# ROLES OF TNF-α SIGNALLING AND p38 MAP KINASE ACTIVATION IN THE RESPONSES TO GROWTH PLATE INJURY IN YOUNG RATS

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**BY**

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# THESIS SUMMARY

Growth plate cartilage is responsible for bone lengthening in children and yet it has limited abilities to regenerate. After injury, a bone bridge often forms at the injury site, which may lead to growth arrest or angulation of the involved bone. Although our previous study has demonstrated inflammatory, fibrogenic, and osteogenic cellular responses after growth plate injury, the underlying molecular mechanisms remain unclear. Pro-inflammatory cytokine TNF-α inhibits osteoblast differentiation *in vitro* and yet TNF signalling is essential for bone fracture healing. In addition, the production and signal transduction of TNF-α and IL-1β require activation of p38 MAP Kinase, which is often induced by stress and tissue injury. Currently roles of TNF-α signalling and p38 activation in the bony repair of injured growth plate cartilage are unknown.

In this study, firstly, the changes in expression of pro-inflammatory cytokines, growth factors and chondrogenic/osteogenic markers, and patterns of p38 activation were examined in injured proximal tibial growth plate of young rats. Results showed up-regulated expression in cytokines TNF-α, IL-1β and TGF-β1, but down regulated expression in cartilage marker Col-2a, osteogenic markers cbfa1 and osteocalcin during the immediate inflammatory phase. Consistent with TNF-α and IL-1β expression pattern, up-regulation of p38 activation was also seen during the inflammatory response. While Col-2a and chondrogenic factor Sox9 mRNA levels were not altered during the subsequent fibrogenic response, fibrogenic growth factors FGF-2 and PDGF-B expression were up-regulated. In addition, expression of osteocalcin as well as bone remodelling regulatory factors IGF-I, TNF-α, FGF-2 and TGF-β1 was induced during bone bridge formation and maturation stage. Changes in expression of these cytokines, p38 and growth factors suggest their possible roles in regulating the inflammatory, fibrogenic, osteogenic, and remodelling events for bony repair of the injured growth plate. Furthermore, we also found a significant inverse correlation between TNF-α and cbfa1 expression levels, suggesting a negative

relationship between TNF-α and cbfa1 in this *in vivo* model. Therefore these observations encourage us to further examine the roles of TNF-α signalling in p38 activation and in the subsequent bony repair of the injured growth plate.

In the second study, effects of TNF-α inhibitor on growth plate injury responses, and the regulatory effects of TNF-α and p38 signalling on proliferation and migration of cultured rat bone marrow mesenchymal cells (rBMMC) were examined. We are the first to identify that TNF-α signalling is required to mediate p38 activation induced by growth plate injury. TNF-α inhibition reduced mesenchymal infiltration and cell proliferation and FGF-2 expression at the injured growth plate. Consistently, TNF-α increased proliferation and migration of rBMMC *in vitro*, which required the action of p38. On the other hand, TNF-α inhibition up-regulated expression of cbfa1 and osteocalcin, and increased trabecular bone formation at the injury site. In conclusion, TNF-α signalling is required to induce mesenchymal cell proliferation and migration at the growth plate injury site and in cell culture through the p38 MAP kinase pathway; on the other hand, TNF-α signalling suppresses bony repair of the injured growth plate by inhibiting bone cell differentiation.

# DECLARATION

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Published works are:

1) **Zhou FH**, Foster BK, Sander G, Xian CJ. Expression of proinflammatory cytokines and growth factors at the injured growth plate cartilage in young rats. **Bone** 2004; 35(6): 1307-1315.
2) **Zhou FH**, Foster BK, Zhou XF, Cowin AJ, Xian CJ. TNF-α mediates p38 MAP kinase activation and negatively regulates bone formation at the growth plate injury site in rats. **Journal of Bone Mineral Research** 2006;21(7): 1075-1088.

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# ABBREVIATIONS

| Abbreviation | Full name |
|---|---|
| ABC | Avidin-Biotin Conjugate |
| αMEM | alpha-modified Minium Essential Medium |
| BMPs | Bone Morphogenic Proteins |
| BSA | Bovine Serum albumin |
| cbfa1 | Core binding factor a1 |
| cDNA | complementary DNA from mRNA |
| c-fms | M-CSF receptor |
| CFU-F | Colony Forming Units-Fibroblast |
| cm, mm, μm | centimetre, millimetre, micrometre |
| $CO_2$ | Carbon dioxide |
| Col | Collagen |
| DAB | 3'3'-diamino-benzidine tetrachloride |
| DEPC | DiEthyl PyroCarbonate |
| DNA | DeoxyriboNucleic Acid |
| DTT | DiThioThreitol |
| ECL | Enhanced Chemiluminescence |
| ECM | Extracellular Matrix |
| FBS | Fetal Bovine Serum |
| FGF | Fibroblast Growth Factor |
| H&E | Haematoxylin & Eosin |
| h, min, s | hour, minute, second |
| $H_2O_2$ | Hydrogen peroxide |
| HCl | Hydrogen Chloride |
| HRP | Horse-Radish Peroxidase |
| HSC | Hematopoietic Stem Cell |
| IGF | Insulin Growth Factor |
| IGF-BPs | IGF binding proteins |
| Ihh | Indian hedgehog (ihh) |
| IL | Interleukin |
| KD | kilo Daltons |
| kg, g, mg, μg, ng | kilogram, gram, milligram, microgram, nanograms |

| | |
|---|---|
| l, ml, µl | Litre, millilitre, microlitre |
| M | Molar |
| M, mM, µM, nM | Molar, milliMolar, microMolar, nano Molar |
| MAP Kinase | Mitogen Activated Protein Kinase |
| M-CSF | Macrophage-Colony Stimulating Factor |
| MMP | Matrix MetalloProteinase |
| MOPS | 3-[N-morpholino] propanesulfonic acid |
| mRNA | messenger RiboNucleic Acid |
| MSC | Mesenchymal Stromal Cell |
| MW | Molecular Weight |
| NaCl | Sodium Chloride |
| NaOH | Sodium hydroxide |
| °C | Degrees Celsius |
| OCIF | OsteoClastogenesis Inhibitory Factor |
| OD | Optical Density |
| ODF | Osteoclast Differentiation Factor |
| OPG | OsteoProteGerin |
| PAGE | PolyAcrylamide Gel Electrophoresis |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase chain Reaction |
| PDGF | Platelet derived Growth Factor |
| pH | Hydrogen ion concentration |
| PMN | Polymorphonuclear |
| PPR | ParaThyroid Hormone related Peptide -Receptor |
| PTHrP | ParaThyroid Hormone (PTH) related Peptide |
| RANK | Receptor Activated Nuclear factor Kappa B |
| RANK-L | Receptor Activated Nuclear factor Kappa B-Ligand |
| rBMMC | rat Bone Marrow-derived Mesenchymal Cells |
| RER | Rough Endoplasmic Reticulum |
| RT | Reverse Transcription |
| SDS | Sodium DodecylSulphate |
| Sox | Sex reversal Y-related high-mobility group box protein |
| TBS | Tris Buffered Saline |
| TGF | Transforming Growth Factor-β |
| TNF | Tumour Necrosis Factor |

| | |
|---|---|
| TRAP | Tartrate Resistant Acid Phosphatase |
| VEGF | Vascular Endothelial Growth Factor |

# CHAPTER 1

# LITERATURE REVIEW & PROJECT AIMS

## 1.1 Introduction

The growth plate is a cartilaginous structure located at both ends of growing long bones in children, and is responsible for bone longitudinal growth through endochondral ossification, which involves replacement or conversion of cartilage tissue by bone tissue.

The poor ability for cells in the growth plate to regenerate after injury in young children can be problematic for their continuous growth. Damage to the growth plate may result in the formation of bony tissue (or bone bridge) at the injury site as a common injury repair response mechanism to growth plate cartilage wounding. Bone bridge formation may lead to growth arrest with a short or angulated limb depending on the area of fracture in the growth plate. Since they have not reached skeletal maturity, fractures in children are more frequent and occur much easier after seemingly insignificant traumas, such as falls during sports and play, compared to adults (Xian and Foster, 2006). It has been previously reported that growth plate disruptions make up about 15 % of all skeletal injuries in children, 1.4% of which have resulted in some type of growth arrest (Kao and Smith, 1997; Mizuta *et al.*, 1987). Current treatment for growth arrest is through surgical correction through limb lengthening processes, which are highly painful, and invasive, and often do not effectively induce growth plate repair.

There have been many studies investigating the molecular and cellular mechanisms involved in healing following bone fractures, however, the mechanism in bone bridge formation following growth plate fracture is not as well defined. In order to develop a strategy to prevent bone bridge formation and to find a target to develop a biological therapy for growth plate repair, it is important to understand in detail the molecular and cellular mechanisms underlying the formation the bone bridge in injured growth plate. Previously studies from our laboratory using a rat growth plate injury model have identified several injury responses following growth plate injury, including inflammatory, fibrogenic and osteogenic responses prior to bony bridge formation at growth plate injury

sites (Xian *et al.*, 2004). However the molecular regulation of these responses remains unclear. Since pro-inflammatory cytokine TNF-α has been reported to influence bone formation, remodelling, and chondrocyte apoptosis (Aizawa *et al.*, 2001; Bertolini *et al.*, 1986), and its production and transduction require the activation of p38 mitogen activated protein kinase (MAPK) (Kumar *et al.*, 2001; Lee *et al.*, 1994) that is often induced by environmental stress and tissue injuries (Gao *et al.*, 2002; Obata *et al.*, 2000; Schafers *et al.*, 2003), the current PhD project focused on studies on the roles of TNF-α signalling and p38 activation in the injury responses of the injured growth plate.

In this introductory chapter, a brief review on structure and function of immature long bone, bone growth mechanisms, cellular events of bone and growth plate fracture repair, and molecules involved in skeletal development and repair will be presented. In addition, the aims and hypothesis of this project will be introduced.

## 1.2 Immature Long Bone Structure

The immature long bones, formed during embryonic development, childhood and adolescence, consist of the epiphysis, physis (growth plate), metaphysis and diaphysis (**Figure 1.2.1**) (Kerr, 1999). These four unique and constantly changing structural components originate as a result of endochondral ossification. The epiphysis is located at the ends of long bones with a covering of articular cartilage. Just below the epiphysis is the growth plate, which facilitates the longitudinal growth of the bone. The metaphysis is the area where mineralised growth plate matrix has been newly replaced by endochondral bone. In between the metaphyses is the diaphysis, which is the major portion (or shaft) of each long bone and where the marrow cavity is formed. The outer region of the immature long bone consists of the thick fibrous periosteum, which is loosely connected to the periosteal cortical bone (outer shell) of the metaphysis and diaphysis. The osteogenic

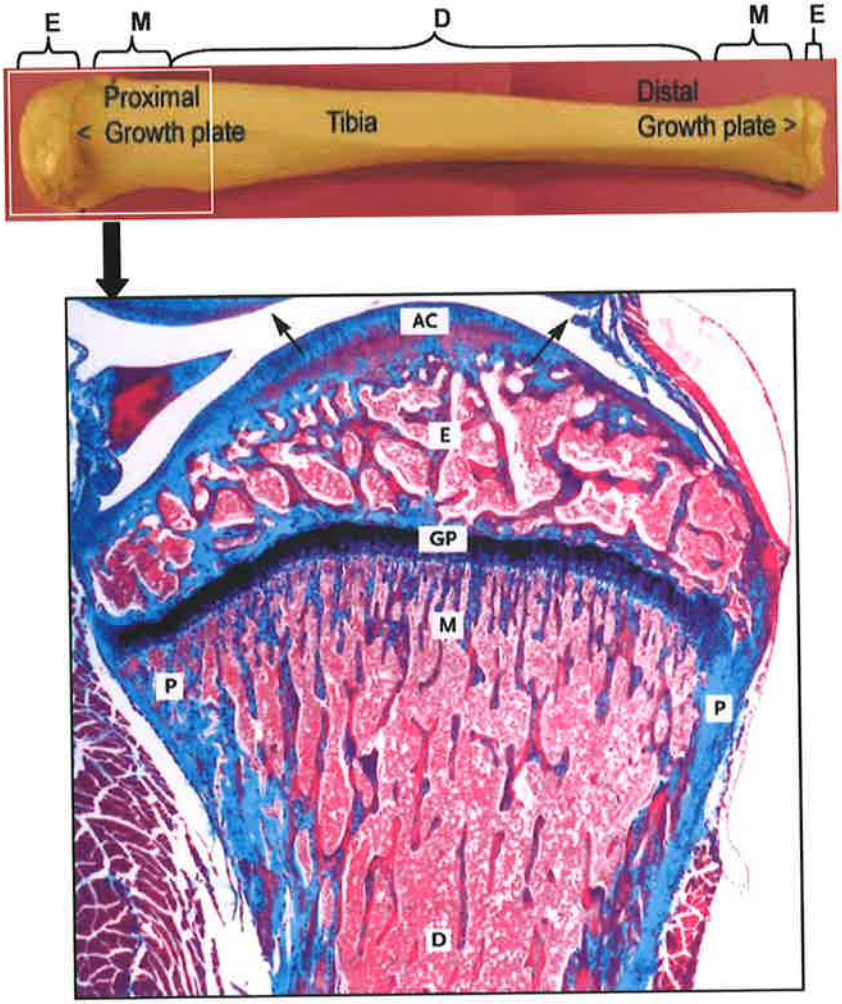


**Figure 1.2.1. Tibial long bone structure.**

In this immature tibial bone, the proximal and distal growth plates are located at the ends between the epiphysis E and the metaphysis M, and the diaphysis D is situated between the metaphysis. The histology image of proximal tibia (boxed area) further illustrates the morphology of these different regions and the location of the periosteum P adjacent to the metaphysis and diaphysis. Above the growth plate GP, the epiphyseal bone is covered by articular cartilage AC, which is responsible for its enlargement in the direction of the arrows. The bone image is kindly provided by Dr CJ Xian, and the histological image is adapted from Kerr JB (1999) Atlas of functional histology.

potential of the periosteum facilitates intramembranous ossification of the periosteal bone (Pechak *et al.*, 1986).

### 1.2.1 Epiphysis

In the immature long bone, the epiphysis is the end of each long bone, which consists of a bony area in the centre and cartilage tissue on the outer edge (**Figure 1.2.1**). During embryonic development and at birth this area is entirely a cartilage structure with calcified cartilage in the centre. During childhood the epiphysis centre is vascularized and gradually replaced by bone area with trabecular bone formation and marrow cavity, making it the secondary centre of ossification (Iannotti *et al.*, 1994).

