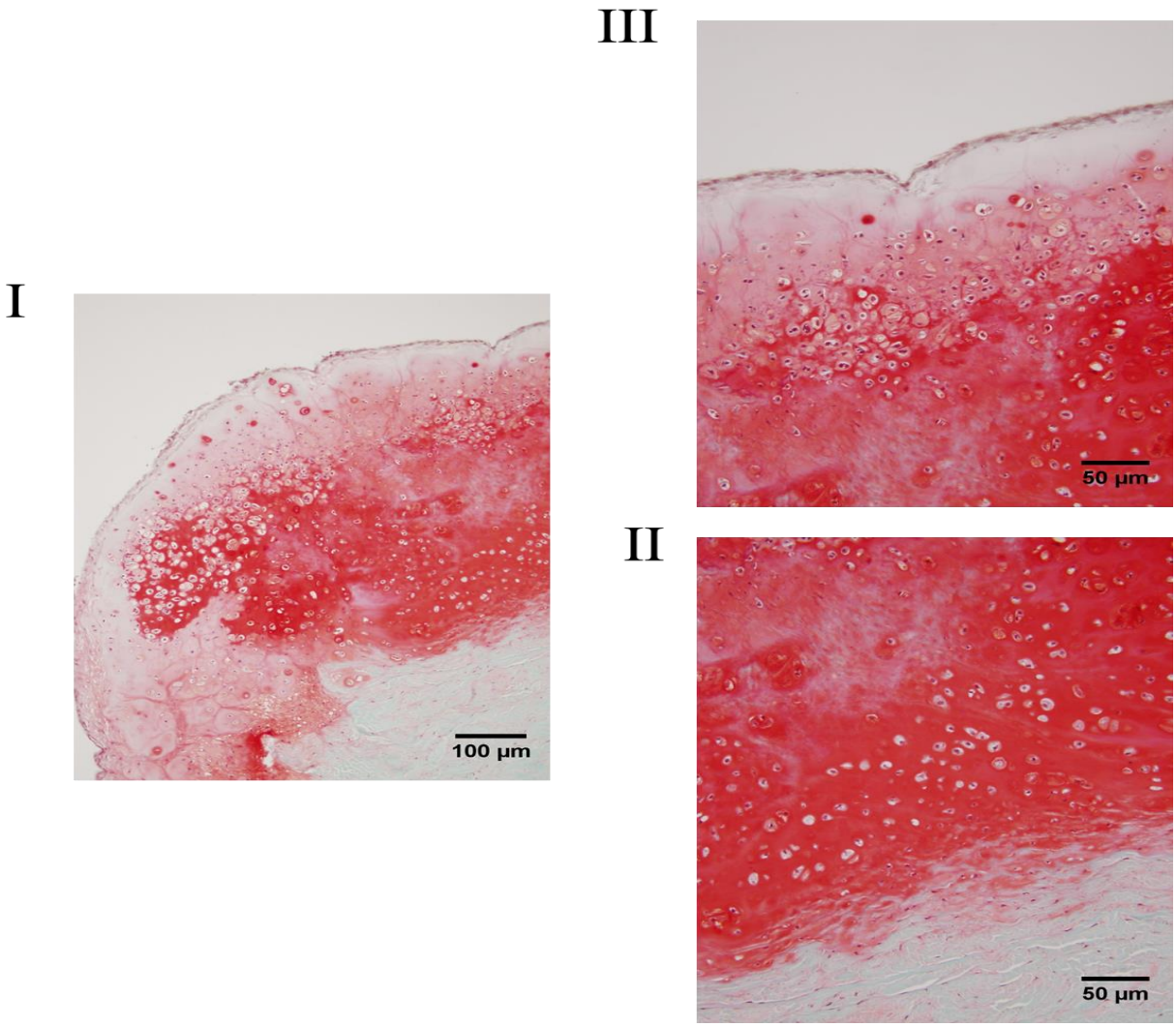

Figure 4-31: Zonal features in cartilage tissue induced by 6 weeks of BMP2 followed by osteogenic culture in OM+T3

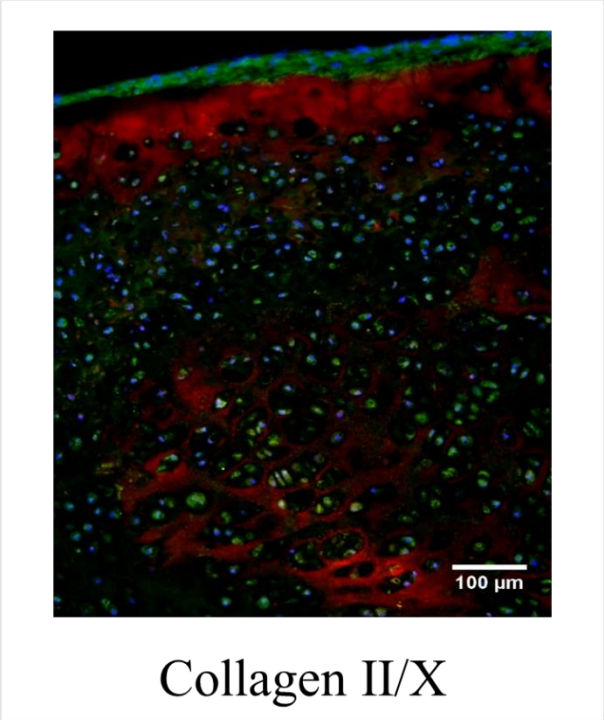

Figure 4-32: Immunohistochemical localization of collagen II/Collagen X in cartilaginous tissue induced after 6 weeks of BMP2 followed by osteogenic culture in OM+T3

4.2.4 Switch Treated Chondrogenic Periosteal Explants in Osteogenic Media Types

Osteogenic induction of Switch treated chondrogenic samples appeared to result in a general increase in the frequency of mineralization (Figure 4-16). Despite an average increase in the number of samples which were mineralized in osteogenic media following 6W Switch treatment, 8W Switch OM+BMP was the only group to improve the frequency of mineralization (f_M=0.80) from the chondrogenic stage of culture (6W Switch) (Figure 4-19). The higher frequency of mineralization was accompanied by an increase in the area of mineralized tissue which developed (Figure 4-18). Switch samples cultured in OM+BMP appeared to undergo a progression of maturation from those induced for six weeks in chondrogenic media (6W Switch), displaying not only higher incidences of mineralized tissue phenotypes (‘calcified’ and ‘osseous’), but ‘hypertrophy’ as well (Figure 4-19). Comparing the effects of osteogenic media types, OM+T3 appeared to similarly shunt samples along the path of endochondral ossification. Samples had high frequencies of ‘resting’, ‘proliferative’, ‘hypertrophic’ and ‘calcified’ cartilage phenotypes, however they did not undergo ossification (Figure 4-19). Despite the apparent maturation of cartilaginous tissue, the frequency of mineralization for 8W Switch OM+T3 was equal to that of the 6W switch condition with a low amount of mineralized tissue (Figure 4-19, Figure 4-20). Osteogenic media alone (8W switch OM), while showing similar frequencies of ‘resting’,

‘proliferative’, and ‘osseous’ tissue phenotypes, had lower frequency of ‘hypertrophic’ tissue and no ‘calcified’ tissue present (Figure 4-19). The area of produced mineralized tissue was also the lowest of all groups induced under the switch treatment (Figure 4-18).

Samples chondrogenically differentiated under the Switch treatment did undergo some bone tissue formation after two weeks culture in osteogenic media (OM; Figure 4-2). Despite the apparent ossification, progressive maturation of chondrogenic phenotypes of endochondral bone formation was not present. Osseous tissue was present at the tissue extremity, staining slightly for collagen I and with morphological characteristics similar to that of bone trabeculae (Figure 4-34 II). Cells were encased within branching collagenous structures which overlaid the remnants of a GAG positive matrix (Figure 4-33). Underlying the osseous tissue were cell clusters residing in a GAG and collagen X positive matrix (Figure 4-33 III-IV, Figure 4-34 I). The cartilaginous tissue bulk (GAG positive) was comprised of these cell clusters or single cell entities occupying their own lacuna (Figure 4-33 I-II). This matrix stained positive for collagen II in areas coincident with low cellularity (single cells per lacuna), such as at the base of the neotissue (cambium) and in some peripheral regions (Figure 4-34 I). Collagen X stained extracellularly throughout a majority of the matrix and was co-expressed with collagen II in some areas (Figure 4-34 I). Non-uniformity along the periosteum surface potentially indicated the periosteal tissue was imperfectly excised, with the cambium only partially lifted.

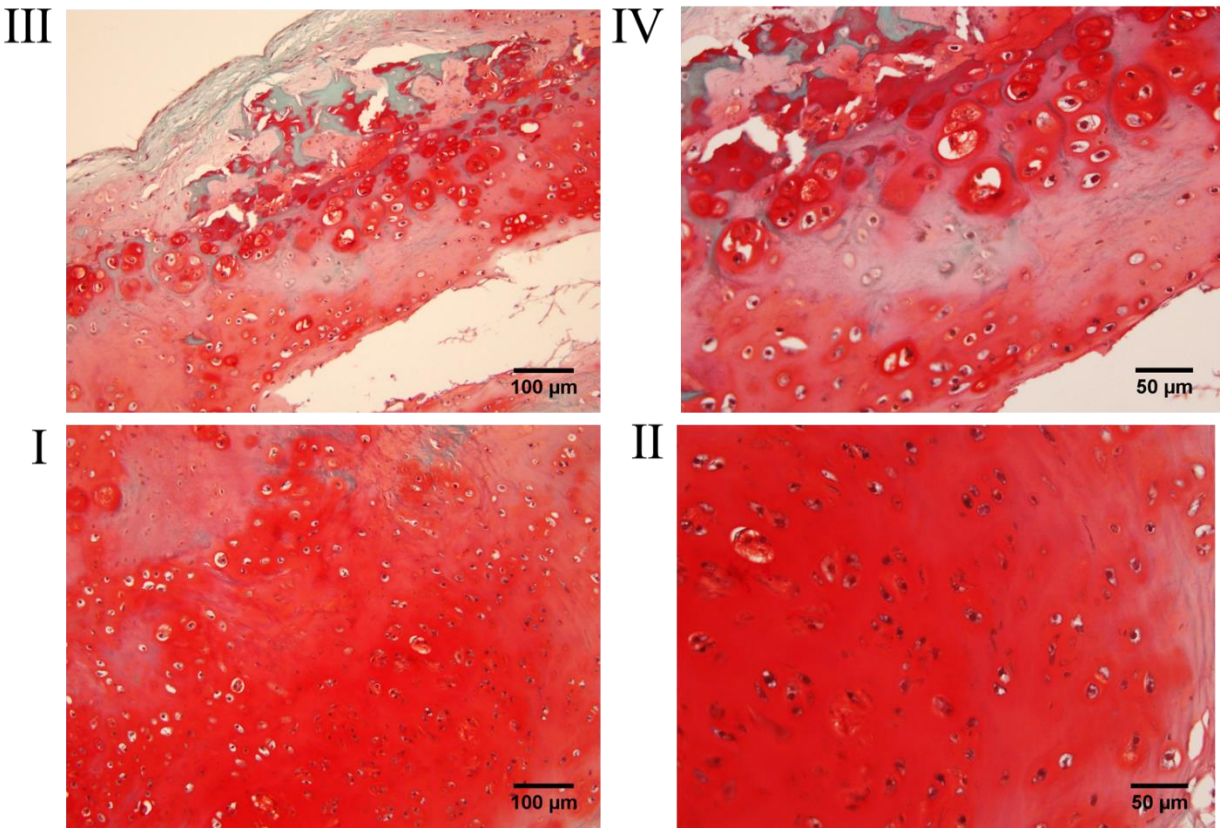

Figure 4-33: Zonal features in cartilage tissue induced by 6 weeks Switch treatment followed by osteogenic culture in OM

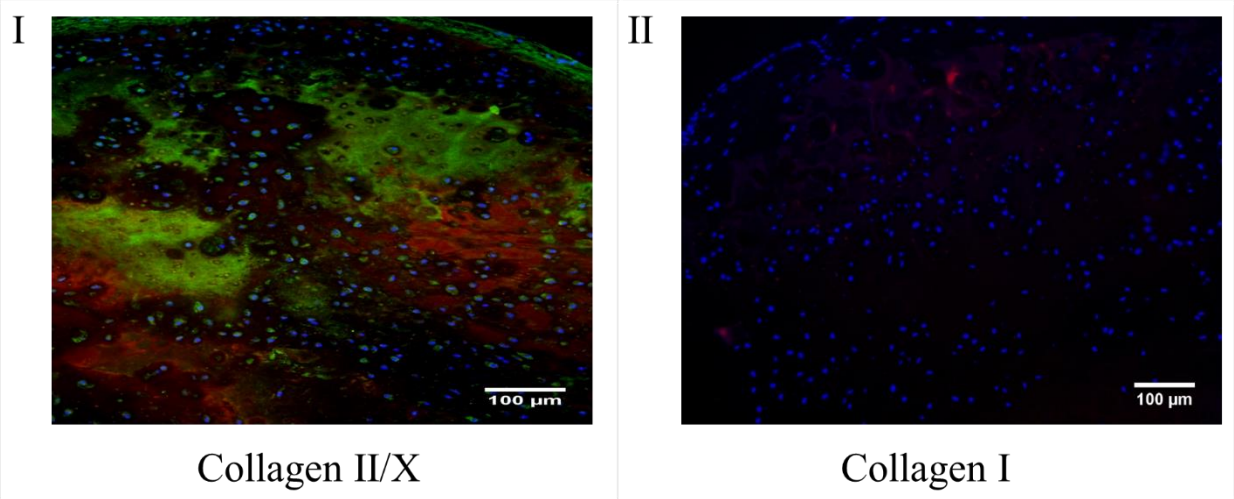

Figure 4-34: Immunohistochemical localization of collagen II/collagen X and collagen I in cartilaginous tissue induced after 6 weeks Switch treatment followed by osteogenic culture in OM

Switch treated chondrogenic periosteal explants cultured in osteogenic media containing BMP2 (8W switch OM+BMP) showed evidence of ossification (Figure 4-2). Under this treatment, 'reserve', 'proliferative', 'hypertrophic', and 'osseous' zones were observed, similar to the 3 week BMP group (Figure 4-35). Formation of osseous tissue in the 3W BMP group was mostly uniform along the length of the periosteal surface; however, this was not the case for the 8W Switch OM+BMP group. Bone-like tissue (Figure 4-35 III-IV, Figure 4-36 II) was observed in two specific areas; beneath which was the appearance of a fully re-established growth plate structure. While these areas contained features of the growth plate such as a 'reserve zone' (cambium adjacent) and an early 'hypertrophic' zone (outermost region), the central tissue bulk was void of both collagen II and collagen X, and generally unordered at a cellular level (Figure 4-36 I-II).