The permanent cartilage on the outer edge of the epiphysis, which does not appear to be capable of calcification and ossification is the articular cartilage. Articular cartilage provides the joints with an excellent friction surface, lubrication and wear-characteristics required for continuous gliding motion (Mankin *et al.*, 1994). In addition it functions to absorb mechanical shock and distributes the applied load on the bony structure of the epiphysis. Articular cartilage is made up of a sparse population of specialised cells called chondrocytes, embedded in a complex extracellular matrix (ECM) consisting mainly of proteoglycan aggrecan, collagens (predominantly collagen-II), and water. Although articular chondrocytes only occupy less than 10% of the total tissue volume, these cells are essential for the production and maintenance of their surrounding cartilage matrix (Mankin *et al.*, 1994).

Immediately below the articular cartilage is the epiphyseal cartilage which is made up of poorly organised hyaline cartilage and contributes to radial expansion of cells around the secondary ossification centre through the process of chondrocyte cell division, cell hypertrophy, matrix calcification, vascular invasion and the replacement by new bone (Forriol and Shapiro, 2005; Iannotti *et al.*, 1994). The transverse and spherical growth of

the epiphysis centre is what determines the shape and form of the joint surface. This process is much slower than long bone lengthening through the physis or growth plate.

### 1.2.2 Growth Plate

During early embryonic development the limb bud is entirely chondrogenic. As the limb bud initiates formation of a long bone, the cartilage structure is gradually replaced by bone and marrow cavity in the primary ossification centre, which is in the diaphysis, and in the epiphysis (second ossification centre). The cartilage structure connecting the two ossification centres is the growth plate (physis) (**Figure 1.2.1**). Unlike the epiphysis plate, the physis is an organised structure with several distinct layers of chondrocytes with different morphology and state of maturity, and it functions to facilitate the elongation of the immature long bone (Brighton, 1978; Brighton, 1987; Brighton, 1987; Buckwalter *et al.*, 1985). The specific zones of the growth plate will be described in section 1.3. Adjacent to the physis is a fibrous area, which regulates latitudinal expansion of the plate in width. This area consists of the ossification groove of Ranvier (discovered by Ranvier 1889) and the perichondral ring of LaCroix (discovered in 1951), which will also be discussed in detail in section 1. 3.

### 1.2.3 Woven and Lamellar Bone

During skeletal development there are two types of bone formed: woven and lamellar. Woven type is primitive bone found during pre and post-natal development and fracture repair (Kaplan *et al.*, 1994). In the immature bone woven bone is located in the metaphyseal region described below in section 1.2.4. Woven bone, which is coarse-fibered, contains no uniform orientation of the collagen fibres and cells and has varied mineral content. Its structural properties allow the mechanical behaviour of woven bone to be similar regardless of the orientation of the applied forces. On the other hand, the lamellar

bone is mature bone formed 1 month after birth and is actively replacing the woven bone as it is resorbed during postnatal development (Kaplan *et al.*, 1994). Unlike woven bone, mature lamellar bone consists of collagen fibres and bone cells, which are highly organized and allow its mechanical behaviour to differ depending on the orientation of the applied forces, with its greatest strength parallel to the longitudinal axis of the collagen fibres. The woven and lamellar structures are combined to form trabecular (spongy or cancellous) and cortical (dense and compact) bone.

### 1.2.4 Metaphysis

The metaphysis is an area of newly formed bone, which starts at the last intact transverse septum of each cartilaginous column of the calcified cartilage or hypertrophic zone at the end of the physis (**Figure 1.2.1**) (Kaplan *et al.*, 1994; Kerr, 1999). There are two areas of bone formation in the metaphysis. First being the primary spongiosa, where primary immature woven or trabecular bone is formed by bone cells (osteoblasts) lining the longitudinal bars of calcified cartilage matrix and producing bone matrix after vascular invasion and removal of the transverse septum (Junqueira *et al.*, 2005). This process of bone formation is called endochondral ossification, which will be looked at in later sections. As the primary spongiosa comes to an end, the existing proteoglycan cartilage matrix template is progressively replaced with trabecular bone by osteoblasts. The functions of the metaphysis are characterised by anaerobic metabolism (hence lack of oxygen and low oxygen tension) and vascular stasis, which result from low blood flow and the arteriovenous loops at the cartilage bone junction (Iannotti *et al.*, 1994).

The second area of bone formation in the metaphysis is called the secondary spongiosa. It is where internal bone remodelling occurs where the primary woven bone and calcified cartilage is removed by bone resorbing cells, osteoclasts, and replaced by mature lamellar bone by osteoblasts (Xian and Foster, 2006). As long bone extends in length, this

area will become part of the enlarging diaphysis. It is these immature and mature regions of bone formation along with the physis that enable the lengthening of long bones.

Although bone is cancellous or spongy within the metaphysis as well as the epiphysis, the outer shell protecting these areas is made up of harder compact bone called the cortical bone. The cortical bone is externally remodelled to obtain the flared shape of the metaphysis and its diameter is further narrowed to meet the diaphysis shaft, hence the process is called funnelization (Iannotti *et al.*, 1994; Junqueira *et al.*, 2005). Formation of the cortical bone is due to the coalescence of peripheral endochondral trabecular bone from the physis with intramembranous bone from the inner osteogenic (cambial) layer of the periosteum (Cadet *et al.*, 2003). Membranous ossification process is where mesenchymal cell or osteoprogenitor cells are recruited to differentiate into osteoblasts and produce bone (Lee *et al.*, 2000; Xian *et al.*, 2004).

### 1.2.5 Diaphysis

This area is the primary ossification centre during embryonic development and later forms the marrow or medullary cavity (**Figure 1.2.1**). Unlike the epiphysis and metaphysis where the major bone structure is held up by thick spongy bone and thin outer cortex shell, in the diaphysis shaft almost the entire thickness of the bone that covers this area is compact cortical bone. There is only a small proportion of trabecular bone facing the bone marrow cavity in the diaphysis. Cortical bone has four times the density of cancellous bone, and is also capable of resisting bending and torsional forces and compressive loads (Kaplan *et al.*, 1994). On the another hand, the porous trabecular bone has approximately 20 times more surface area per unit volume and thus containing more active osteoblasts, bone lining cells and osteoclasts per unit volume, which allow it to undergo a higher metabolic turnover rate eight times greater than that of cortical bone (Kaplan *et al.*, 1994; Singh, 1978). In addition, the trabecular bone forms a three-dimensional branching lattice

network oriented along lines of stress and is structurally suited for the resistance of compressive loads (Goldstein *et al.*, 1990; Kaplan *et al.*, 1994). The enlargement of the overall diameter of the diaphyseal cortical shaft is a result of endosteal remodelling and periosteum-mediated, membranous, appositional bone formation.

### 1.2.6 Periosteum

Periosteum, the connective tissue membrane that is loosely attached to the long bone shaft, comprises of two tissue layers. While the fibrous outer layer of the periosteum is less cellular and provides fibrous attachment to the perichondrium layer of physis, muscles, tendons and ligaments, the inner or cambium layer is more vascular and contains undifferentiated mesenchymal cells with osteochondrogenic potential which support periosteal bone formation and fracture repair (McKibbin, 1978; Scott-Savage and Hall, 1980). Periosteum is much thicker and elevated in the diaphysis and metaphysis regions of the immature bone compared to mature bone. Exhibiting greater osteogenic potential than that of an adult, the periosteum during embryonic and postnatal development is involved in the radial growth of the long bone through direct differentiation of osteoprogenitor cells into osteoblasts (Pechak *et al.*, 1986). In addition, the chondrogenic potential of periosteum also was shown to decrease with increase in age (O'Driscoll *et al.*, 2001). The cellular properties of the inner cambium layer have increased the interest in the use of periosteum tissue as sources of osteochondroprogenitor cells for cartilage/bone repair or tissue engineering (Nakahara *et al.*, 1991; Nakahara *et al.*, 1991; O'Driscoll and Fitzsimmons, 2001; Wirth *et al.*, 1994). This will be discussed in 1.6.4.

## 1.3 Growth Plate Structure

Growth plates or physis appear during early stages of embryonic skeletal development. This is observed in Col-2-GFP (collagen-II conjugated green fluorescence

protein) transgenic mice, where the cartilaginous structure of the growth plate was present at e14.5 within the long bones (Cho *et al.*, 2001). The growth plate in human is formed between the epiphyseal and metaphyseal regions by week 8 of gestation at ends of the long bone, and proceeds to facilitate bone elongation until skeletal maturity in adults (Iannotti *et al.*, 1994).

The growth plate is composed of a cartilaginous component, surrounded by a fibrous structure and bounded by a bony metaphyseal component, which are responsible for the latitudinal and longitudinal growth and remodelling of the developing bone, respectively. The cartilaginous component of the growth plate principally consists of three distinct zones, and cells known as chondrocytes are identified within these zones (Iannotti *et al.*, 1994; Weisser *et al.*, 2002). From the resting zone through the proliferative zone to the hypertrophic zone, the maturity state of the chondrocytes within each zone increases from undifferentiated immature stage through proliferating stage to differentiated mature stage (**Figure 1.3.1**).

### 1.3.1 Resting Zone

The resting or germinal zone is histologically characterised by single or paired round cells scattered in an abundant matrix (**Figure 1.3.1).** Chondrocytes existing in the resting zone are relatively inactive in cell or matrix turnover. Initially, as prechondrogenic mesenchymal cells begin to differentiate into immature chondrocytes in this zone, they start to produce cartilage specific collagen-II (Col-2) (see section 1.4.7.1) at relatively low levels, along with aggrecan, a cartilage-specific sulfated proteoglycan, and several minor collagens including collagen-IX and -XI (Col-9 and –11) (Buckwalter, 1998; Sandell *et al.*, 1998).

Langenskiold (1998) reviewed strong evidence indicating the involvement of cells from the growth plate germinal layer in the interstitial latitudinal growth of the growth plate. More recent functional studies of the resting zone in the growth plate suggest that

this zone is an important contributor to endochondral bone formation at the growth plate as it contains stem-like cells that give rise to clones of proliferative chondrocytes (Abad *et al.*, 2002). In addition, damage to the area within the resting zone may result in closure of the growth plate (Salter and Harris, 1963).

### 1.3.2 Proliferative Zone

The proliferative zone is histologically characterised by longitudinal columns of flattened cells closely clustered together (**Figure 1.3.1**). The uppermost progenitor cell in each column give rise to the daughter cells lined up along the axis of the bone (Hunziker, 1994). In this zone, chondrocytes are stimulated and regulated by growth factors, hormones and signalling molecules to proliferate, and undergo chondrogenesis (Beier *et al.*, 2001; de Crombrugghe *et al.*, 2001; Jingushi *et al.*, 1995; Shum and Nuckolls, 2002). In addition to cellular division, the proliferative zone also functions in matrix production, including proteoglycan aggregates and collagen-II (Col-2). From *in vitro* studies reviewed in Buckwalter (1998), there is strong evidence suggesting that large cartilage-proteoglycan aggregates (aggrecans) inhibit matrix mineralization (Howell *et al.*, 1969). Furthermore, disruption of the large aggrecan structures or reduction in their concentration and size eliminated the inhibition (Boskey *et al.*, 1992). This function is consistent with several reports indicating that large aggrecan synthesis and turnover appeared to be highest in the proliferative and pre-hypertrophic zones, where cartilage calcification does not occur, and were reduced or absent in the cartilage calcification regions of lower hypertrophic zones (Mundlos *et al.*, 1991 Buckwalter, 1998 #490; Shapses *et al.*, 1994). However, compared to Col-2 expression levels, aggrecan and collagen-XI mRNAs were detected in much lower quantities in a study with bovine growth plate (Sandell *et al.*, 1994). The primary function of the cartilage matrix is to maintain the integrity of cellular architectural pattern

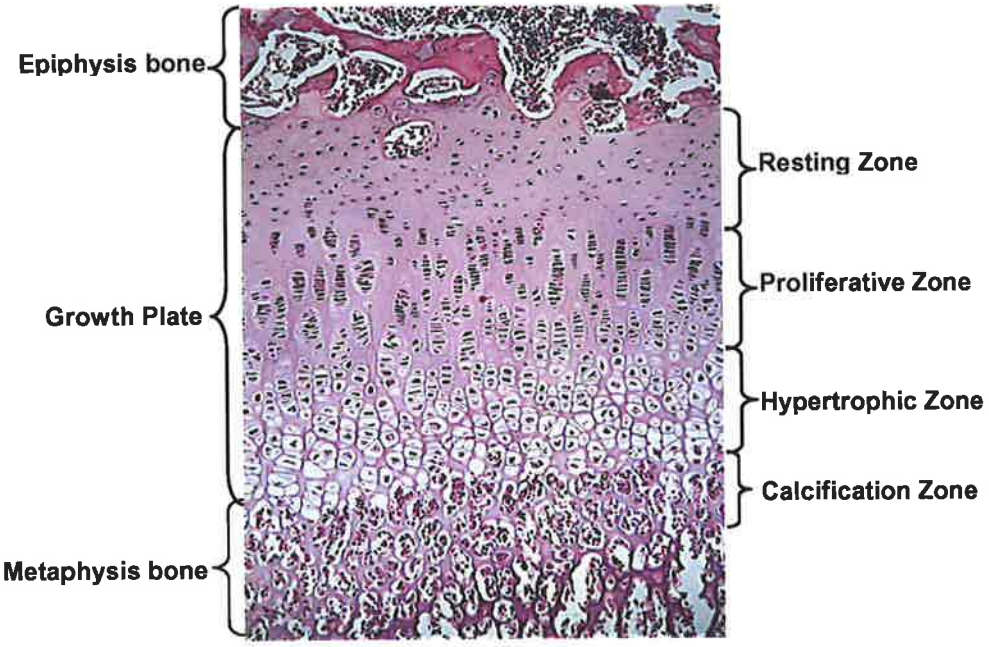


**Figure 1.3.1. Growth plate histology stained by hematoxylin and eosin.**

The growth plate, situated between the epiphyseal and metaphyseal bone, consists of the resting zone with inactive chondroprogenitors, the proliferative zone with actively proliferating chondrocytes, and the hypertrophic zone with mature, hypertrophic and terminally-differentiated chondrocytes in a matrix undergoing calcification.

and to promote directionality of cells in the growth plate. Together, these processes of cell proliferation and matrix production in the growth plate contribute to longitudinal bone growth.