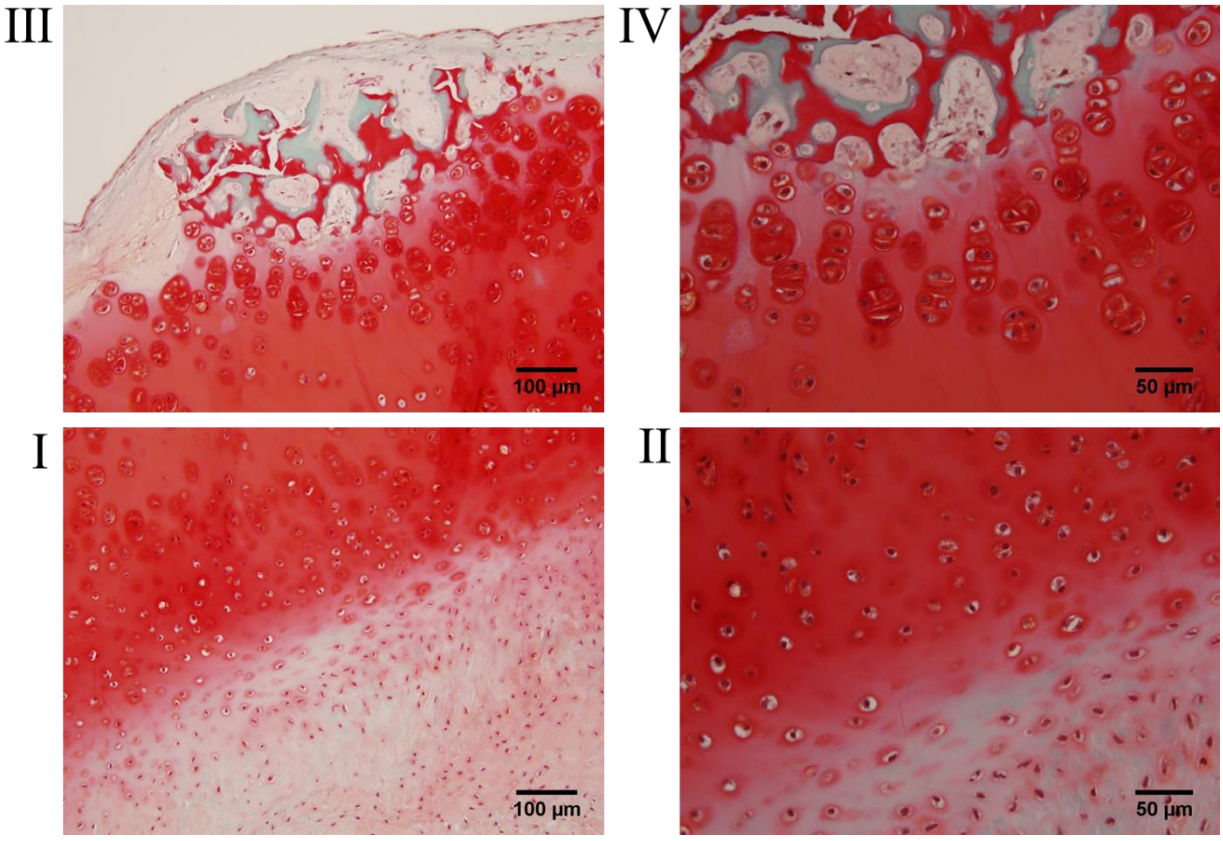

Figure 4-35: Zonal features in cartilage tissue induced by 6 weeks of Switch followed by osteogenic culture in OM+BMP

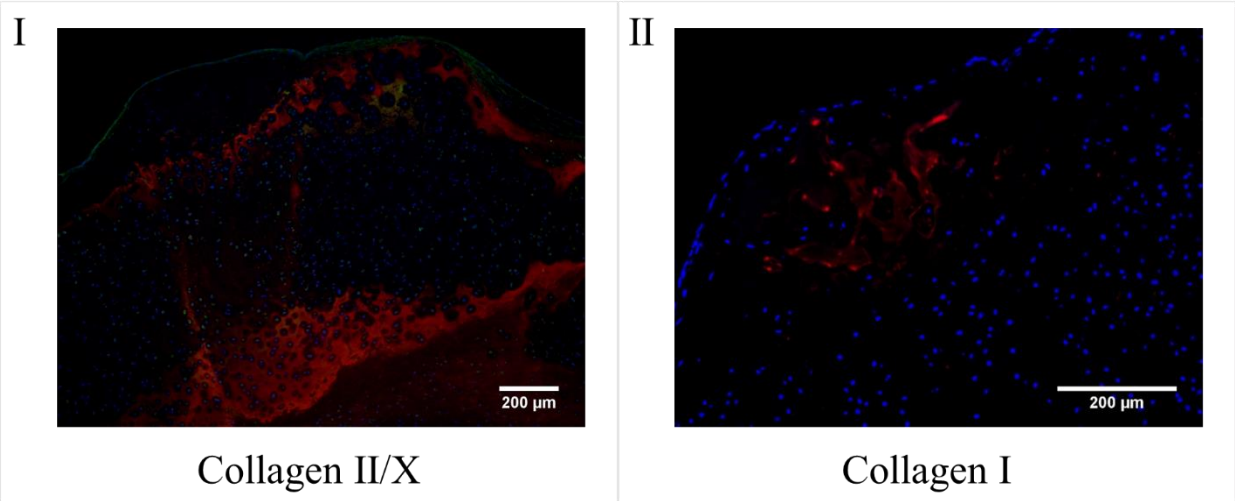

Figure 4-36: Immunohistochemical localization of collagen II/collagen X and collagen I in cartilaginous tissue induced after 6 weeks Switch treatment followed by osteogenic culture in OM+BMP

Subsequent to two weeks of culture in osteogenic media with T3 (8W Switch OM+T3), Switch treated explants did not show evidence of osseous tissue formation (Figure 4-2). The cartilaginous tissue had a 'reserve' zone that gave way to a region of chondrocyte clusters, which stained positive for intracellular collagen X, and occupied a GAG positive and collagen II negative matrix (Figure 4-37, Figure 4-38). At the outermost region, some organization was observed, with these cell clusters appearing to show a small degree of alignment, with putative columnar formation and orientation perpendicular to the periosteum surface (Figure 4-37 II). Collagen X appeared extracellularly in the interterritorial matrix surrounding these oriented cell clusters (Figure 4-38). Overlying these stack-like cell formations was a GAG positive matrix that was calcifying. This area featured formation of matrix struts which appeared to be undergoing remodelling with collagenous tissue lining the edges of the GAG remnants (Figure 4-37 II). Collagen I was not observed within the tissue structure (Figure A-6).

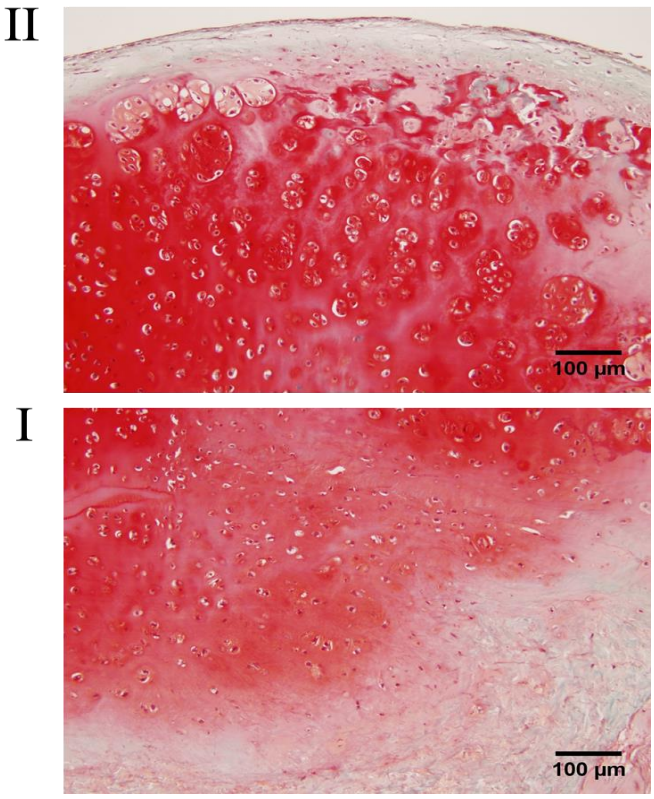

Figure 4-37: Zonal features in cartilage tissue induced by 6 weeks Switch treatment followed by osteogenic culture in OM+T3

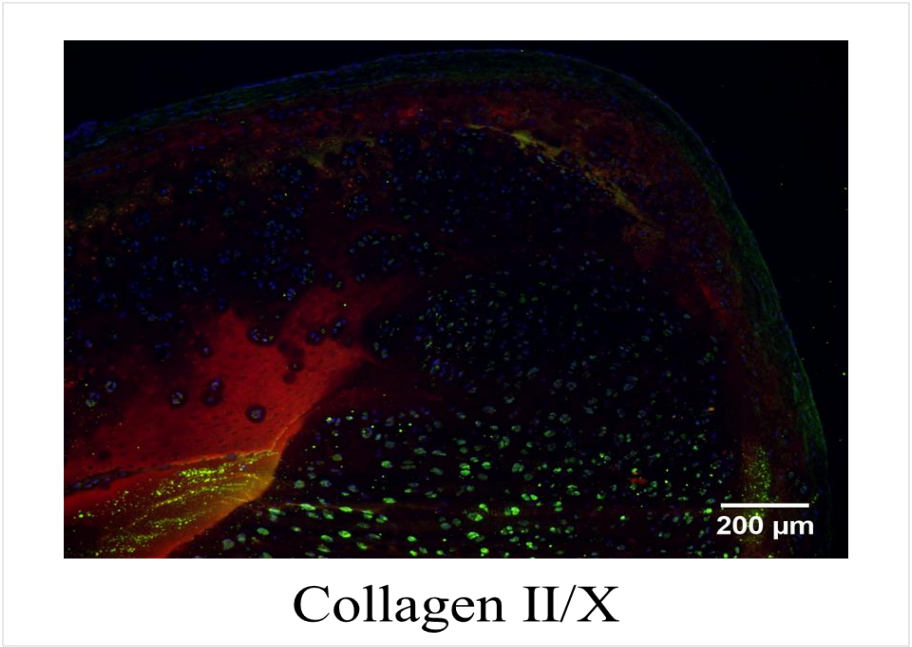

Figure 4-38: Immunohistochemical localization of collagen II/ collagen X in cartilaginous tissue induced after 6 weeks Switch treatment followed by osteogenic culture in OM

Chapter 5 Discussion

Periosteal explants were utilized as a substratum for tissue engineering of endochondral bone constructs *in vitro*. Successive application of chondrogenic and then osteogenic media to the explants was used to emulate the endochondral route of ossification wherein a cartilage template undergoes mineralization to form bone tissue.

5.1 Differential Actions of TGFβ1 and BMP2 During Explant Chondrogenesis

During the chondrogenic phase of induction, both TGFβ1 and BMP2 supplemented chondrogenic media were capable of inducing chondrogenesis in explants. Averages of safranin O positive samples indicate little to no difference in the ability of either growth factor to elicit a chondrogenic response; the growth factor affected the phenotype of the cartilaginous tissue produced rather than the ability to chondrogenically differentiate. Disparities in the *in vitro* action of TGFβ1 and BMP2 have been well established and are indicative of their divergent involvement in the processes of cartilage and bone formation *in vivo* [195], [196]. While both growth factors are considered chondrogenic, TGFβ1 has been further shown to have chondroprotective properties whereas BMP2 has been reputed to be hypertrophic and osteoinductive [197].

Three weeks of culture was sufficient to produce chondrogenic tissue in explants and longer induction times (six weeks) did not increase likelihood of samples undergoing chondrogenesis. Instead, increasing the time of growth (including during the osteogenic phase) led to changes in the phenotypes of cartilaginous tissue and, in some instances, the amount of tissue which developed. The changes in cartilage which developed over time under the action of either growth factor indicate their activities were cell lineage specific.

5.1.1 TGFβ1 Induced Immature Cartilage and Inhibits Endochondral Ossification

Under the action of TGFβ1, periosteal explants produced immature cartilage, comprised mainly of resting and proliferative tissue. Extension of the chondrogenic culture period served to mature the tissue to a degree; hypertrophic, calcified, and osseous phenotypes were seen more frequently in samples at six weeks, however these tissue types comprised only a small fraction of the cartilage bulk, which is not duly represented when considering the frequency plots alone. Histological assessment of samples showed that the cartilage was structurally organized, with phenotypically distinct regions spanning the full tissue depth. A growth-plate-like zonal arrangement was observed with one phenotype giving way to the next most mature (resting to osseous), beginning at the cambium and ending at the extremity of the

neotissue. By six weeks of TGFβ1 induction, recapitulation of this structure typically occurred up to the proliferative or early hypertrophic stage.

The chondrogenic and chondroprotective effects TGFβ1 exerted in the differentiation of periosteal explants correspond with the activity of this growth factor *in vivo* and were further corroborated by *in vitro* studies. During limb morphogenesis, transient expression of TGFβ1 within mesenchyme condensations stimulates the key chondrogenic transcription factor *Sox9*, whose action not only induces chondrocyte differentiation and proliferation, but also inhibits cellular hypertrophy [112], [198], [199]. The protocol for the *in vitro* chondrogenic differentiation of MSCs was originally developed based on this process, with high density cultures (pellet, aggregate) attempting to replicate the cellular environment during limb bud formation and addition of exogenous TGFβ1 further promoting cartilaginous lineage commitment [93]. Indeed, TGFβ1 has since seen ubiquitous use as a chondrogenic factor for *in vitro* differentiation of a variety of different cell types and culture systems including periosteal explants [63], [200]. Under TGFβ1, cartilaginous differentiation occurred as early as one week in culture with definitive expression of chondrogenic markers (aggrecan, Collagen II) typically by three weeks [60], [93], [95].

Downstream of initial chondrogenesis, TGFβ1 plays further roles as a regulator endochondral ossification. Conditional deletion of TGFβII receptors resulted in a shortening of appendicular bones in both embryonic and adult mice which was attributed to accelerated hypertrophic differentiation, and delayed terminal hypertrophy during long bone development and growth [201], [202]. This implicates that TGFβ1 acts as anti-hypertrophic agent. These chondroprotective actions are similarly supported by its involvement in the repair and maintenance of the articular cartilage phenotype [203]. Disruptions in TGFβ1 signalling at diarthrodial joints has been associated with onset of osteoarthritis as characterized by increased hypertrophy and encroachment of underlying subchondral bone [204]–[206]. Addition of TGFβ1 to cultures of articular chondrocytes *in vitro* improved both collagen II and proteoglycan production again demonstrating its function in upregulating synthesis of chondrogenic matrix components [207].

It is proposed here that zonal strata within the cartilaginous neotissue were formed as a result of downstream signalling induced by exogenous application of TGFβ1 in periosteal cultures; TGFβ1 was responsible for initial chondrogenesis but its constitutive action impeded terminal hypertrophy and the endochondral process through potentially a PTHrP-dependent mechanism. It is believed that ‘resting’ tissue was initially induced as the MSC population of the cambium underwent chondrogenesis. Based on the process of tissue turnover at the growth plate, a ‘proliferative’ zone was developed over time as a pool of maturing cells exited their quiescent, resting state to continue along the route of endochondral ossification. In accordance with this analogy, subsequent zones (hypertrophic, calcified, osseous phenotype) were re-established as a fraction of each cellular phenotype moved to the next phase of

maturity. The most immature cells were therefore to be found at the base of the neotissue whilst the most mature cells were at the outermost edge of the neotissue. In this model, the 'resting' zone was maintained by stem-like progenitors within the region and supported by MSCs of the underlying cambial layer. The overall high preservation of the resting zone in all TGFβ1 groups may support this theory.

It is thought that either periosteal cells or cells from the 'resting' zone produced PTHrP under the action of TGFβ1, resulting in the formation of a self-regulating tissue and preventing overall tissue maturation. Relevant to the observed results, the perichondrium has been shown to modulate the effects of TGFβ1 during endochondral ossification of the developing skeleton; removal of perichondrial tissue from mouse embryonic metatarsal bone rudiments abrogates the antihypertrophic action of TGFβ1 [204], [208], [209]. In an organ culture study conducted by Serra et al., PTHrP expression was stimulated in the perichondrium of mouse embryonic metatarsal bones by TGFβ1, resulting in suppression of hypertrophy [210]. Hypertrophy was not inhibited in PTHrP-null mice, indicating the effects of TGFβ1 are mediated in a PTHrP-dependent manner. The developmental and functional relationship between perichondrium and periosteum supports the idea that periosteum may act in a similar fashion. Perichondrium initially delimits the cartilage anlage during limb morphogenesis, becoming periosteum upon ossification of the underlying tissue [51]. In this thesis, we considered the tissue used to be the periosteum; however it may more correctly be termed the perichondrium due to its localization over the growth plate. Regardless, the periosteum has shown similar involvement as the perichondrium in regulation of the endochondral process and thus may be involved in this process in this culture system [211]. MSCs undergoing TGFβ1 induced chondrogenesis displayed a transient cartilage phenotype *in vitro* therefore the periosteum was thought to be crucial in developing this zonal structure and deterring endochondral ossification, whether as a producer of PTHrP or as a continual source of MSCs for the 'resting' zone [212].

Postnatally, PTHrP expression shifts from the perichondrium to the resting zone within the growth plate [130], [213]. Localization of TGFβRI/II indicates cells of the resting and proliferative zone are receptive to TGFβ stimulus [214], [215]. Indeed, PTHrP production was induced in upper sternal chondrocytes of chicks under exogenous addition of TGFβ isoforms *in vitro* [216]. A study conducted by Abad et al. supports the notion that resting zone chondrocytes may be the cell population responsible for the secretion of anti-hypertrophic factor, as realignment of the resting zone within the growth plate led to alteration of direction of ensuing proliferative columns and delayed the onset of hypertrophy [217].

Considering this model, we assumed that the full endochondral process may eventually occur over time. Exhaustion of the MSC population within the periosteum and progenitors of the 'resting' zone would potentially cause senescent changes in the tissue similar to growth arrest and epiphyseal fusion wherein the cartilaginous tissue is fully replaced by bone [120]. Alternatively, withdrawal of TGFβ1 chondrogenic media may have served to accelerate this process; an initial period of chondrogenesis under

TGFβ1 may only be required to initiate endochondral ossification in periosteal cultures. This scenario not only occurs during limb morphogenesis but also during osteophyte formation [218]. TGFβs participate in the early development of osteophytes, subsequently dwindling in effect to be overtaken by BMP2 action during later stages of maturity [219], [220]. The 'switch' condition attempted to emulate a similar process and did appear to result in improvement in endochondral phenotype.