### 1.3.3 Hypertrophic Zone

The hypertrophic zone is characterised by its chondrocytes, which are five to ten times the size of proliferative chondrocytes (Hunziker, 1994) (**Figure 1.3.1**). In the upper hypertrophic zone, cells undergo differentiation into mature hypertrophic chondrocytes with an increase in cell diameter and volume. As these chondrocytes continue to increase in size, they progressively start to deteriorate in the mid hypertrophic zone. Ultimately they undergo apoptosis in the lower hypertrophic zone, where the matrix compartment between the chondrocyte columns become mineralised (Iannotti *et al.*, 1994). The main function of the hypertrophic zone is in matrix calcification. In addition, the differentiation of chondrocytes accompanied with the increase in cell size contributes to longitudinal growth (Farnum *et al.*, 2002; LuValle and Beier, 2000). Besides collagen-2 (Col-2), which continues to express at high levels, collagen-10 (Col-10) is specifically localised to mature chondrocytes and released into the matrix in the hypertrophic zone (Sandell *et al.*, 1998; Tchetina *et al.*, 2003) (see section 1.4.7.2). Unlike the negative role aggrecans play in cartilage mineralization, Col-10 is known to support cartilage matrix calcification (Kielty *et al.*, 1985; Kirsch and von der Mark, 1990).

### 1.3.4 Groove of Ranvier and Perichondrial Ring of LaCroix

The fibrous structure surrounding the periphery of the growth plate consists of an inner ossification groove of Ranvier, a wedged shape groove of cells merging with the resting zone, and outer ring of fibrous tissue, the perichondrial ring of LaCroix. Within the groove of Ranvier there are three cell types: osteoblast type cells forming the bony portion

of the perichondrial ring surrounding the outside of growth plate and connecting to the periosteum; chondrocyte type cells contributing to the lateral expansion of the growth plate; and fibroblast type cells covering the groove and anchoring it to the perichondrium proximal to the germinal zone. These actively dividing cells contribute to an increase in the diameter of the physis (Iannotti *et al.*, 1994).

How the groove of Ranvier contributes to width expansion of the physis has been debated in literature history (Langenskiold, 1998). Originally in 1889, Ranvier postulated that cells in the ossification groove originate from cartilage and differentiate to osteoblasts to form the periosteal bone. However Lacriox in 1951 found that the growth plate grows in width by apposition of cells from the neighbouring connective tissue of the perichondrium, and disputed the interstitial growth theory. Later the apposition growth hypothesis was rejected with literature demonstrating the bone forming layer of periosteum contained cartilage specific maker collagen-II (Col-2) mRNA, which suggest that the cells in the groove originate from the germinal growth plate layer (Langenskiold *et al.*, 1993; Sandberg and Vuorio, 1987).

The perichondrial ring of LaCroix structure varies greatly among and within different species, and with age of animals, however its basic structure is a fibrous collagenous network. The Ring of LaCroix is located adjacent to groove of Ranvier on the outer periphery of the growth plate, and is continuous with the fibrous portion of groove of Ranvier and the periosteum of metaphysis (Iannotti *et al.*, 1994). Therefore, it functions as a strong mechanical support at the bone-cartilage junction to resist compressive forces on the growth plate

## 1.4 Cells and Matrix Proteins of Cartilage and Bone

In cartilage the major cell type is chondrocytes, which are derived from the mesenchymal lineage (**Figure 1.4.1**). Although chondrocytes vary in morphology, function and

production of collagens at different stages of maturity, their primary function is in the formation and maintenance of cartilage. In addition, in the bone there are various cell types with different morphology, functions and location involved in the process of bone formation, resorption and repair. Osteoblasts, osteocytes and bone lining cells also derived from the mesenchymal lineage are the 3 main cell types involved in bone formation, whereas osteoclasts derived from the hematopoietic lineage are bone destroying cells and function in the removal of bone matrix during bone remodelling (**Figure 1.4.1**). The cells of cartilage and bone are responsible for the production of the various collagens and matrix proteins required for maintaining the integrity of these two tissues.

### 1.4.1 Chondrocytes

Chondrocytes are cells specific for cartilage formation. Chondrocytes are located in matrix lacunae and secrete the extracellular matrix (ECM), which consists of collagen fibrils (predominantly collagen-II (Col-2) by all chondrocytes, and collagen-X (Col-10) by hypertrophic cells in the growth plate cartilage), hyaluronic acid, proteoglycan aggrecans and small amounts of several glycoproteins (Buckwalter, 1998; Sandell *et al.*, 1998). Electron microscopy reveals that active proliferating chondrocyte have abundant rough endoplasmic reticulum (RER) and well developed Golgi complex, features of a typical protein secretory cell. In addition, chondrocytes have larger and more indentations and protrusions to increase their surface area for facilitating nutrient exchange with the ECM (Junqueira *et al.*, 2005).

There are three types of cartilage: hyaline cartilage, elastic cartilage and fibrocartilage, each with different purpose and location. Hyaline cartilage is the most common type and is the initial skeleton during embryonic development. In immature bone hyaline cartilage serves as the growth plate, and after bone maturation the articular

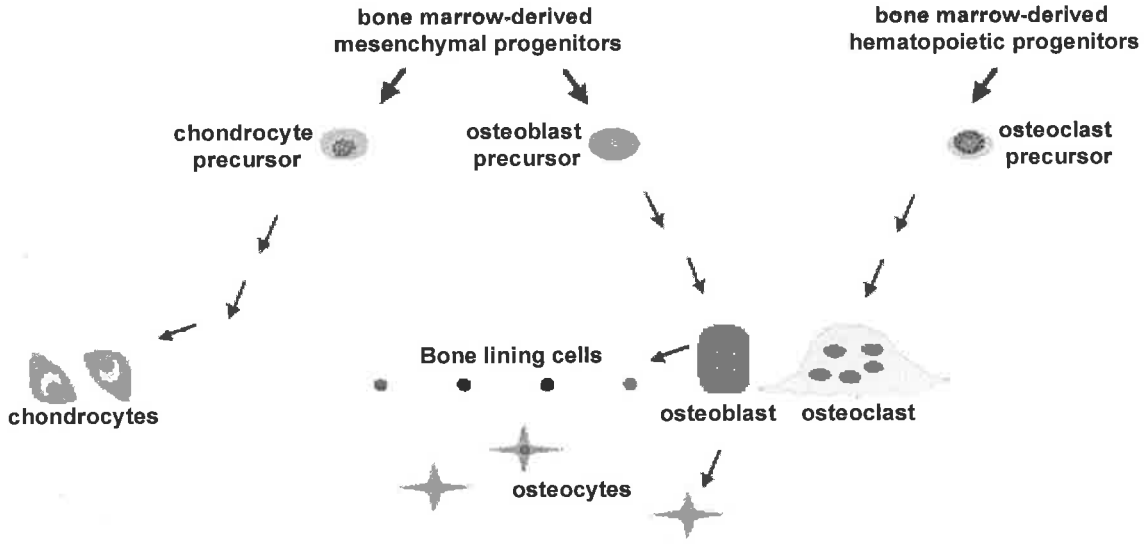


**Figure 1.4.1. Origins of cartilage and bone cells**

Bone marrow-derived mesenchymal progenitors can give rise to either chondrocyte or osteoblast precursors, which result in their differentiation into cartilage forming chondrocytes or bone forming osteoblasts, respectively. Osteoblasts further differentiate into less active bone lining cells or become osteocytes trapped in the new osteoid. On the other hand, bone destroying osteoclasts are derived from bone marrow hematopoietic progenitors.

cartilage is the only hyaline type left. All cartilage types are avascular and aneural tissues with a high matrix to cell ratio. Therefore cartilage is nourished by diffusion of nutrients from capillaries in adjacent perichondrium. In the case of articular cartilage, where perichondrium is absent, nutrients are transported by means of synovial fluid from joint cavities (Junqueira *et al.*, 2005).

As mesenchymal progenitors (with elongated fibroblastic morphology) undergo condensation and chondrocyte differentiation, they become round with an irregular surface. Chondrocytes appear in clusters of up to 8 cells originated from mitotic division of one chondrocyte. In the case of growth plate proliferating chondrocytes accumulate side by side in parallel columns. As chondrocytes reach hypertrophy, their cell diameter increase dramatically (5 times the size of proliferative cell), which also contribute to the function of the growth plate in bone lengthening (Hunziker, 1994).

### 1.4.2 Osteoblasts

Active osteoblasts have cuboidal to columnar shape and, are located at the surfaces of newly formed bone tissue (Junqueira *et al.*, 2005). These osteoblastic cells line up side by side in a row to actively participate in the synthesis of organic components and deposition of inorganic components of the unmineralised extracellular matrix called osteoid. Histological analysis has characterised osteoblasts with intense basophilic staining in the cytoplasm and high alkaline phosphatase distributed over the outer surface of cell membrane (Aubin, 1998; Hancox, 1972; Kaplan *et al.*, 1994). In addition, the ability to produce collagen-I (Col-1) (see section 1.4.7.3), osteopontin (OP) and bone matrix protein osteocalcin (see section 1.4.7.4), and the capacity to mineralise the extracellular matrix (ECM) are all attributes of the osteoblastic phenotype (Aubin *et al.*, 1995; Jaiswal *et al.*, 1997; Peter *et al.*, 1998; Shi *et al.*, 1996). Furthermore, electron microscopy reveals, beside the nucleus, abundant levels of the rough endoplasmic reticulum (RER) and a prominent

Golgi complex in the cytoplasm of osteoblasts. These are features of a cell that manufactures and secretes proteins that make up the osteoid (Kaplan *et al.*, 1994; Ross *et al.*, 1989).

As osteoblasts become less active they either encase themselves in the osteoid to become osteocytes or take on the flatter form of bone lining cells which remain on the bone surface. During bone remodelling, parathyroid hormone (PTH) together with local cytokines stimulates osteoblasts to release mediators such as M-CSF and RANKL (see section 1.5.3) that activate osteoclast differentiation (Lee and Lorenzo, 1999; Teitelbaum, 2000). The function and structure of osteocytes, bone lining cells and osteoclasts will be further discussed in section 1.4.3, 1.4.4, and 1.4.5 below, and some of the cytokines and growth factors released by osteoblasts, which are involved in regulating bone remodelling will be discussed in section 1.7.

### 1.4.3 Osteocytes

Osteocytes are derived from osteoblasts that are no longer synthesising collagens. They are described as flat almond shaped cells surround by mineralised bone matrix, where only one osteocyte is found in each lacuna, which is situated between lamellae of mineralised bone matrix (Junqueira *et al.*, 2005; Noble and Reeve, 2000). Electron microscopy also reveals that osteocytes have a higher nucleus to cytoplasm ratio and fewer organelles (Aarden *et al.*, 1994; Kaplan *et al.*, 1994). Unlike osteoblasts, osteocytes are metabolically inactive and are not required to produce and secrete a large amount of bone matrix proteins; therefore they have a significantly reduced RER and Golgi apparatus in the cell cytoplasm. However there is evidence that osteocytes can still produce collagens, control bone mineralisation and carry out bone resorption (Aarden *et al.*, 1994; Noble and Reeve, 2000). Osteocytes have numerous long branching cytoplasmic processes that project through the canaliculi to establish contact via gap junctions with processes of

adjacent osteocytes, bone lining cells and osteoblasts to communicate strain and stress signals, exchange nutrients and regulate the overall metabolism of the tissue (Aarden *et al.*, 1994; Junqueira *et al.*, 2005; Noble and Reeve, 2000). There is recent evidence indicating roles of osteocytes in regulating responses to mechanical loading of bone, and in the apoptosis of osteocytes to modulate bone remodelling processes (Gerstenfeld, 1999; Noble *et al.*, 2003; Noble and Reeve, 2000; Terai *et al.*, 1999). It is possible that the osteocytes are responsible for sensing bone stress where if undue stress is detected, they favour bone deposition, whereas if a lack of stress is detected, they favour bone resorption (Cowin *et al.*, 1991).

### 1.4.4 Bone Lining Cells

As osteoblasts become less active they take the form of elongated or flattened cells known as bone lining cells, which are also referred to as surface osteocytes (Marks and Hemey, 1996). Unlike osteocytes, these cells are found on newly synthesized unmineralised bone surface after bone formation has completed. However, similar to osteocytes, bone lining cells are found to be rarely involved in cell division and also contain cytoplasmic extensions that penetrate the mineralised matrix to come into contact with the osteocytes (Lian and Stein, 1996; Noble and Reeve, 2000). The function of bone lining cells remains controversial, however there is recent evidence suggesting that bone lining cells are capable of reverting back to osteoblastic phenotype under certain circumstances such as induction of bone formation with PTH (Dobnig and Turner, 1995) or mechanical loading on bone (Chow *et al.*, 1998). In addition, bone lining cells may also initiate osteoclastic bone resorption by dissolving the collagenous osteoid, which appears to permit osteoclast attachment (Bord *et al.*, 1996; Kaplan *et al.*, 1994).

### 1.4.5 Osteoclasts

Osteoclasts are multinuclear cells arising from mononuclear precursors from the hemopoietic cell lineage that are stimulated to proliferate, differentiate and fuse together (Udagawa *et al.*, 1990). The distinctive features of osteoclasts that distinguish them from macrophages are their ability to secrete tartrate resistant acid phosphatase (TRAP), which serves as an excellent marker for osteoclasts (Udagawa *et al.*, 1990), and to resorb bone (Ross *et al.*, 1989). Electron microscopy reveals numerous mitochondria and lysosomes within the osteoclast, which are located beyond but in close proximity of the bone surface. The nucleus of osteoclast is polarised and away from the bone surface with profiles of RER, multiple stacks of Golgi saccules and many vesicles arising from the Golgi in the same region.

The major resorptive organelle of the osteoclast is a region containing numerous plasma membrane infoldings forming microvillous type structure called the ruffle borders, which is surrounded by the clear zone to give the osteoclast surface membrane a smooth characteristic as it lies directly in regions of bone resorption called Howship's lacunae (Ross *et al.*, 1989; Teitelbaum, 2000). Ruffle borders appear only when the activated cell is attached to bone. High dose administration of estrogen, an anti-resorbing agent, to mice led to failure of osteoclasts attaching to bone surfaces and the subsequent formation of ruffle borders which further prevented successful bone resorption (Gruber *et al.*, 2001). Therefore, the attachment of osteoclasts to bone surface and the formation of ruffle borders are essential processes for bone resorption. In addition, the irregular foldings of ruffle borders allow small particles to be easily trapped and resorbed, and increase resorption area.