5.1.2 BMP2 Induces a Transient Cartilage and Promotes Endochondral Ossification

Chondrogenic differentiation with BMP2 induced the formation of mature cartilage and mineralized tissue in periosteal explants. At three weeks, some samples had undergone the entirety of the endochondral process and formed osseous tissue. The structural organization of the cartilage seen under TGFβ1 action was again re-established up to later stages of maturity (hypertrophy, calcification, or ossification). Lengthening of the culture period to six weeks increased the frequency of hypertrophy but decreased the incidences of ossification in the samples. The hypertrophic region accounted for a vast majority of the tissue bulk and thus a rise in the developed tissue area was observed from three to six weeks. Disorganization within the proliferative and hypertrophic regions led to an overall lack of structural tidiness. Cells were not arranged in stacked, unidirectional alignments as previously seen but instead were clustered randomly within their matrices. The zonal configuration of tissue phenotypes was still roughly maintained; however with the least mature tissue cambium adjacent and the most mature tissue localized to the outer periphery.

In this periosteal culture system, BMP2 is believed to act as a hypertrophic and osteoinductive agent which agrees with the literature in respect to its *in vivo* and *in vitro* activities. BMP2 was initially identified as a bone regenerative agent; ectopic injection promoted bone formation through both intramembranous and endochondral mechanisms [221]–[223]. This alludes to its involvement in directing cellular fate as both chondrogenic and osteogenic lineages may be induced through its activity [53]. However, while BMP2 is capable of initiating chondrogenesis, in contrast to TGFβ1, it appears to conduct the transient progression of chondrocytes through endochondral ossification rather than maintain cartilage homeostasis. In the early limb bud, BMP2 signalling is required downstream of TGFβ1 to continue the process of chondrocyte maturation through regulation of the hypertrophic transcription factor *runx2* [218], [224]. Localization at the growth plate reveals a BMP action gradient, demonstrating its interceding role in chondrocyte maturation with expressional activity ramping from the resting to hypertrophic zone [225]. Indeed, exogenous addition of BMP2 to fetal metatarsal bone organ cultures caused rapid longitudinal growth which was attributed to enhanced proliferation and hypertrophy [226]. BMP2 mediated MSC chondrogenesis is characterized by accelerated hypertrophic onset *in vitro* with earlier and elevated expression of collagen X and ALP as compared to alternative chondrogenic agents

[227], [228]. Osteogenic differentiation of MSCs can also be stimulated *in vitro* using BMP2. This method simulates intramembranous ossification, where a monolayer culture is employed in conjunction with application of media containing BMP2 [89], [90]. *In vivo*, commitment to an osteogenic versus chondrogenic lineage under BMP2 action is a poorly understood phenomenon. Three main transcription factors believed to regulate skeletal tissue fate are *Sox9, Runx2,* and *osterix* all of which are activated by BMP2 [122], [229]. The balance between the expression of each factor is thought to direct lineage commitment. Prevalence of *Sox9* promotes chondrogenesis with concomitance of *Runx2* encouraging hypertrophy, while *Runx2* together with *osterix* stimulates osteogenesis [69], [71]. Until recently, osteogenic differentiation was believed to be a separate and distinct event from chondrogenic differentiation; however, transdifferentiation of hypertrophic chondrocytes to osteoblasts has been substantiated, indicating plasticity of cells beyond their initially believed commitment [116], [230], [231].

While a full recapitulation of endochondral ossification was seen at three weeks under the action of BMP2, this may be the result of locational differences between samples and consequently variability of the resident cellular population. BMP2 appears to exert its effects based on the cellular phenotype, with studies indicating that chondroprogenitors at a particular stage of maturity are more susceptible to its actions [144], [232]. It is possible that the periosteal cells within three week BMP2 samples may have been more advanced in terms of their commitment and thus were 'primed' to begin the endochondral process. In comparison, samples utilized for analysis at later time points may not have contained this initial cellular reservoir and therefore underwent a slower start to eventual maturation.

The pleiotropic action of BMP2 in regulating chondrogenic and osteogenic cellular differentiation may have been necessary in producing the full endochondral structure as observed in periosteal explants after three weeks of culture. It is here proposed that chondroprogenitors at the particular state in which BMP2 exerts its hypertrophic effects are also capable of osteogenic differentiation; BMP2 action on the chondroprogenitor gives rise to chondrogenic osteoprogenitors. Osteogenic differentiation of these progenitors may not occur through the same mechanism as typical intramembranous ossification but rather through the mechanism of transdifferentiation. A study conducted by Erenpreisa and Roach may support the idea of a chondrogenic osteoprogenitor, as apoptosis within a culture of hypertrophic zone chondrocytes negatively selected for cells which, upon subsequent asymmetric divisions developed into osteoblasts [233]. Chondrocyte-derived osteoblastic progenitor cells were localized *in vivo* to the lower hypertrophic zone; however, as the mechanism of commitment to the osteogenic rather than hypertrophic lineage is unknown, their true origin remains a mystery [234]. Furthermore, the periosteum has previously been shown to house cells with which can be routed down either differentiation pathway under the direction of BMP2 [53]. This notion would explain the presence of hypertrophic and osteogenic cells at three weeks under BMP2 and would support the idea that the lack of osseous tissue in six week BMP2

samples is due to the delay associated with first establishing responsive chondroprogenitors. Six week BMP2 samples may however, contain chondrogenic osteoprogenitors capable of producing osseous tissue if given the correct stimuli.

5.1.3 Switch Treatment Promotes the Endochondral Process Similarly to BMP2 Treatment

The switch treatment resulted in the generation of mature cartilaginous tissue which was predominantly proliferative and hypertrophic. The high rate of hypertrophy contrasts the effects observed under the action of TGFβ1 but was comparable to those treated with BMP2. In particular, switch treatment samples appeared very similar to those assessed at three weeks under BMP2. The rigid hierarchical endochondral structure, which was observed at three weeks under BMP2 but lost at six weeks, was preserved in switch samples and recapitulated up to a late stage (hypertrophy, calcification). Furthermore, switch and three week BMP2 samples produced approximately proportionate amounts of chondrogenic tissue. Despite the apparent likeness between both groups, switch treated samples lacked the extensive ossification observed at three weeks under BMP2. The decrease in the incidences of the resting phenotype along with the high frequency of proliferative and hypertrophic tissue types would indicate that the tissue is undergoing a shift from an immature to a mature state with exhaustion of the source MSCs or progenitors [204].

The switch condition did show improvements in terms of furthering the endochondral process however these samples were not quite as advanced as the samples at three weeks under BMP2 alone. The similarity between the two groups however may support the notion that a particular, mature chondroprogenitor responds more readily to a BMP2 stimulus [232]. Thus, a short chondrogenic pre-induction with TGFβ1 may be helpful in improving the endochondral process, given the variability in cell population between samples.

5.2 Lack of Ossification in Cartilaginous Explants During Osteogenic Induction

Osteogenic media did little to stimulate ossification in cartilaginous periosteal explants. Both the average frequency of mineralization and area of produced mineralized tissue were higher during the chondrogenic induction phase as compared to during the osteogenic induction phase. Although the propensity of samples to mineralize was generally unaffected, differences in tissue phenotypes were seen subsequent to the osteogenic phase of induction which were influenced by both the pre-culture method and the osteogenic media type. The chondrogenic phenotypes developed during chondrogenic pre-induction responded differently to each osteogenic media type. Separate constituents were believed to be responsible for propagating the effects of each osteogenic media type. It is thought that dexamethasone within osteogenic media alone (OM), BMP2 in osteogenic media with BMP (OM+BMP), and T3 in

osteogenic media with T3 (OM+T3) were the main additives which impacted the responses during osteogenic induction phase.

5.2.1 TGFβ1 Induced Cartilage Matures Upon Addition of T3 to Osteogenic Media

Following the osteogenic induction phase, osteogenic media with T3 (OM+T3) matured the cartilaginous tissue produced under TGFβ1 action, with a comparatively higher frequency of hypertrophic, calcified, and osseous tissue in samples. Osteogenic media with BMP (OM+BMP) appeared to have similar effects as OM+T3 but was less potent in its ability to affect these changes in TGF induced samples and did not advance the overall maturity of chondrogenic tissue. Osteogenic media alone (OM) seemed to maintain the immature state of cartilaginous tissue induced under TGFβ1 action. Interestingly, samples cultured in OM, produced the largest amount of chondrogenic tissue of all TGFβ1 induced groups. Although OM and OM+BMP were worse than OM+T3 at inducing mineralization in samples, they produced greater amounts of mineralized tissue comparatively.

The maintenance of the immature status of cartilaginous tissue observed under osteogenic media alone (OM) in TGFβ1 induced chondrogenic explants may be attributed to the action of dexamethasone. The context of dexamethasone use tends to dictate its activity; the effect of this glucocorticoid have been various and are often divergent. In particular, dexamethasone has been systematically applied during chondrogenesis and osteogenesis of MSCs *in vitro* with the capability to augment the differentiation of each distinct lineage based on microenvironment and synergistic cues [235]–[238]. In a counterintuitive fashion, the *in vivo* action of dexamethasone has led to the development of skeletal system related pathologies including growth retardation in children and osteoporosis in adults [239], [240]. Dexamethasone-induced growth inhibition in adolescents was found to be due to decreased proliferation of stem-like cells within the growth plate (resting and proliferative zones), likely as a result of diminished signalling in the growth hormone/insulin-like growth factor–I (GH/IGF-I) axis [126], [241], [242]. Following cessation of glucocorticoid treatment, a phenomenon known as ‘catch-up growth’ occurs which is characterized by a period of accelerated growth, indicating that the proliferative potential of these stem cells is conserved [125], [243]. Prevention of proliferation due to dexamethasone present in osteogenic media may have resulted in continued presence of resting chondrocytes and their stem-like progenitors within TGFβ1 induced cartilage cultures, thus resulting in the secretion of PTHrP and delayed hypertrophy. This may be further supported by the high chondrogenic tissue volume present in the OM supplemented samples, as PTHrP is also known to upregulate *Sox9* expression and stimulate cartilage matrix production [244]. The action of dexamethasone appears to be specific to immature chondrocytes – specifically resting, proliferative and pre-hypertrophic cells. Dexamethasone has little action during hypertrophy and subsequent stages of endochondral ossification, however in prehypertrophic

chondrocytes it prevents alkaline phosphatase (ALP) activity and mineralization [245]. This supports the idea that the phenotype of chondrocytes within the cartilage following chondrogenic pre-culture dictated the subsequent effects of the osteogenic media.

The choice of the same osteogenic media utilized in *in vitro* osteoblastogenesis to promote mineralization and osteogenesis within TGFβ1 chondrogenic periosteal cultures was, in retrospect, ill-advised. The specific action of dexamethasone on chondrocytes should have been considered, rather than its role as a mediator of osteogenesis for MSCs. Removal of dexamethasone from the osteogenic media may therefore improve the progression of endochondral ossification and promote mineralization of terminal hypertrophic chondrocytes in TGFβ1 induced cartilaginous explants.

Improved progression of endochondral ossification was dependent on addition of T3 and BMP to the osteogenic media. As previously described, BMP2 has hypertrophic and osteoinductive properties, therefore the maturation of TGFβ1 induced cartilage under OM+BMP is to be expected. T3 similarly is involved in maturation during chondrocyte differentiation: thyrotoxicosis during childhood causes rapid bone lengthening and advances bone age, consequently culminating in decreased height due to early fusion of the epiphyseal plate [246]. Its actions appear to be focused in resting and proliferative zone chondrocytes, where it restricts proliferation and accelerates hypertrophy [247]. While the advancement of chondrocyte phenotype was also expected under T3, the improvement in tissue maturation in comparison to BMP2 was unanticipated. Interestingly, T3 lies upstream of BMP2 in the hypertrophy signal cascade [248]. Its actions have been directly linked to BMP2 expression where the addition of the BMP2 antagonist, *Noggin*, to T3 induced chondrocyte culture abolished the expression of the hypertrophic marker collagen X [249], [250]. The results of the present study suggest that T3 may not be working through an indirect, BMP2-mediated pathway but rather through direct repression of PTHrP or *sox9* [250]. The specificity of T3 to particular chondrocyte phenotypes may further support this, as TGFβ1 induced chondrogenesis led mostly to the differentiation of the targeted immature cells. This would also indicate that TGFβ1 induced cartilage cultures do not contain a large population of cells responsive to BMP2 mediated promotion of the hypertrophy and terminal differentiation at six weeks. Here a potential discrepancy was observed, as the switch condition positively responded to BMP2 following three weeks of TGFβ1 induction. One possibility is suggested as to why this may occur. Prolonged exposure to TGFβ1 may cause the hypertrophic response to BMP2 to be sluggish and thus BMP2 may even act to promote the chondrogenic lineage. In articular cartilage (permanent cartilage), BMP2 has been shown to increase the synthesis of cartilage matrix components and only upon long term application does it result in deleterious effects [251]–[253]. Thus, six week differentiation under TGFβ1 in combination with the shorter culture period in osteogenic media with BMP2 may not lead to

advancement of endochondral ossification. A longer exposure to BMP2 following six weeks TGFβ1 chondrogenic induction may facilitate maturation however.

5.2.2 BMP2 Induced Cartilage Undergoes Ossification in Osteogenic Media

Only osteogenic media alone (OM) appeared to advance the progression of endochondral ossification following six weeks chondrogenic induction with BMP2. Samples cultured in OM showed an increase in both the incidences of mineralization and the amount of mineralized tissue produced from six weeks chondrogenic induction. Nevertheless, the amount of mineralized tissue produced remained inferior to that of three week culture in BMP2. Despite having lower overall frequencies in resting, proliferative, hypertrophic, and calcified tissue, the trend in tissue phenotypes was similar and remained generally high for samples under OM osteogenic induction as compared to those chondrogenically induced. Indeed, samples at six weeks under BMP2 and after a further two weeks in OM were comparatively alike, showing a similar disorganization of the proliferative and hypertrophic regions as well as a proportionately high tissue volume. Addition of BMP2 to the osteogenic media appeared (assessed by the frequency plots) to greatly inhibit the endochondral process, with samples mainly of resting phenotype with few to no indications of other phenotypes. Osteogenic media with T3 seemed to also hinder endochondral progression, with a larger number of samples displaying early cartilage phenotypes (resting and proliferative) and less of mature phenotypes (hypertrophic, calcified, osseous). Samples induced for three weeks remained superior in terms of producing endochondral bone tissue.

While the effects of osteogenic media on the BMP2 induced chondrogenic cultures agree with the literature with respect to the activity of dexamethasone, the results of addition of BMP2 and T3 to osteogenic media appear to be aberrant. After six weeks of chondrogenic differentiation under BMP2, the cartilage was mainly hypertrophic. As previously discussed, dexamethasone primarily affects resting and proliferative zone chondrocytes with little action downstream following hypertrophy [245]. Thus, the ossification which occurred was to be expected upon addition of a phosphate donor to cultures. Continued action of BMP2 and T3 should have promoted terminal hypertrophy and calcification in samples. As seen in the histological images of these samples, the full endochondral program does appear to be continued in the presence of osteogenic media with BMP or T3. The frequency plots instead show that the produced tissue was mainly resting or proliferative. This dichotomy between outcome measures may have been due to error, specifically in sample harvest. Overall, a low number of samples actually produced tissue in OM+BMP2 and OM+T3 groups following BMP2 induced chondrogenesis. Upon removal of samples which underwent chondrogenesis poorly or ectopically within these groups, the sample size is too small to truly make any real posits, however based on literature, the endochondral process should be advanced with addition of either BMP2 or T3.

5.2.3 Switch Treatment Samples Mature Following Culture in Osteogenic Media with BMP2

Subsequent to the osteogenic induction phase, osteogenic media with BMP (OM+BMP) matured the cartilaginous tissue produced under the switch treatment, with a comparatively higher frequency of hypertrophic, calcified, and osseous tissue in samples, along with a higher amount of mineralized tissue produced. The area of chondrogenic tissue produced was no greater than under any other osteogenic media type. The area of mineralized tissue, however, was greater than what was produced at six weeks under the switch condition. Osteogenic media with T3 matured the cartilaginous tissue as well, with higher incidences of hypertrophic and calcified tissue. Neither chondrogenic nor mineralized tissue area increased following the osteogenic phase of induction in samples cultures in osteogenic media with T3. A similar morphological structure to that which developed during the chondrogenic phase under the switch treatment was observed for both OM+BMP and OM+T3.

The improvement in tissue mineralization of switch induced samples may be the result of a longer period of culture in BMP2. This is as expected based on the previous assertions regarding the switch treatment: a short term TGFβ1 induced chondrogenic 'priming' step functions to improve the BMP2 mediated progression of endochondral ossification. T3 was found to be similarly capable of improving tissue maturity although without a concurrent increase in mineralized tissue. As T3 action is upstream of BMP2, it is possible the effects of T3 were simply slower than those of a direction addition of BMP2 [249].

5.3 Limitations of the Study

Despite the high number of samples which underwent chondrogenesis, the variability in response between all groups suggests that explants may have differed in their chondrogenic potential. This is postulated to be due either to locational disparities in the excised tissue utilized in experimentation or issues with the harvesting technique. Locational differences may have influenced culture outcomes to an extent; however these issues can largely be accounted for and were discussed previously. An imperfect harvest technique, however, may also have been responsible for the presence of osseous and calcified tissue within TGFβ1 induced cartilage at early stages of differentiation as chondrogenic or osseous tissue remnants may have been excised with along with periosteum.