Osteoclasts bind to bone surface via integrins and begins the resorption process by isolating an area of bone under the region of cell attachment. The pH of the local environment is lowered through the production of hydrogen ions by osteoclasts, which

increase solubility of bone minerals and liberate the calcified ground substance (Kaplan *et al.*, 1994). In addition, the organic components of bone matrix such as collagen-I are removed by acidic proteolytic enzymes such as collagenase and TRAP secreted by lysosomes in the osteoclasts (Junqueira *et al.*, 2005; Teitelbaum, 2000).

### 1.4.6 Bone Marrow Cells

The undifferentiated mesenchymal cells reside within the bone canal, endosteum and periosteum and marrow can be extracted and cultured as plastic adherent mononuclear colony forming cells (Friedenstein *et al.*, 1976) and can be differentiated to bone and cartilage forming cells. On the other hand, the plastic non-adherent hemopoietic stem cells from the bone marrow, circulating blood or more recently in gastrointestinal tract (Lynch *et al.*, 2006) give rise to various red and white blood cells and osteoclasts (Douay and Giarratana, 2005; Lai and Kondo, 2006; Udagawa *et al.*, 1990).

#### *1.4.6.1 Mesenchymal Stromal cells*

Bone marrow-derived mesenchymal stromal cells (MSCs) easily separated from hemopoietic cells in the bone marrow aspirate by their ability to adhere to plastic culture plates were previously identified as colony forming units-fibroblast (CFU-F) (Friedenstein *et al.*, 1976). MSCs can be characterised by the presence of a consistent set of cell surface marker proteins (SH2 and SH3), CD 44, CD 71 (Haynesworth *et al.*, 1992), and the absence of hematopoietic linage markers (Colter *et al.*, 2000; Pittenger *et al.*, 1999). Characterised by their ability to expand and proliferate in the presence of serum in culture, pluripotent MSCs have the potential to differentiate into non-marrow cells with osteogenic, chondrogenic, adipogenic (Pittenger *et al.*, 1999; Prockop, 1997; Tsutsumi *et al.*, 2001), myogenic (Moscoso *et al.*, 2005; Wakitani *et al.*, 1995), or neuronal (Kopen *et al.*, 1999; Sanchez-Ramos *et al.*, 2000; Woodbury *et al.*, 2000) phenotypes when exposed to

appropriate stimuli in differentiation media. In addition, this ability of MSCs to differentiate into many different cell types has been well characterised in a variety of animal species including; human (Pittenger *et al.*, 1999), rat (Javazon *et al.*, 2001; Peter *et al.*, 1998), mouse (Phinney *et al.*, 1999; Sun *et al.*, 2003), rabbit (Tsutsumi *et al.*, 2001), dog (Kadiyala *et al.*, 1997), cat (Martin *et al.*, 2002), sheep (Zhang *et al.*, 2004) and pig (Moscoso *et al.*, 2005). Currently the mechanisms and the mitogenic factors that are required to stimulate MSC proliferation are unknown. However some of the factors that are known to stimulate MSC growth include PDGF, EGF, TGF-β and FGF-2 (Gronthos and Simmons, 1995; Tsutsumi *et al.*, 2001). In addition, a previous study using rat MSC culture has suggested that low oxygen tension environment of MSCs *in vivo* is important to stimulate cell proliferation and bone differentiation (Lennon *et al.*, 2001).

During fracture repair, primitive mesenchymal cells are recruited to the injury site and have the capability to differentiate into either chondrogenic or osteogenic phenotype depending on the type of fracture and mechanical environment. If a fracture is stabilized such as during distraction osteogenesis where fractures are held stable by external fixation frames, or in drill hole model and marrow ablation model where the process of bone healing is examined without movement, mesenchymal cells will differentiate into osteoblastic cells producing bone (Chiba *et al.*, 2001; Kuroda *et al.*, 2005; Thompson *et al.*, 2002). On the other hand if there is motion at the site of injury, MSCs will differentiate to a chondrogenic phenotype producing a cartilage, which provides intermediate stabilisation of the fracture segments (Le *et al.*, 2001; Probst and Spiegel, 1997). At the moment the mechanism by which mesenchymal cells interpret and respond to these changes in the local mechanical environment is unclear.

The morphology of MSCs can range from fibroblast-like cells with elongated spindle cell bodies to large flat cells. However unlike primary skin fibroblasts, MSCs have the ability to differentiate into different cell types of the mesenchymal tissue such as

osteoblasts, chondrocytes and adipocytes under different culture conditions. They were originally considered to be important as feeder cells, which secrete cytokines that stimulate the growth of hematopoietic stem cells (HSCs) (Prockop, 1997). Currently MSCs have also attracted a lot of interest because of their multilineage potential, and possible use for tissue engineering and cell therapy.

### *1.4.6.2 Hematopoietic Stem Cells*

The major purpose of a functional bone marrow is its ability to produce hematopoietic stem cells (HSCs) that give rise to continuous source of progenitors of red blood cells, platelets monocytes, granulocytes and lymphocytes which are essential to renew the circulating blood cells every few days or months, and osteoclasts for bone remodelling (Udagawa *et al.*, 1990). HSCs can be characterised by several markers including lipopolysaccharide receptor CD14, CD34, and the leuckocyte common antigen CD45 (Lynch *et al.*, 2006; Malik *et al.*, 1998; Pittenger *et al.*, 1999).

These also are the cells that are recruited into injury site immediately after bone fracture and growth plate injury to cope with the transient inflammatory response (Arasapam *et al.*, 2006; Chung *et al.*, 2006; Joyce *et al.*, 1991; Kon *et al.*, 2001). In addition, during bone remodelling when the existing bone matrix is being removed and new matrix is being laid down, monocyte-lineage cells originated from HSCs are recruited to the site of resorption where they proliferate and differentiate to mononuclear osteoclasts that fuse to become osteoclasts when stimulated by macrophage-colony stimulating factor (M-CSF) and RANK-L (receptor for activation of nuclear factor kappa B Ligand) produced by osteoblasts or MSC microenvironment (Kaplan *et al.*, 1994; Teitelbaum, 2000). This has been observed through co-culture of HSCs with osteoblasts or mature monocytes with MSCs (Udagawa *et al.*, 1990).

### 1.4.7 Matrix Proteins in Cartilage and Bone

They are many types of extracellular matrix proteins produced by chondrocytes and osteoblasts such as collagens and proteoglycans, which function to support the integrity of the cartilage and bone tissue structure, and in determining the size, shape and strength of these tissues. This Section will focus discussion on proteins that are better studied in bone growth and fracture repair, including collagen-II, -X and -I (Col-2, -10, -1), and non-collagen bone matrix protein osteocalcin. Since, Col-2, -10, -1 and osteocalcin are expressed at different stages of chondrocyte and osteoblast differentiation and maturation from undifferentiated mesenchymal progenitor cells during embryonic development and bone fracture repair (Ferguson *et al.*, 1999; Gerstenfeld *et al.*, 2003; Sandberg *et al.*, 1993; Sandell *et al.*, 1998), these proteins serve as ideal markers for bone/cartilage repair studies.

#### *1.4.7.1 Collagen -II*

Collagen is a family of molecules with a triple helical domain in between the globular domains at the amino and carboxyl termini, where each collagen member varies in composition and serves specialised function. Collagen-II (Col-2) (first characterised in avian cartilage) is the predominant collagen in cartilage, which comprises 80-90% of the collagen content, and functions to resist tensile stress (Miller and Matukas, 1969). *In-situ* hybridisation and Northern blot studies (Sandell *et al.*, 1994) have demonstrated that Col-2 mRNA was expressed by chondrocytes throughout entire growth plate zones, where levels were low in most immature chondrocytes, and gradually increased in chondrocytes at the proliferative and hypertrophic zones. Similarly, RT-PCR quantitative analysis of type II collagen mRNA expression in distinct zones of bovine growth plate collected by microdissection showed higher levels of Col-2 mRNA expression in the proliferative and hypertrophic zones (Tchetina *et al.*, 2003).

Some skeletal genetic disorders such as a variety of achondrodysplasias are as a result of mutations in the Col-2 gene, reviewed by Shum and Nuckolls (2001) (Shum and Nuckolls, 2002). These mutations in Col-2 will result in disruption of endochondral bone formation, which may contribute to dwarfism, joint deformities, and other skeletal abnormalities. For example, a transgenic line of mice with inactive Col-2 gene displayed a lack of endochondral long bone and growth plate, but did not show signs of developmental abnormalities in other organs and intramembranous bones (Li *et al.*, 1995). This indicates the importance of Col-2 for normal long bone formation.

Col-2 mRNA synthesized by chondrocytes is a marker commonly used to determine chondrocyte differentiation and serves as a good indicator of endochondral ossification during fracture healing (Einhorn, 1998; Sandell *et al.*, 1998). In addition, Col-2 is also a tool used to assess chondrogenesis of dedifferentiated chondrocytes (de la Fuente *et al.*, 2004), and of mesenchymal progenitors derived from periosteum, bone marrow and trabecular bone in vitro (Johnstone *et al.*, 1998; Nakahara *et al.*, 1991; Noth *et al.*, 2002).

In certain stabilised bone/growth plate fracture healing models for intramembranous ossification without the intermediate cartilage component, Col-2 expression was undetected (Lee *et al.*, 2000; Thompson *et al.*, 2002). However, although cartilage formation was not histologically visible in a stably fixed drill hole model for examining bone healing, a faint Col-2 mRNA signal was detected in majority of osteoblasts lining the newly formed trabecular bone by in-situ hybridisation (Chiba *et al.*, 2001). This is further supported by investigations indicating transient expression of Col-2 mRNA in osteoblasts in embryonic calvarial bone (Nah *et al.*, 2000) and in fracture callus (Hughes *et al.*, 1995). Even though Col-2 is predominantly a cartilage matrix protein secreted by chondrocytes, its expression by other cell types such as osteoblasts cannot be ruled out. Therefore, Col-2 alone appears to be not enough to accurately access formation of cartilage in bone/growth plate repair models or in vitro chondrogenesis. Accordingly,

alcian blue, an acid based blue dye that stains sulphated proteoglycan aggregates abundantly produced in the cartilage extracellular matrix (ECM), is routinely employed. In addition, examination of cells based on morphology and distribution is also useful to distinguish chondrocytes from osteoblasts.

### *1.4.7.2 Collagen -X*

Collagen-X (Col-10) mRNA is specifically expressed by chondrocytes late in their maturation in the hypertrophic zone of growth plate at similar levels to Col-2 mRNA prior to ossification (Sandell *et al.*, 1994; Tchetina *et al.*, 2003; Wang *et al.*, 2004). In addition Col-10 is also found in the hypertrophic regions of fetal preosseous cartilage such as sternal analage, and in fracture callus, which are undergoing endochondral ossification (Ferguson *et al.*, 1999; Jingushi *et al.*, 1992; Schmid and Conrad, 1982). Furthermore, Col-10 is expressed by chondrocytes in the deep zone and calcified zone of the articular cartilage close to second ossification centre (Schmid and Linsenmayer, 1985). On the other hand, Col-10 is not synthesized in structural cartilage such as nasal and costal cartilage, as Col-10 may be only specific to endochondral ossification in transitional cartilage (Grant *et al.*, 1985; Sussman, 1988).

Since Col-10 is uniquely expressed by hypertrophic chondrocytes in cartilage during endochondral ossification, it has been used to determine chondrocyte maturation and endochondral ossification process in growth plate injury (Lee *et al.*, 2000; Xian *et al.*, 2004), bone fracture repair (Einhorn, 1998) and in vitro models for endochondral ossification (Reiter *et al.*, 2002). However, recently, Mwale *et al.* (2006) have reported the expression of Col-10 earlier than Col-2 (early chondrogenic marker) during chondrogenic differentiation of MCS, suggesting that caution may need to be taken when using Col-10 as a marker for chondrocyte maturation in *in vitro* experiments (Mwale *et al.*, 2006).

Col-10 is involved in the mineralisation of cartilage matrix (Kielty *et al.*, 1985; Kirsch and von der Mark, 1990). Abnormal compartmentalization of cartilage matrix components in mice lacking Col-10 further suggests that it is important in providing support for the degraded cartilage matrix, and an easily resorbed foundation for the deposition of bone matrix (Kwan *et al.*, 1997). Mutations within the Col-10 gene have been reported to be responsible for certain human chondrodysplasias, such as spondylometaphyseal dysplasias, and Schmid metaphyseal chondrodysplasia, that display skeletal deformities including compression of hypertrophic growth plate, decrease in newly formed bone, and leukocyte deficiency in bone marrow (Jacenko *et al.*, 1993; Warman *et al.*, 1993). On the contrary, in another study on transgenic Col-10 null mice, Rosati *et al.* (1994) reported that these Col-10 deficient mice developed normally with no skeletal pathology (Rosati *et al.*, 1994). Therefore, the difference in this study and those reporting abnormalities might be due to a mutated Col-10 rather then the absence of it.

#### *1.4.7.3 Collagen -I*

Collagen-I (Col-1) is the most ubiquitous collagen present in bone, skin, tendon, ligament and other soft tissues (Sussman, 1988). Col-1 is a well-characterized fibrillar collagen consisting of two $\alpha 1$ and one $\alpha 2$ polypeptide chains. As the predominant collagen of bone produced by osteoblasts, Col-1 makes up approximately 90% of the total bone organic matrix (Kaplan *et al.*, 1994). In addition, in-situ hybridisation of avian embryonic skeletal tissue has revealed distribution of Col-1 mRNA in perichondrium and periosteum (Nalin *et al.*, 1995). It is also expressed by undifferentiated mesenchymal progenitor cells prior to chondrogenesis and osteogenesis (Kosher *et al.*, 1986; Noth *et al.*, 2002; Sandell *et al.*, 1994; Sandell *et al.*, 1998). In culture, chondrocytes undergoing dedifferentiation (a process where cells lose cell interaction and become unstable) assume a Col-1-positive

phenotype similar to that of their undifferentiated mesenchymal cell precursors, where expression of collagen II (Col-2) is switched back to Col-1 (Benya *et al.*, 1977).

Although Col-1 mRNA was not detected in any chondrocytes of bovine growth plate by in situ (Sandell *et al.*, 1994), it was localized in a group of cells with mesenchymal phenotype attached to calcifying cartilage septa in the region of vascular invasion. Furthermore recent microarray analysis of rat growth plate zones identified the Col-1 mRNA expression at the hypertrophic zone (Wang *et al.*, 2004). These results may allow us to postulate that at the hypertrophic zone as some cells undergo apoptosis and become calcified, some cells may lose their chondrogenic phenotype and regain Col-1 production.

During bone fracture repair, spatial Col-1 mRNA expression was detected in the mesenchymal progenitor cells and osteoblasts undergoing intramembranous ossification at the adjacent periosteum, and in hypertrophic chondrocytes and osteoblast undergoing endochondral bone formation at the bone injury site (Einhorn, 1998; Jingushi *et al.*, 1992). The temporal expression analysis of Col-1 mRNA further showed Col-1 up-regulation starting from early osteogenic through to remodelling during bone fracture healing (Cho *et al.*, 2002; Kon *et al.*, 2001). In addition, Col-1 expression was also detected in mesenchymal osteoprogenitor cells of fibrous tissue prior to bone formation at the bone injury site, and is further up-regulated during the osteogenic and remodelling events of some intramembranous bone healing models (Chiba *et al.*, 2001; Kuroda *et al.*, 2005). Furthermore the expression of Col-1 by cells with a chondrocyte phenotype in fracture callus may suggest an ongoing change in expression of collagen (Einhorn, 1998).

Mutations in regions of Col-1 result in osteogenesis imperfecta, a genetic disease with decrease bone mass and bone fragility. For example, transgenic mice with a partially deleted Col-1 $\alpha$1 chain have spontaneous fractures and display reduced bone collagen and mineral production (Pereira *et al.*, 1993). In addition, other mutations in the Col-1 $\alpha$2 chain have been shown to display some form of osteoporosis, where there is more bone

resorption than formation resulting in bone fragility (Spotila *et al.*, 1994; Spotila *et al.*, 1991). This indicates that Col-1 is an important bone matrix protein for bone maintenance.

*1.4.7.4 Osteocalcin*

Osteocalcin, also known as α-carboxyglutamic acid containing protein (bone Gla protein), is a 5.7KD, vitamin K-dependent bone matrix protein. The carboxylation of three glutamic acid residues on osteocalcin due to vitamin K dependent posttranslational modification converts this protein into a calcium and mineral binding protein (Kaplan *et al.*, 1994). Although osteocalcin has been reported to be uniquely synthesized by osteoblasts and osteocalcin transcripts are detected throughout trabecular bone of growth plate, perchondrium and periosteum (Ferguson *et al.*, 1999), it has also been identified in the hypertrophic region of growth plate (Tchetina *et al.*, 2003; Xian *et al.*, 2004).

Osteocalcin accounts for 10-20% of non-collagen protein of the bone, and is a marker of mature osteoblasts. Osteocalcin gene is uniquely expressed during osteogenic induction of mesenchymal progenitor/ osteoprogenitor cells in vitro, indicative of osteoblastic differentiation (Noth *et al.*, 2002; Sun *et al.*, 2003). In addition, osteocalcin is often used to examine the osteogenic event during repair of bone fractures and growth plate injuries in rodent models. For example, up-regulation in osteocalcin expression has been shown to coincide with the osteogenic event of fracture healing in murine model (Cho *et al.*, 2002). Furthermore, osteocalcin mRNA was detected in the periosteum and in mesenchymal cells in the fracture hematoma three days post fracture. Osteocalcin was also found strongly expressed on the periphery of soft callus, and within osteoblasts of newly formed woven bone but not in cartilage islands six days post-fracture (Ferguson *et al.*, 1999). In rat growth plate drill-hole injury model, osteocalcin was detected by immunohistochemistry in osteoblasts, bone lining cells and osteocytes trapped in the osteoid at the injury site during bone bridge formation and maturation (Xian *et al.*, 2004).

Although osteocalcin is used as an indicator of bone formation in *in vivo* and *in vitro* studies, its function is not clear. Osteocalcin is thought to play a role in mineralization of bone matrix, as rats treated with vitamin K inhibitor, Warfarin, showed reduction in osteocalcin levels in bone and abnormalities in the mineralization of several cartilages (Price, 1989). However the treatment did not affect the overall bone structure, which suggests osteocalcin may not be involved in maintaining bone integrity and density. Furthermore, osteocalcin–deficient mice developed higher bone mass and bones of improved functional quality without impairing bone resorption (Ducy *et al.*, 1996). This contradicts previous *in vitro* studies suggesting the role of osteocalcin in attracting osteoclasts to the site of bone resorption (Glowacki and Lian, 1987; Malone *et al.*, 1982).

## 1.5 Bone Formation

Bone formation during skeletal development occurs through two distinct processes. Endochondral ossification is a process where an intermediate cartilaginous framework such as the growth plate of long bones is replaced by bone matrix by osteoblasts in the metaphysis. Intramembranous ossification is the direct bone formation process on a collagenous framework from bone cells derived from mesenchymal precursor cells in the periphery of long bones (periosteum) and in skull and facial bone development. Bone remodelling is essential to maintain strong healthy bone structure and function. In addition, regulation between osteogenesis and osteoclastogenesis is critical to ensure appropriate balance between bone formation and bone resorption during the remodelling event.

### 1.5.1 Endochondral Ossification

The first step of endochondral ossification during skeletal development is the aggregation and condensation of mesenchymal cells into cartilaginous limb buds, which requires transcription factor Sox9 (described in detail in section 1.7.3.1). This begins at

approximately embryonic day 12 (e12) in mice, and the expression of Col-2 and detection of a cartilaginous matrix within the future skeletal element indicates that these cells are committed to a chondrogenic lineage (Ferguson *et al.*, 1999). These chondrocytes will proliferate and then reach hypertrophy, and ultimately undergo apoptosis, while the cartilage extracellular matrix (ECM) including collagens and proteoglycans is mineralised and resorbed. As the vascularisation of the calcified cartilage occurs, the resorbed cartilage ECM is replaced by newly formed bone, which is the final stage of endochondral ossification (**Figure 1.5.1**).

The degradation and vascularization of cartilage depend on the action of matrix metalloproteinases (MMPs). MMP13 has been shown to regulate remodelling of the hypertrophic cartilage matrix (Johansson *et al.*, 1997), and MMP9 mediates vascular invasion in hypertrophic cartilage callus (Colnot *et al.*, 2003; Vu *et al.*, 1998). In addition, vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis by stimulating invasion of blood vessels into the hypertrophic growth plate cartilage, and is involved in promoting endochondral bone formation of long bones (Ferrara and Davis-Smyth, 1997; Gerber *et al.*, 1999; Zelzer *et al.*, 2002)). VEGF transcripts were abundant in hypertrophic and apoptotic chondrocytes during limb formation and fracture healing (Colnot and Helms, 2001; Ferguson *et al.*, 1999; Tatsuyama *et al.*, 2000). In addition, regulation of VEGF expression during bone development requires function of transcription factor core binding factor (cbfa1) (described in detail in section 1.7), since cartilage angiogenesis and up-regulation of VEGF expression in hypertrophic chondrocytes and VEGF receptor expression in the perichondrium did not occur in cbfa1 deficient mice (Zelzer *et al.*, 2001). During postnatal development, the endochondral process continues through the growth plate, which allows replacement of cartilage with bone, leading to bone elongation. A similar process is induced again in fracture cartilaginous callus

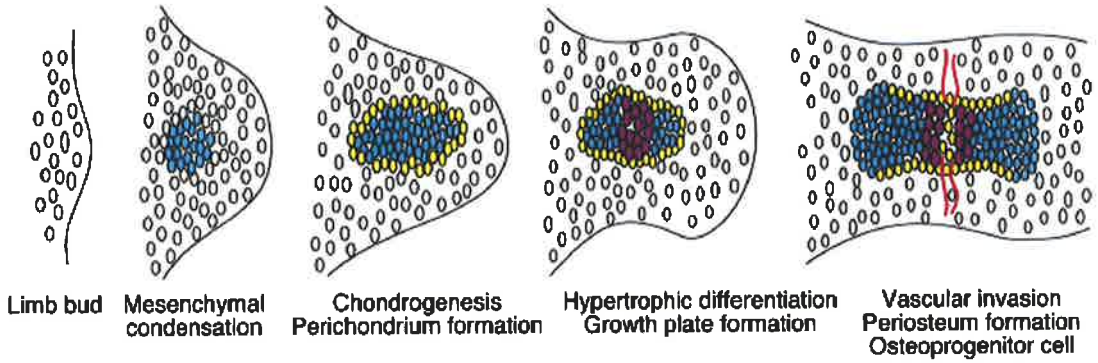


**Figure 1.5.1. Illustration of endochondral bone process during skeletal development.**

A mesenchymal condensation is firstly formed with chondroprogenitors expressing Sox9 and Col-2 (blue). Secondly, chondrogenesis involve proliferation of chondrocytes, followed by hypertrophic differentiation with mature hypertrophic chondrocytes expressing Col-10 (purple). A perichondrium (yellow) is formed around the developing growth plate. Finally, endochondral bone formation occurs with vascular invasion of growth plate and formation of primary ossification centre containing osteogenic cells expressing Col-1 (yellow). A periosteum (yellow), which gives rise to the membranous cortical bone is also developed adjacent to the endochondral bone. (Extracted from Ornitz DM & Marie PJ (2002) Gene Dev (16: 1446-1465).

during fracture repair (however it is more disorganised compared to growth plate) (Gerstenfeld *et al.*, 2003).

The regulatory mechanism of enchondral ossification process is complex and involves signalling of a large group of endocrine/paracrine/autocrine factors such as hormones, transcription factors, growth factors and vitamins (Ferguson *et al.*, 1999; Forriol and Shapiro, 2005; Tchetina *et al.*, 2003; Wang *et al.*, 2004; Xian and Foster, 2006). Only the functional roles of parathyroid hormone (PTH)-related peptide (PTHrP), and indian hedgehog (ihh) in signalling the endochondral ossification process (reviewed in de Crombrugghe *et al.*, 2001; Shum and Nuckolls, 2002) will be discussed here. Through genetic studies, signalling of PTH/PTHrP and its receptor PPR has been identified to be essential for the phosphorylation or activation of chondrogenic transcription factor Sox-9, and to promote bone elongation through increasing the size of the proliferative zone and preventing chondrocyte maturation from prehypertrophic chondrocytes to the hypertrophic chondrocytes (Huang *et al.*, 2001; Karaplis *et al.*, 1994; Lanske *et al.*, 1996; Weir *et al.*, 1996). The spatial mRNA expression pattern of PTHrP, where it is up-regulated in the early proliferative zone and prehypertrophic zone but then down-regulated in hypertrophic zone, is consistent with its function in normal bone growth (Tchetina *et al.*, 2003). The expression of PTHrP is dependent on ihh, a secreted polypeptide expressed by hypertrophic chondrocytes (St-Jacques *et al.*, 1999), and signalling of ihh at the hypertrophic zone by its patched receptor stimulates PTHrP expression. PTHrP then binds to its receptor PPR, which is highly expressed on prehypertrophic chondrocytes to inhibit the maturation of proliferating and prehypertrophic chondrocytes into hypertrophic chondrocytes (van der Eerden *et al.*, 2000; Vortkamp *et al.*, 1996).

Indian hedgehog is also needed for normal chondrocyte proliferation and for the establishment of characteristic columns of proliferating chondrocytes in the growth plate through a pathway independent of PTHrP (Karp *et al.*, 2000). In addition, studies in ihh

null mice have shown that ihh signalling is required for cbfa1 expression and osteoblast differentiation of mesenchymal cells from the periosteum and perichondrium in only endochondral bone but not in membranous bone (St-Jacques *et al.*, 1999). Therefore, since the function of ihh is uniquely specific, it is often used as a marker for endochondral bone formation (Ferguson *et al.*, 1999; Lee *et al.*, 2000).

### 1.5.2 Intramembranous Ossification

Intramembranous ossification, as depicted in **Figure 1.5.2**, is a process involving direct differentiation of condensed mesenchymal cells into cbfa1-positive osteoprogenitor cells and then into mature osteoblasts that express bone proteins collagen-1 (Col-1) and osteocalcin, and mediate bone matrix synthesis along the bone margins. Once bone matrix synthesis is complete, these osteoblasts eventually die by apoptosis or become inactive osteocytes that are embedded in the bone matrix. Bone types that are formed through this mechanism include the flat bones of the skull, parts of the clavicles, and the diaphyseal cortex (Erlebacher *et al.*, 1995; Ornitz and Marie, 2002). This direct bone formation process without a cartilage intermediate and also involved during both bone and growth plate fracture repair is mainly controlled by bone transcription factor cbfa1 (Otto *et al.*, 1997), and is characterised by up-regulation in expression of osteogenic markers such as cbfa1, osteocalcin, and alkaline phosphatase and lack of expression in chondrogenic markers such as Col2, Col-10 and ihh (Le *et al.*, 2001; Lee *et al.*, 2000; Thompson *et al.*, 2002; Xian *et al.*, 2004). The process of intramembranous bone healing and the involvement of cbfa1 in controlling osteoblast differentiation are described in section 1.6 and 1.7, respectively.

### 1.5.3 Bone remodelling

This process occurs continuously in the normal skeleton and it differs depending on

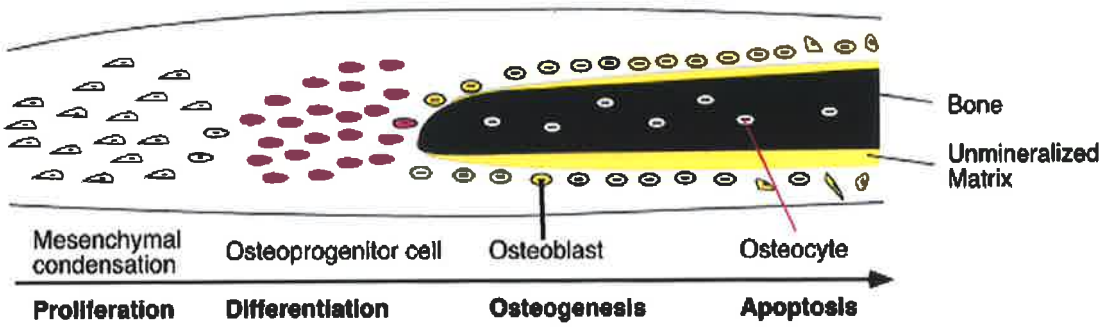


**Figure 1.5.2. Illustration of intramembranous ossification process.**

Mesenchymal cells are differentiated into cbfa1-expressing osteoprogenitor cells (pink), followed by their progression into mature osteoblasts that express bone matrix proteins Col-I and osteocalcin (yellow), and deposit and mineralise bone matrix. These osteoblasts either undergo apoptosis or become osteocytes embedded in the bone. Extracted from Ornitz DM & Marie PJ (2002) Gene Dev (16: 1446-1465).

location. Remodelling of woven bone can occur on the surface of the trabeculae as the required cells are near the blood supply, whereas in compact bone they are not, and osteoclasts are required to tunnel through the hard bone via remodelling systems known as cutting cones or cutter head (**Figure 1.5.3**). The pathways generated by the osteoclasts for the penetration of blood vessels are accompanied by the recruitment and differentiation of endothelial cells and mesenchymal cells into osteoblasts, which leads to new osteoid production of lamellar bone in the resorption cavity (Einhorn, 1998; McKibbin, 1978).