The interpretation of the frequency plots may be somewhat misleading as there may be a discordance between the frequency of tissue phenotypes and amount of tissue present. While a frequency measure gives a good indication of the tendencies of cellular action under certain treatments, there was no thresholding as it was an ordinal classification system. For example, in a particular sample even if hypertrophic tissue accounted for a very small fraction of the tissue bulk, its presence automatically

counted in the frequency distribution. A better method would have been to assess phenotype frequency along with the corresponding phenotypic tissue area; however this was logistically difficult to perform in this study.

Chapter 6 Conclusions and Recommendations

6.1 Conclusions

Tissue engineered bone has been investigated for use in orthopaedic procedures following a decline in tissue donation and a rising global need for osseous grafts [3], [4], [254]. Recently, the strategic paradigm in bone tissue engineering has shifted wherein a biomimetic rather than a modular approach is utilized. Intramembranous ossification has thus been superseded by endochondral ossification as the mechanism of choice for *in vitro* development of bone as it emulates the *in vivo* pathway of bone tissue formation during limb morphogenesis [26]. The endochondral mechanism of bone tissue engineering has been touted as the superior method, resulting in the production of larger bone volumes and without the complications associated with implantation of intramembranous constructs [35], [39]. Periosteum tissue contains a reservoir of cells with multi-lineage potential which contribute to processes involving endochondral ossification *in vivo* such as fracture repair, osteophyte formation, and growth plate maintenance [51], [143]. The present study featured the timed application of chondrogenic and osteogenic media to periosteal explants in order to develop endochondral bone tissue *in vitro*.

The results of experimentation can be summarized into several main conclusions. The overarching finding is that periosteum tissue can give rise to endochondral bone *in vitro*. Although not unexpected based on the known capacity of periosteum cells and periosteum tissue to produce chondrogenic tissue *in vitro*, endochondral bone formation has only been observed after *in vivo* implantation of hypertrophic constructs [255]–[257]. The development of endochondral bone tissue was thought to be dependent upon generation of a chondrogenic osteoprogenitor capable of either hypertrophy or osteogenesis. The growth factors utilized (TGFβ1 and BMP2) were postulated to induce periosteal cells to become osteochondral progenitors but differentially effect their maturation and downstream commitment. Following long term culture in TGFβ1, the induced cartilage tissue was maintained in an immature state. This indicated its actions ensuing chondrogenesis were primarily chondroprotective and thus undesirable in furthering the endochondral ossification. Culture in BMP2 was found to accelerate cartilage tissue maturation, and its application was essential for the production of osseous tissue. BMP2 was thought to have hypertrophic and osteogenic action on a population of cells (termed chondrogenic osteoprogenitors) which were brought to a chondrogenic yet bi-potent state through the action of either factor. Thus, culture of explants in BMP2 following a short-term TGFβ1 pre-induction positively influenced the endochondral process. An initial period of chondrogenic stimulation in which these chondrogenic osteoprogenitors was produced appeared to mediate the osteogenic-hypertrophic action of BMP2. Whether the ‘switch’ condition or simply long-term BMP2 culture produced a better endochondral response is still unknown. It was possible that the presence of osseous tissue in short term

BMP2 cultures was due to the presence of these chondrogenic osteoprogenitors in the periosteal explants which did not require chondrogenic stimulation to become bi-potent.

The osteogenic media utilized to promote ossification in constructs was inappropriate for use on chondrogenic periosteal explants. Different constituents within each osteogenic formulation had targeted effects on distinct cellular phenotypes and thus the chondrogenic phase of induction influenced the osteogenic induction. Endochondral ossification was believed to be inhibited in TGFβ1 induced cartilage by addition of osteogenic media (OM) due to the action of dexamethasone which preferentially targets resting and proliferative chondrocytes to prevent hypertrophy. Dexamethasone is not known to influence cells downstream of the resting and proliferative zone, thus following culture in osteogenic media (OM), BMP2 and switch treated explants much resembled their chondrogenic counterparts at six weeks. Osteogenic media (OM) therefore hinders maturation in TGFβ1 induced cartilage and simply maintains BMP2 and 'switch' induced cartilage. TGFβ1-induced cartilage was capable of maturing upon addition of T3 and BMP2 to osteogenic culture media; however the response was less impressive in comparison to the 'switch' treatment (at six weeks). This may suggest that if the culture period under TGFβ1 is lengthened, so too should the successive culture in hypertrophic or osteogenic media (T3 or BMP2) for an equal maturation. Osteogenic media with T3 and BMP similarly progressed the maturation of the 'switch' treatment, and potentially BMP2 induced cartilage. These outcomes indicate that BMP2 and T3 act similarly as during the chondrogenic phase to further the endochondral process but in combination with the osteogenic media, did not have the overall desired effect. The process of construct mineralization was as good (if not better) during the chondrogenic phase than as during the osteogenic phase. Mineralization of endochondral explants can therefore occur even in chondrogenic media and alteration to the formulation to promote this event may not be necessary.

6.2 Recommendations

Although the literature supports most of the study results, the conclusions of this thesis were based on qualitative outcomes measures and therefore need to be further substantiated with larger sample sizes and additional quantifiable data. In particular, the effects of BMP2 should be clarified, as there were several discrepancies involving its actions. The decreased osteogenic action of BMP2 over a longer period of time was attributed to the presence of chondrogenic osteoprogenitors within explants. This should be further investigated, as a shorter BMP2 culture period would be beneficial and a longer culture in BMP2 consequently wasteful. In addition, the tailoring of the media supplementation regime should be further refined. While the idea of a successive application of media types proved correctly able to direct the endochondral process, the conditions which led to ultimate bone formation should be elucidated. A study is therefore proposed in which the pre-induction should be optimized based on growth factor and

time. BMP2 and TGFβ1 should be assessed on their ability to prime cells to undergo endochondral ossification in order to determine whether BMP2 alone is sufficient to promote the full process. It would be interesting to attempt to fixate on the particular cell state or phenotype in which further BMP2 action can direct osteogenic or the chondrogenic lineage development.

Methods to produce the largest amount of bone tissue *in vitro* should be identified. Therefore, in addition to optimizing the pre-induction, the length the following culture period in BMP2 to produce substantial bone should be considered. Along this same thread, as a strategy to produce larger bone volumes, it should be determined whether extension in the secondary culture in BMP2 could induce the endochondral phenotype in long term TGFβ1 induced cultures. Whether periosteum requires exhaustion of its MSC population prior to full ossification of the induced cartilage tissue could be examined as well. The necessity of mineralizing media should be investigated. Although the cultures were capable of mineralizing under chondrogenic conditions, the addition of a phosphate donor to the media may improve the amount and quality of produced osseous tissue. A reformulation of osteogenic media should be attempted following upstream optimization. This is suggested to involve the removal of dexamethasone and a decrease in βGP concentration in order to prevent non-specific mineralization.

Appendix 1

7.1 Collagen I Staining in Periosteal Explants

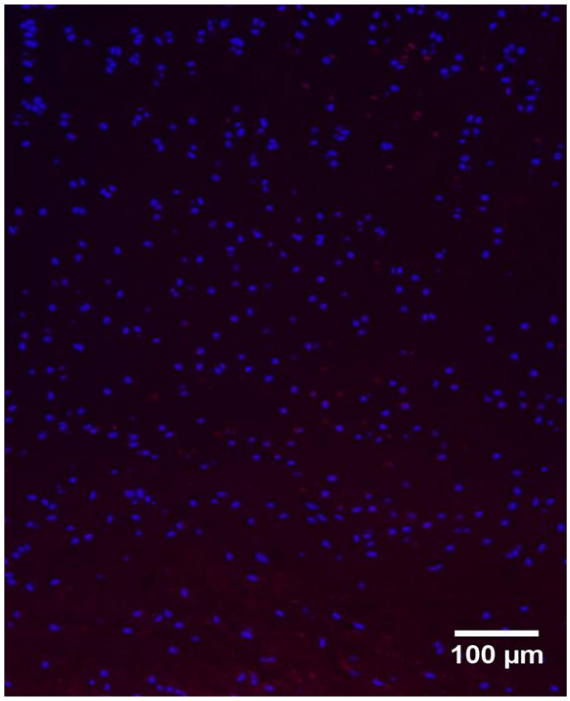

Figure A-1: Immunohistochemical localization of collagen I in cartilaginous tissue induced by 6 weeks culture in TGFβ1.