The two major cell types required for bone remodelling are osteoclasts and osteoblasts (described in section 1.4). The functions of these cell types are tightly regulated to ensure that the appropriate amount of bone resorption by osteoclasts is accompanied by simultaneous laying down of new bone matrix by osteoblasts. However, as demonstrated from human and murine genetic studies, osteoclast and osteoblast functions are regulated by independent pathways, since in the absence of osteoclast function, the osteoblast function was not affected and bone matrix was deposited normally (Karsenty, 1999). This was also the case in the study with osteopenic transgenic mice where bone resorption occurred normally while bone formation was completely arrested (Corral *et al.*, 1998). Never the less, the correct balance between the amount of osteoclastogenesis and osteogenesis during bone remodelling is essential for maintenance of healthy functional bone.

Although the signalling pathway of osteoblasts and osteoclasts are completely independent from each other, it is generally believed that the osteoblast activity and osteoclast activity are linked during the bone remodelling. The main signalling molecules responsible for controlling osteoclastogenesis during normal bone remodelling are M-CSF, receptor activator of NF-kappaB (RANK) ligand (RANKL) (also called osteoclast differentiation factor ODF) and osteoprotegerin and osteogenesis during bone remodelling is essential for maintain healthy functional bone. M-CSF produced by stromal cells

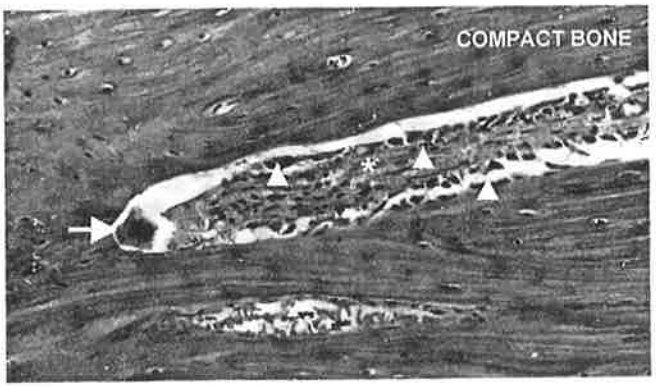


**Figure 1.5.3. Compact bone remodelling system known as cutting cones**.

Also known as cutter head, this system involve osteoclasts (arrow) tunnelling through the cortical compact bone to generate the way for in-growth of blood vessels which is followed by the infiltration of undifferentiated spindle like mesenchymal cells (*) that have the capacity to differentiate into osteoid-producing osteoblasts (arrow heads). Adapted image from McKibbin B (1978) J Bone Joint Surg (Br) (**60B**: 150-162)

and osteoblasts is an essential growth factor to activate the osteoclast differentiation process of mononuclear cells that are committed to osteoclast lineage by binding to their M-CSF receptor (c-fms) (**Figure 1.5.4**). This was demonstrated in mice with inactive M-CSF mutations, where they developed macrophages normally but lack osteoclast formation (Yoshida *et al.*, 1990). In addition, osteoclast differentiation factor RANKL, synthesised by bone marrow stromal cells, osteoblasts and T cells, and found as a soluble molecule in the bone environment (Karsenty, 1999; Teitelbaum, 2000) is required to interact with its receptor (RANK) expressed on osteoclast precursors (Lacey *et al.*, 1998) to stimulate osteoclast differentiation and activation, thus inducing the mineralised bone resorption process (**Figure 1.5.4**). This was further demonstrated in separate genetic studies using 1) RANKL deficient mice (Kong *et al.*, 1999); and 2) transgenic mice expressing soluble receptor RANK (Hsu *et al.*, 1999), where both indicated that these mutant mice lack osteoclast formation and developed severe osteopetrosis due to loss of osteoclast function.

On the other hand, OPG, a decoy receptor for RANKL, inhibits osteoclast differentiation by blocking the RANKL-RANK interaction (Simonet *et al.*, 1997; Tsuda *et al.*, 1997; Yasuda *et al.*, 1998; Yasuda *et al.*, 1998) (**Figure 1.5.4**). This molecule is important in controlling the number of osteoclasts formed and rate of bone resorption, as mice deficient in OPG expression developed osteoporosis, a disease characterised by a relative increase of bone resorption over bone formation (Bucay *et al.*, 1998; Mizuno *et al.*, 1998). In addition, over expression of OPG in transgenic mice resulted in loss of osteoclast function and development of non-lethal osteopetrosis over a long period of time (Simonet *et al.*, 1997).

Correct regulation of M-CSF/c-fms, and RANK-L/RANK or OPG signalling pathways will ensure the appropriate amount of bone resorption accompanied by bone formation

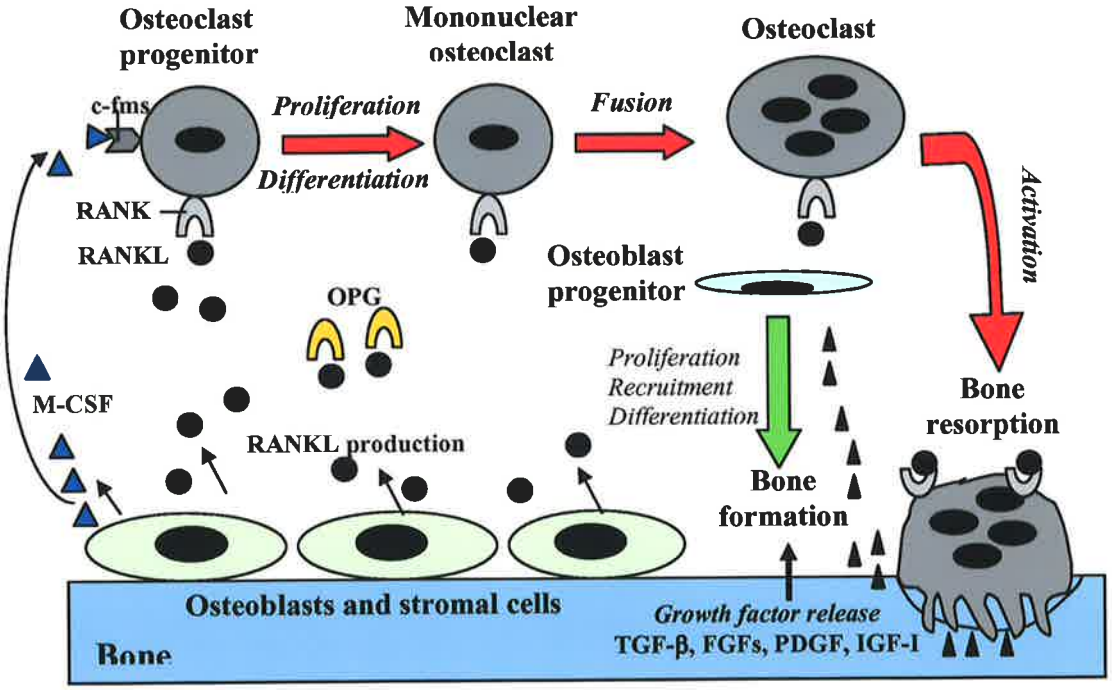


**Figure 1.5.4. Signalling activities between osteoclast and osteoblast during bone remodelling.**

Osteoblasts and stromal cells produce RANK-L and M-CSF which are required to stimulate osteoclast differentiation by binding to their respectively receptors RANK and cfms, present on mononuclear cells committed to ostoeoclast lineage. The RANK-L /RANK signalling is required to further stimulate osteoclast activation, thus inducing the osteoclast bone resorption process. The proteolytic degradation of the bone matrix induced by osteoclasts results in the release and activation of growth factors stored in the bone reservoir such as TGF-β, FGFs, PDGF and IGF-I to further stimulate recruitment, proliferation and differentiation of osteoprogenitor cells to become osteoid forming osteoblasts. A soluble decoy receptor of RANK, OPG, tightly regulates the osteoclast differentiation process by binding to the free RANK-L and antagonising RANK function , which inhibits osteoclast formation. Illustration was kindly provided by Dr. CJ Xian, adapted from Xian CJ and Foster BK (2006) Fractures in children ($6^{th}$ edition), pg 21-50.

occurs during the normal remodelling event. The growth factors such as TGF-β, FGFs, PDGF and IGF-I which are activated and released from the bone matrix during bone resorption by osteoclasts to promote bone formation, along with the main transcription factor cbfa1 essential for regulating osteoblast differentiation, will be discussed in section 1.7.

## 1.6 Bone and Growth Plate Fracture Repair

In general, wounding healing processes of damaged tissues involve: 1) a coagulation phase that occurs within a few minutes of injury to reduce blood loss which involves the formation of a hematoma; 2) an inflammatory phase that lasts several hours to days to remove necrotic debris; 3) a granulation phase that occurs for several days to recruit fibroblasts to produce fibrous tissue; 4) a scar formation phase that last for years (even longer for children) which involves remodelling of scar (Ogden, 2000). Unlike skin wounds due to burns and deep cuts where repair results in formation of fibrous scar tissue, the repair process of bone fracture healing results in the regeneration of the original tissue (which in this case is bone). In the case of growth plate fracture, rather than regeneration of growth plate cartilage, the natural healing process often results in the formation of bony tissue at the injury site, which resembles the direct membranous bone formation process during fracture healing (Chiba *et al.*, 2001; Lee *et al.*, 2000; Xian *et al.*, 2004). The injury responses to bone fracture healing as well as to growth plate injury will be described in this section. In addition, the current treatments for growth plate injury-induced bone growth deformity and future strategies in regeneration of growth plate will be briefly discussed.

### 1.6.1 Bone Fracture Healing Responses

The bone fracture healing or regeneration process in both adults and children may be divided into three sequential but overlapping phases: 1) inflammatory phase; 2) reparative/callus formation phase; 3) remodelling phase, shown in **Figure 1.6.1**. In

children (depending on their age) the remodelling event is a more prolonged and physiologically more active process compared to same event in adults (Ogden, 2000; Xian and Foster, 2006). From bone fracture/injury studies using animal models, the duration of each event and the specific type of events that take place at particular time points after injury vary between different experiments and bone injury models as described in **Table 1.6.1**.

*1.6.1.1 Inflammation Phase*

Inflammation occurs immediately after any type of tissue injury, which includes skeletal fractures. This phase can last for several days depending on the severity of the injury. As reviewed in Ogden (2000), within minutes after bone fracture a coagulation event occurs through a cascade of clotting (vasoconstriction and hemostasis) mechanisms to reduce blood loss due to bleeding from damaged periosteum, contiguous and external soft tissues. This immediate physiological response leads to the accumulation of a hematoma at the fracture site in the medullary canal, and underneath the elevated periosteum or extraperiosteally if the periosteum is disrupted as shown in **Figure 1.6.1** (Ogden, 2000). The formation of hematoma and fibrin clot is accompanied by influx and chemotaxis of inflammatory cells including polymorphonuclear (PMN) leukocytes (neutrophils), lymphocytes, platelets, wandering histocytes, mast cells and monocytes at the fracture site to facilitate cleaning up and degradation of damaged tissues (necrotic debris), foreign bodies and bacteria, as usual in an acute inflammation response (McKibbin, 1978). Enzymes, mainly collagenases such as MMPs, are synthesised by inflammatory cells and PMNs to digest and break down debris. Monocytes in the injury site also fused to form activated macrophages, which are some of the main phagocytes of debris, bacteria and dead tissues. As the fibrin clot is organised into granulation/fibrous tissue surrounding the fracture site, different growth factors and

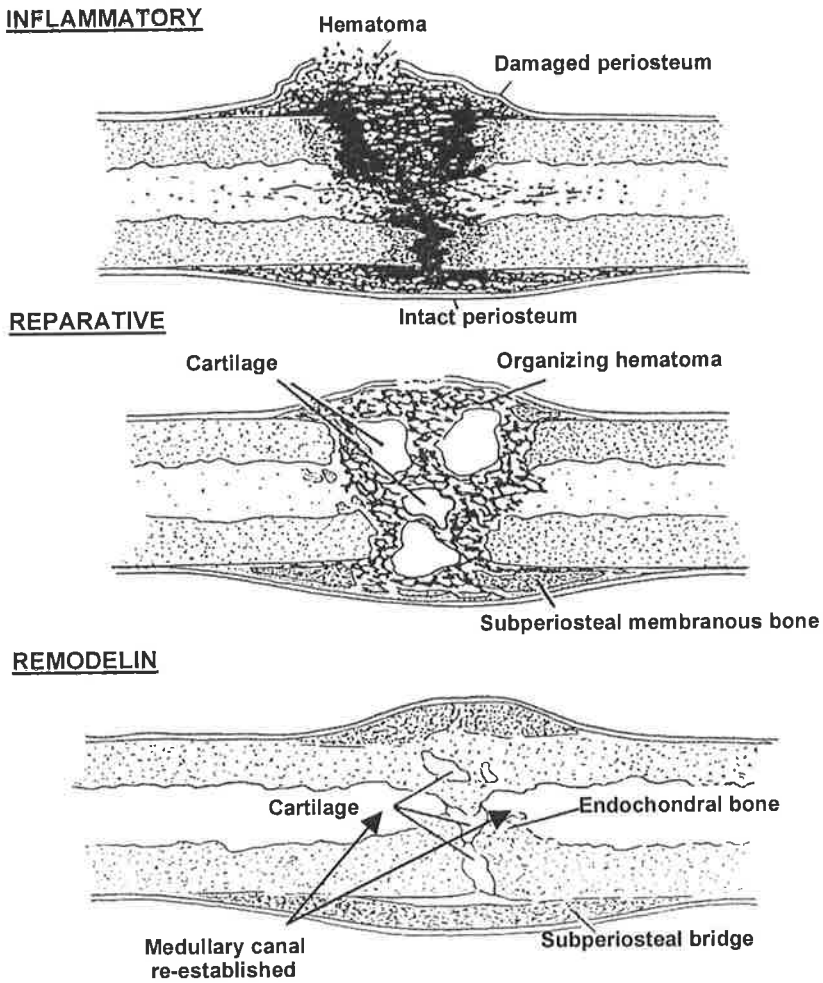


**Figure 1.6.1. Graphic representation of long bone fracture healing events.**

During the inflammatory phase the hematoma filling the fracture site is organised into fibrous tissue. During the reparative phase the organizing hematoma is replaced by cartilage-like soft fracture callus, which will undergo endochondral ossification, as the hard callus at the periosteum forms the subperiosteal bone via intramembranous ossification. During the remodelling phase, cartilage material is replaced by new endochondral bone and is further remodelled to the existing cortical structure, as the medullary canal is re-established. Diagram is adapted from Ogden JA (2000) Skeletal injury in the child (3rd edition), pg 243-268.