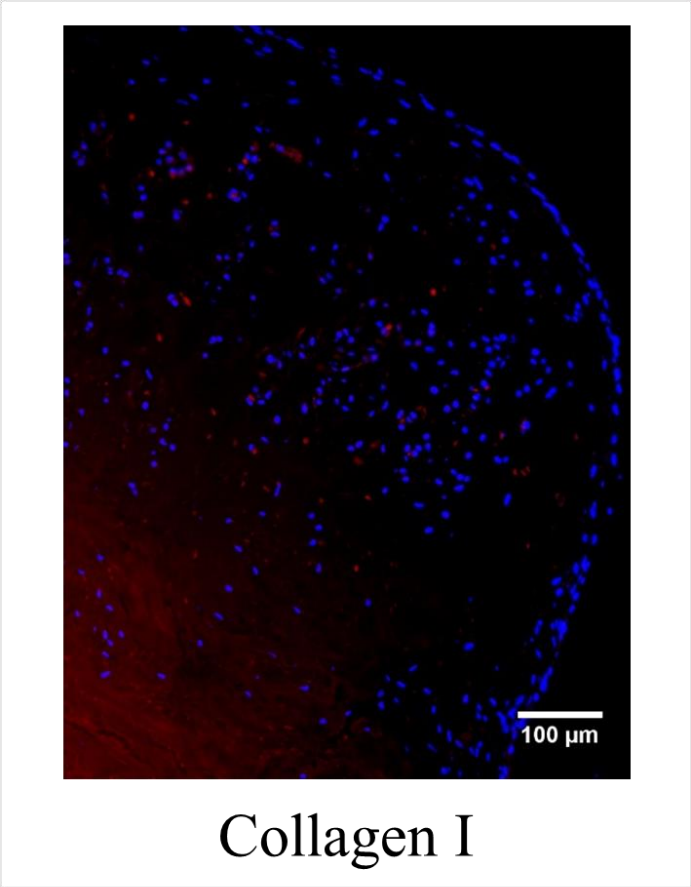

Figure A-2: Immunohistochemical localization of collagen I in cartilaginous tissue induced after 6 weeks culture in TGFβ1 followed by osteogenic culture in OM

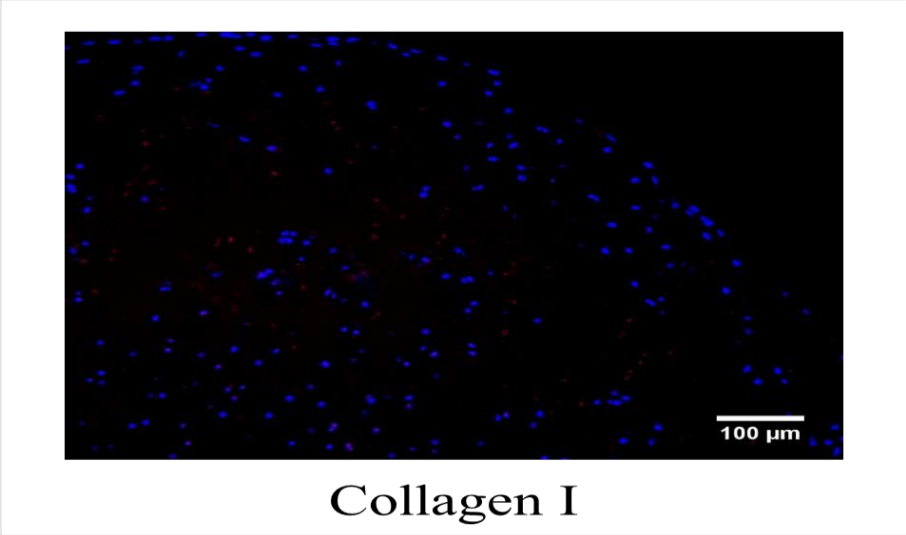

Figure A-3: Immunohistochemical localization of collagen I in cartilaginous tissue induced after 6 weeks culture in BMP2 followed by osteogenic culture in OM

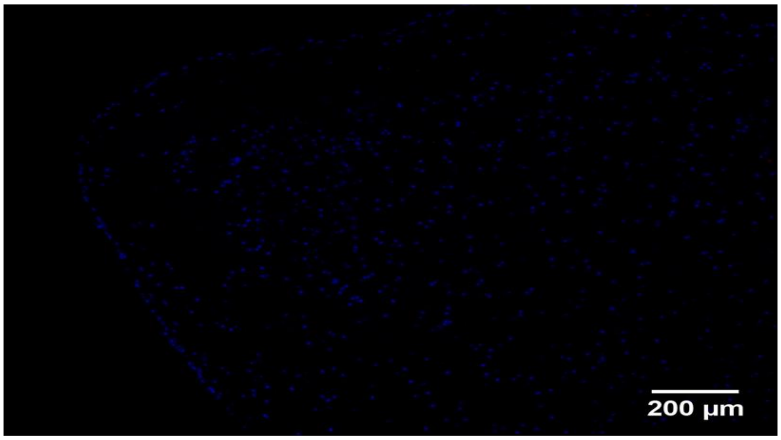

Figure A-4: Immunohistochemical localization of collagen I in cartilaginous tissue induced after 6 weeks culture in BMP2 followed by osteogenic culture in OM+BMP

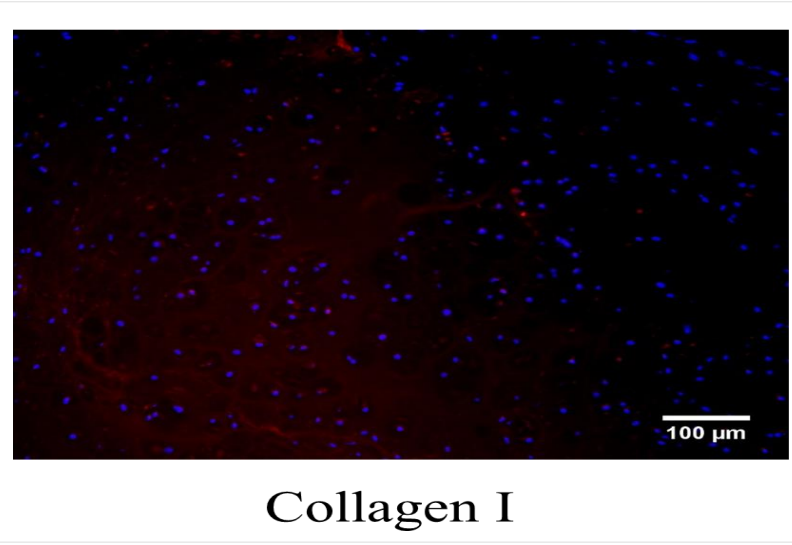

Figure A-5: Immunohistochemical localization of collagen I in cartilaginous tissue induced after 6 weeks culture in BMP2 followed by osteogenic culture in OM+T3

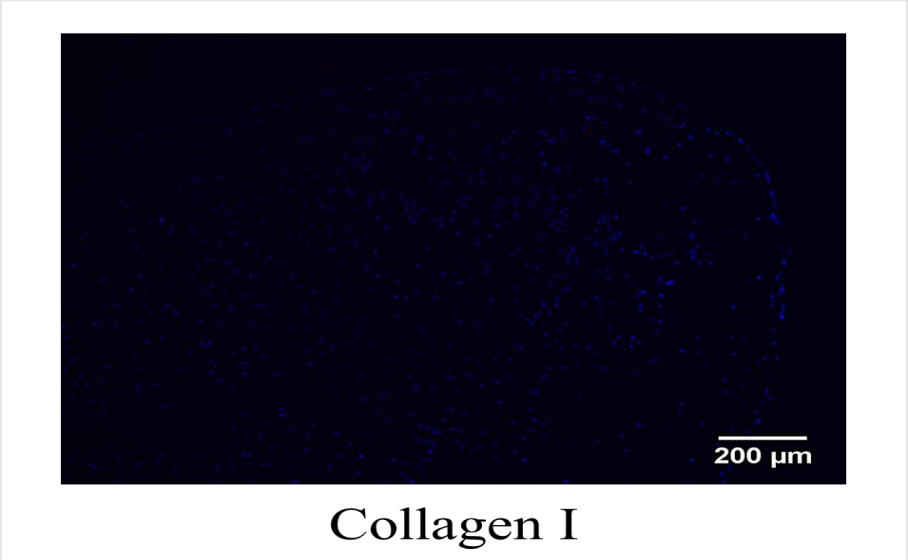

Figure A-6: Immunohistochemical localization of collagen I in cartilaginous tissue induced after 6 weeks Switch treatment followed by osteogenic culture in OM+T3

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