**Table 1.6.1.** Specific injury responses to bone injuries and their variable durations in different animal experimental bone injury models.

| | | | The approximate period of each response after injury reported in the following fractue healing studies | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Type of bone injury model* | *Species* | *Reference* | *Inflammation* | *Granulation* | *Intramembranous* | *Chondrogenesis* | *Endochondral* | *Remodeling* |
| Simple transverse fracture | Rat | Joyce *et al*., 1990 | Day 1-3 | Day 2-7 | Day 3-12 | Day 7-12 | Day 12 on wards | N/D |
| Simple transverse fracture | Murine | Fujii et al., 1999 | Day 2-4 | Day 2-4 | Day 4-7 | Day 7-14 | Day 14-21 | Day 21-28 |
| Simple transverse fracture | Rat | Tatsuyama et al., 2000 | Day 1-3 | Day 2-6 | Day 2-14 | Day 3-10 | Day 7-14 | N/D |
| Simple transverse fracture | Murine | Kon *et al*., 2001 | Day 1-3 | Day 3-7 | Day 7-14 | Day 7-14 | Day 14-21 | Day 21, 28 onwards |
| External fixation of ST | Murine | Thompson et al., 2001 | Day 3 | Day 3-4 | Day 4-21 | none | none | Day 21 onwards |
| Marrow ablation | Rat | Kuroda et al., 2005 | Day 1-5 | Day 3-5 | Day5-10 | none | none | Day 10-14 |
| Drill hole (Diaphyseal) | Rat | Chiba et al., 2001 | Day 3 | Day 3-7 | Day 7-14 | none | none | Day 14-21 |
| Drill hole (growth plate injury) | Rat | Xian et al., 2004 | Day 1-3 | Day 3-7 | Day 7-14 | none | none | Day 14-35 |

ST - simple transverse fracture; N/D - not determined

cytokines produced by inflammatory cells including macrophages and platelets regulate migration and proliferation of fibroblasts, periosteal and mesenchymal cells, and their subsequent differentiation into osteoblasts or chondrocytes in a paracrine and autocrine manner (see section 1.7) (Bolander, 1992; Szczesny, 2002). These newly infiltrated and expanded fibrous-like cells contribute in the production of ground substances and matrix proteins including proteoglycans, collagens, fibronectin and laminin, which make up the cellular matrix for the successive healing process.

### *1.6.1.2 Reparative Phase*

The reparative event varies depending on the type of fracture, mechanical environment (rigidity of fracture fixation), and presence/absence of angiogenesis (Ferguson *et al.*, 1999; Thompson *et al.*, 2002). When simple transverse fractures in the diaphysis or metaphysis of long bones such as the the femur and tibia (created by pure bending) are not treated with sling or cast immobilization, external fixation, or intramedullary fixation procedures, some degree of motion and disruption of angiogenesis occurs. The reparative phase (**Figure 1.6.1**) involves a combination of intramembranous bone formation of hard callus at the adjacent periosteum, and endochondral ossification of external soft callus (fibrocartilage) at the fracture site (Einhorn, 1998; Ogden, 2000; Ostrum *et al.*, 1994)

A standard closed/simple transverse fracture model, which is reliable, reproducible and clinically relevant to fracture healing, was originally developed in rats (Bonnarens and Einhorn, 1984) or mice (Hiltunen *et al.*, 1993). This model, involving insertion of an intramedullary pin into the rat femur or mouse tibia and the production of the fracture in preferably the centre of diaphysis with a blunt guillotine driven by a drop weight, is the most extensively used technique in the research on cellular and molecular mechanisms that regulate fracture repair in small rodent models. Following the initial inflammation and

granulation phase, which lasts for approximately 4-7 days, the mesenchymal cells recruited to and periosteal cells existing at the adjacent periosteum undergo osteoblast differentiation and bone matrix protein synthesis from day 4 post fracture, which leads to immature trabecular woven bone formation by day 7 (Bolander, 1992; Fujii *et al.*, 1999; Joyce *et al.*, 1990; Kon *et al.*, 2001; Tatsuyama *et al.*, 2000). Meanwhile, dense mesenchymal cells in the external soft callus at the fracture site in a relatively anti-angiogenic environment, differentiated into chondrocytes with high levels of chondrocyte marker Col-2 expression detected (Sandberg *et al.*, 1989), a process that persists until the fibrous tissue is replaced by cartilage. This cartilage formation process provides temporary stabilisation of the fracture, and as mature chondrocytes reach hypertrophy and the matrix is mineralised then resorbed, the angiogenic response is re-established, followed by subsequent osteoblast invasion and bone matrix synthesis. Although endochondral bone formation during bone development and bone fracture repair share similar genetic mechanisms (Ferguson *et al.*, 1999), fracture cartilage callus is structurally different from the growth plate cartilage, which further reflects their different functional roles. As demonstrated in **Figure 1.6.2**, chondrocytes in growth plate are more organised compared to those in fibrocartilage of fracture callus, and hypertrophic chondrocytes of growth plate are much larger in volume than those at the chondro/osseous junction in the fracture callus (Gerstenfeld *et al.*, 2003). Growth plate, a highly organised structure, functions in bone elongation through even contributions of cellular proliferation, increasing chondrocyte volume and in matrix deposition, which is a slow process in mammals. Since the cartilage component of the fracture callus functions in stabilising the fracture site and is the template for new rapid bone formation rather than bone lengthening, rapid growth of fracture callus is achieved through predominantly chondrocyte proliferation while increases in cell and matrix volume are less important contributors (Barreto and Wilsman, 1994; Gerstenfeld *et al.*, 2003).

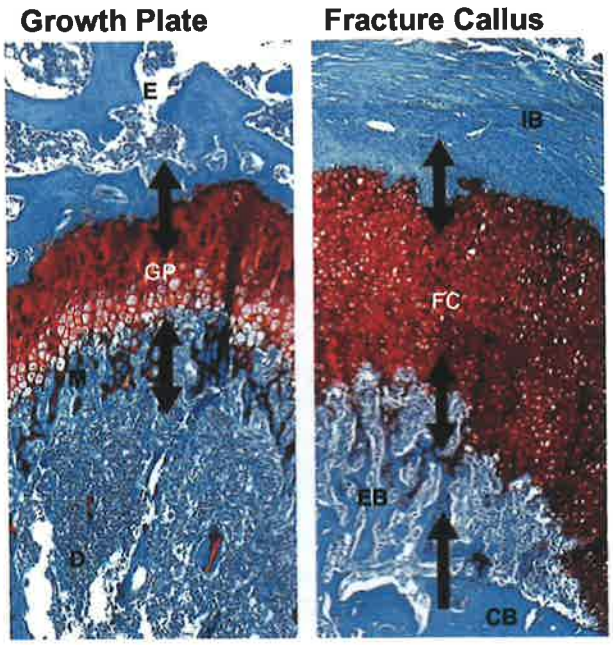


**Figure 1.6.2. Cellular and structural comparison of the postnatal growth plate and fracture callus during their respective endochondral progression.**

The histological sections presented were stained with safranin Orange to distinguish the cartilage structure from the fast green stained bone. Arrows indicate potential sites of interaction with various tissue types. The growth plate **GP** directly below the epiphysis **E** undergoes endochondral ossification to give rise to the metaphyseal bone **M**. Accumulation of marrow space occurs in the diaphyseal bone **D**. The fibrocartilage **FC**, below the adjacent intramembranous periosteal bone **IB** of the fracture callus, undergoes endochondral ossification to give rise to endochondral bone **EB**, which extends to the original cortical bone **CB**. Image adapted from Gerstenfeld LC *et al.* (2003) J Cell Biochem (**88**: 873-884).

In the case when a fracture is rigidly fixed where interfragmentary movement is reduced and angiogenesis is not disturbed, ossification occurs through direct intramembranous route without formation of the cartilage intermediate. The cellular and molecular mechanisms underlying the intramembranous bone regeneration process of a stable fracture have been reported in literature using bone defect healing models such as: 1) Rat femoral marrow ablation model, which involves the disruption of the bone marrow content in the medullary canal through the patellar groove with a 2mm drill bit without damaging the cortical bone (Kuroda *et al.*, 2005; Suva *et al.*, 1993); 2) Rat femoral diaphyseal bone drill hole model, which involved removing the periosteum of diaphysis and electrically drilling a hole with a 1.8mm drill bit through the cortex to the medullary cavity without penetrating the opposite side (Chiba *et al.*, 2001; Mueller *et al.*, 1991); 3) External fixation of mouse tibial transverse fracture model involves fixation of transverse fracture with two custom-made external aluminium rings stabilized by three stainless steel threaded rods, with the frames secured to the tibia by 0.25mm insect pins (Le *et al.*, 2001; Thompson *et al.*, 2002). In these bone defect models, after the immediate initial inflammatory response within 3 days, recruitment of mesenchymal cells (which expressed osteogenic markers (osteocalcin and Col-1) on day 4 to the injury site) proceeds as the hematoma is replaced by fibrous tissue by day 7. Formation of trabecular bone by osteoblasts starts from day 7 until remodelling occurs around day14 but earlier for the marrow ablation model (day 10) (**Table: 1.6.1**). Chondrogenic and endochondral processes were not detected at the injury site in these models, as determined by lack of safranin Orange staining (which stains for cartilage matrix), absence of chondrocyte phenotype and Col-2 and -10 expressions using histological and molecular analysis (Chiba *et al.*, 2001; Thompson *et al.*, 2002).

*1.6.1.3 Remodelling Phase*

The final stage of fracture healing is the remodelling phase, which takes the longest amount of time. This phase allows the bridging of the fracture to be restored to its original state. Bone turnover is also a continuous process of the normal skeleton that occurs in response to normal stresses of body weight, muscle action, and joint reaction forces as well as intrinsic control mechanisms such as the periosteum (Xian and Foster, 2006). Similar cellular mechanisms of bone remodelling are utilised in fracture repair and normal bone turnover. This remodelling process involves simultaneous removal of existing bone matrix by osteoclasts derived from hemopoietic lineage and replacement of new osteoid by osteoblasts recruited from the periosteum or bone marrow with accompanying blood vessels (McKibbin, 1978). The remodelling event during fracture healing is also characterised by increased marrow space within the fracture site (Gerstenfeld *et al.*, 2003).

Remodelling of the fracture callus during simple transverse mid-diaphyseal fracture healing in mouse tibia started on day 14 after fracture, where TRAP positive osteoclasts had been localised on the cartilage and bone trabeculae along with the presence of marrow cells. By Day 21-28 post fracture the area of marrow space tripled as the callus size decreased and the original cortical structure of the bone began to be re-established (Kon *et al.*, 2001). During this event the trabecular bone bridging the fracture segments is replaced by new compact lamellar bone and further remodelled to the existing cortical structure as the medullary canal is restored with marrow space (**Figure 1.6.1**). However, when this type of fracture is stabilised with external fixation frames a much smaller callus that lacks endochondral bone develops at the fracture site, resulting in less remodelling compared to non-stabilised fractures before returning the injured bone to its original shape (Thompson *et al.*, 2002). In the marrow ablation and drill hole bone defect models where marrow contents of the medullary canal were disrupted, the newly woven bone formed at the injury as response to the reparative phase was completely resorbed by multinuclear osteoclasts as

the area was re-established with hematopoietic marrow during the remodelling phase (Chiba *et al.*, 2001; Suva *et al.*, 1993).

All the above described bone injury models indicate that the remodelling phase results in the regeneration and restoration of the original tissue morphology. However in some instances where the fracture is not stabilised properly (particularly in adults) and excess movement and stress are applied to the fracture, the fracture callus does not undergo endochondral ossification and remains in a chondrogenic state similar to permanent articular cartilage, which results in non-union of fracture (Salisbury *et al.*, 2005). In the case where the fracture segments are not aligned properly, residual angulation of the affected bone may occur and remain permanent in adults. However in children, they have a unique capacity for spontaneous correction of the angulation through re-orientation of the physis. Therefore, the younger the child and the closer the fracture to the physis in distance, the higher the capacity of correction (Ogden, 2000).

### 1.6.2 Growth Plate Injury Responses

The growth plate is the weakest area of the growing long bone. Therefore injuries caused by falls in playgrounds and sports-fields resulting in growth plate damage in children is a common occurrence (up to 18% of all children's bone fractures involve growth plate injuries) (Mizuta *et al.*, 1987; Ogden, 1984; Ogden, 2000; Xian and Foster, 2006). Since growth plate, like the articular cartilage, has limited ability to regenerate itself, different types of injuries to the growth plate, depending on the level at which the fracture occurs in the physis and the locations of the fracture planes, can represent a significant problem for the developing long bone particularly in a young child who has a significant remaining growth period. Salter and Harris (1963) identified 5 types of injuries involving the growth

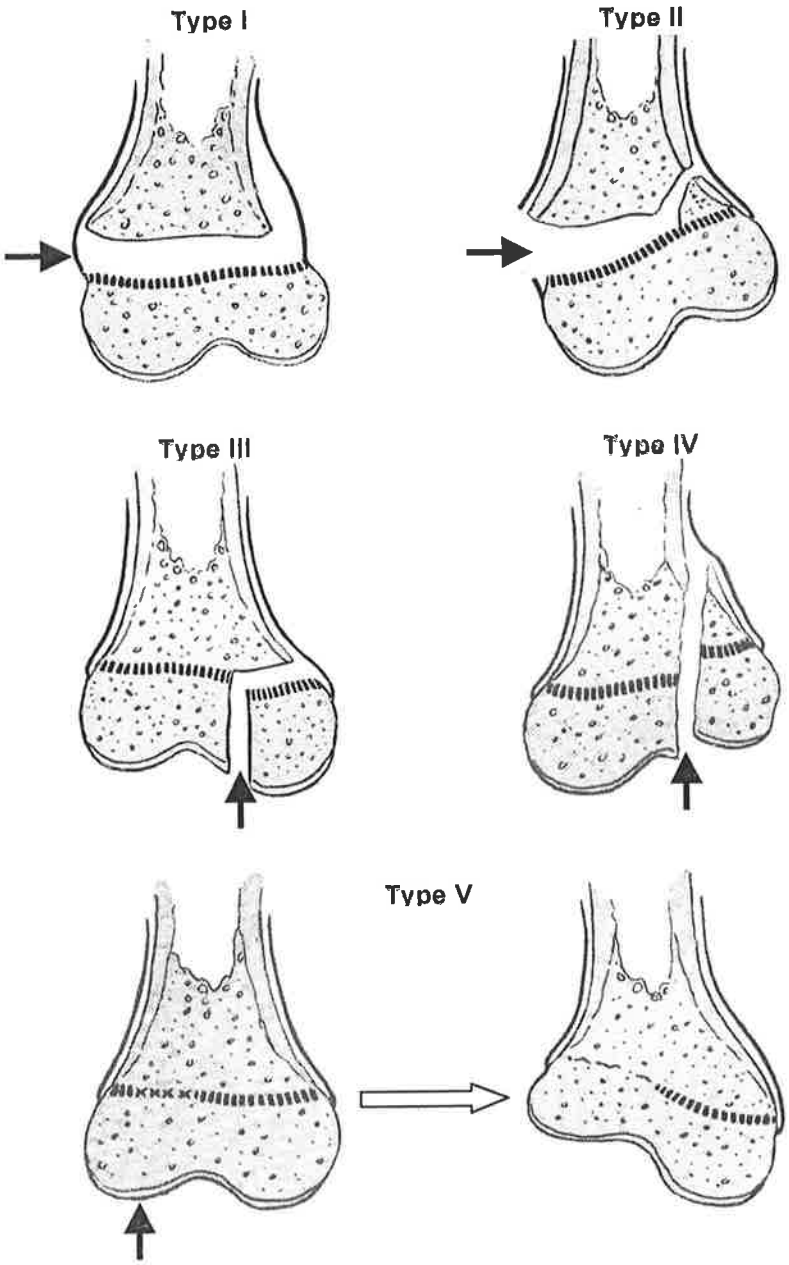


**Figure 1.6.3. Salter and Harris classification of Type I to V growth plate injuries.**

Site of damage is indicated by black arrow. Illustrations adapted from Salter RB and Harris WR (1963), J Bone Joint Surg (Br) (**45A**: 587-622).

plate (as shown in **Figure 1.6.3**). Type I injury involves complete separation of the epiphysis with intact growth plate from the metaphysis with no associated bone fracture, whereas Type II injury has an attached metaphysis bone fragment to the growth plate separated from the metaphysis. Type III injury involves a fracture from the epiphysis joint surface to the growth plate, and then along the hypertrophic zone towards the periphery. Type IV injury involves the fracture from the epiphysis to further extend into the metaphysis. Finally Type V injury occurs when a large compressive force crushes the resting zone cells, which frequently results in premature closure of growth plate (Salter and Harris, 1963). Recent animal experiments with Salter type I physeal fractures showed that when the fracture was contained within the physis where there is no vascular damage, the repair process occur rapidly without major disruption of growth plate structure (Salter and Harris, 1963; Wattenbarger *et al.*, 2002). However when there is a large enough defect and the fracture (particularly Salter Type III and IV injuries) involves the entire width of the growth plate extending from the metaphysis to the epiphysis, the growth plate injury site often has structural disorganization, and formation of vertical bony septa and a bone bridge (Bailey and Dubow, 1981; Lee *et al.*, 2000; Wattenbarger *et al.*, 2002; Xian *et al.*, 2004). When the bone bridge is large enough in the case of Salter Type III and IV injuries, the defect will result in some growth arrest like Salter Type V physeal injury. While growth arrest at the peripheral portions of the physis results in angular deformities, centrally located lesions may cause longitudinal shortening (Ogden, 2000).

Currently the biological mechanisms for regulating the bone bridge formation process are unclear. However, a previous study using a growth plate drill-hole injury model in young mouse tibia has indicated that bone bridge formation does not involve endochondral ossification, since mRNA expressions of Col-2, Col-10, ihh and VEGF, molecules characteristic of endochondral bone formation were not detected by in-situ hybridisation within the disrupted region of the growth plate (Lee *et al.*, 2000). Based on

the murine growth plate injury model, Xian *et al.* (2004) established a proximal tibial drill-hole transphyseal injury model in young rats, to further characterise the injury responses and cellular mechanisms for the bone bridge formation. Interestingly, the injured growth plate histological healing events shown in **Figure 1.6.4** involved recruitment of mesenchymal cells from day 3, osteoblast differentiation and intramembranous formation from day 7, followed by an increase in trabeculae number and size from day 10, and finally bone remodelling with an increase in hematopoietic marrow formation from day 25 and 35 at the injury site (Xian *et al.*, 2004). These events closely resembled those observed in the marrow ablation bone repair model (Suva *et al.*, 1993) and diaphyseal drill hole model (Chiba *et al.*, 2001) (see **Table 1.6.1**). Therefore, using this rat model, Xian *et al* (2004) identified the possible injury responses leading to bone bridge formation after growth plate injury, including the immediate inflammatory phase, the intermediate fibrogenic/granulation phase, and the subsequent osteogenic phase (**Figure 1.6.5**).

In this first stage of cellular response to growth plate trauma, hematoma formation is accompanied by the acute inflammatory phase at the injury site (Arasapam *et al.*, 2006; Chung *et al.*, 2006; Xian *et al.*, 2004). From these studies, an acute transient influx of inflammatory cells at the growth plate injury site as shown in **Figure 1.6.5** including polymorphonuclear neutrophils, monocytes and lymphocytes was seen as early as 8 hours post injury with the inflammatory infiltration peaking by day 1 and subsiding by day 3. In bone fracture repair, this influx of inflammatory cells lasting up to 4 days after injury is not only important for local necrosis and removal of debris and foreign bodies, they are also responsible for the production of various cytokines (interleukins) or growth factors required for regulating subsequent cellular processes of healing (Bolander, 1992; Fujii *et al.*, 1999).

At the injured growth plate, a fibrogenic response occurred from day 3 as shown in

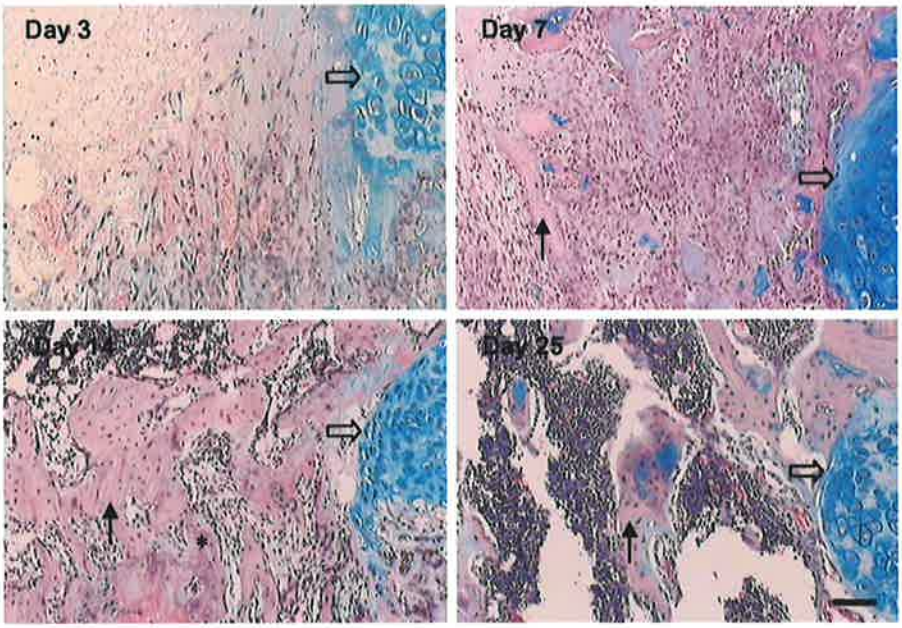


**Figure 1.6.4. Bone bridge formation process at growth plate injury site of rat proximal tibia.**

On day 3 there were no bone trabeculae but mesenchymal infiltrate at the injury site (*). On day 7 Bone bridge trabeculae (indicated by small arrows) appeared at the growth plate injury site (*). On day 14, trabeculae increased in number and enlarged in size and matured on day 25 with well formed marrow. Block arrows indicate adjacent growth plate cartilage. Scale bar = 50 μm. All histology images were kindly provided by Dr. CJ Xian, adapted from Xian CJ *et al.* (2004), J Orthop Res (**22**: 417-426).

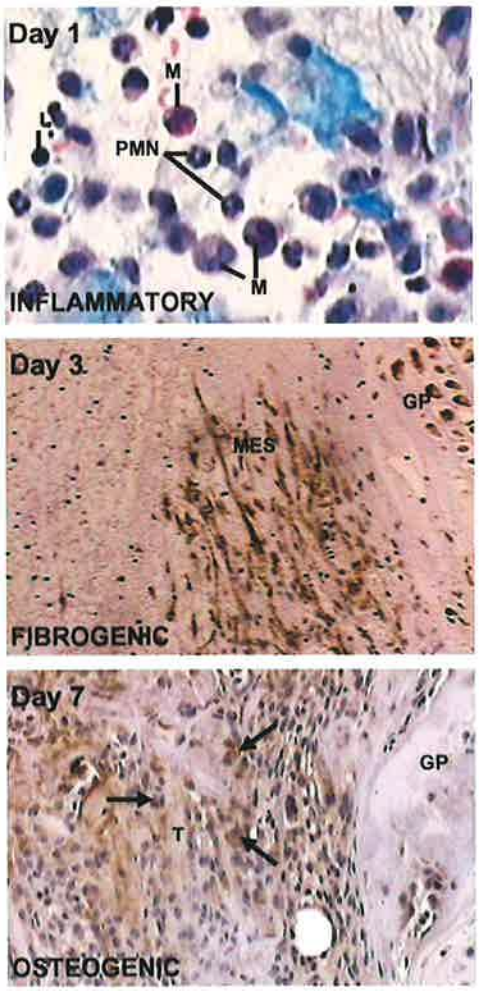


**Figure 1.6.5. The possible injury responses prior to bony bridge formation after growth plate injury.**

The immediate inflammatory phase is identified by the infiltration of inflammatory cells such as monocytes M, neutrophils PMN, and lymphocytes L at the growth plate injury site (*) on day 1. The intermediate fibrogenic phase starts from day 3, where vimentin-positive mesenchymal cells MES begin to migrate into the injury site. The chondrocytes in the adjacent growth plate GP were also vimentin-positive. The subsequent osteogenic event commences from day 7, where some osteocalcin-positive osteoblasts (arrows) surrounding bony trabeculae were localised at the growth plate injury site. The histology image was taken by FH Zhou and the immunohistochemistry images were provided by Dr. CJ Xian adapted from Xian CJ *et al.* (2004), J Orthop Res (22: 417-426).

**Figure 1.6.5**, where infiltration of mesenchymal cells started at the injury site with the mesenchymal population being prominent on days 7 and 10 and subsided on day 14 (Xian *et al.*, 2004). These cells, migrating into the injury site along the edges of the intact metaphysis and epiphysis, were also shown undergoing proliferation as determined by positive BrdU staining. The co-expression of mesenchymal cell marker vimentin with osteoblast differentiation transcription factor core binding factor (cbfa1) on these cells at the injury site prior to bone bridging on day3 and during early osteogenic event on day 7 indicates that they are osteogenic precursors or they are undergoing osteogenic differentiation (Xian *et al.*, 2004). Osteogenic response and formation of bone bridge trabeculae occurred during days 7-25, which involved the formation of osteoblasts from osteogenic precursor cells, maturation of osteoblasts, and formation of bone trabecular and matrix (Lee *et al.*, 2000; Xian *et al.*, 2004).

In addition, prior to and during bone bridging, there was no cartilage proteoglycan metachromatic (alcian blue) staining and no mature chondrogenic marker Col-10 immuno-staining at the injury site, nor was there any up regulation of chondrocyte proliferation detected in the adjacent surrounding growth plate, suggesting a lack of new cartilage formation at the injury site. During bone bridging at the growth plate injury site osteoblast-like cells positive for cbfa1 were detected lining newly formed trabecular bone on days 7 and 10 (Xian *et al.*, 2004). From day 7 onwards, osteoblast differentiation and maturation occurred as indicated by bone matrix protein osteocalcin and alkaline phosphatase immunolocalised on osteoblasts, bone lining cells and osteocytes (**Figure 1.6.5**). These findings suggest that bone bridge formation is through an intramembranous ossification process via the recruitment of mesenchymal derived osteogenic precursor cells, further confirming that the bony bar formation at the growth plate injury site is not through the endochondral bone formation mechanism.

### 1.6.3 Current Treatments For Growth Plate Injury-Induced Bone Growth Defects

Currently there are two surgical approaches to correct growth plate injury-induced bone growth defects: one is by correction of bone length discrepancy by limb lengthening and the other is by removal of bony defect and prevention of bone bridge re-formation. These treatments to date are extremely invasive and painful, mainly involving surgical manipulation of injury-induced defects. One of the oldest and most common clinical procedures used for correction of length discrepancy and angular deformity is through Ilizarov surgery, originally developed by Gavriel Ilizarov in 1951 (Ilizarov and Frankel, 1988). This is a method of distraction osteogenesis, which involves re-fracturing and re-positioning the bone for angular correction, or for formation of new bone in the fracture gap to correct length discrepancy using an Ilizarov external metal frame that pierces through the skin, muscles and bone (Martinez *et al.*, 2002; Xian and Foster, 2006). This procedure is extremely painful, uncomfortable, fracture or infection-prone, and a lengthy procedure, which causes unsightly scars. This may lead to additional surgery to remove infection and to exchange infected pins, longer hospitalisation period, and cosmetic surgery to remove scares. Frames used to be made of stainless steel rings weighing up to 7 kg, but newer models are made of carbon, which though lighter, are equally cumbersome.

Although the Ilizarov procedure for decades was the best option for restoration of bone defects, recent developments in distraction osteogenesis have resulted in hassle-free and less painful alternatives. One method is the use of an intramedullary rod (Fitbone), a fully implantable distraction system, which contains a motorised and programmable sliding mechanism for gradually increasing the distance between two bone segments to allow limb lengthening and bone transport (Baumgart *et al.*, 2005; Singh *et al.*, 2006). This method involves the removal of bone marrow before the device is inserted into the centre of the bone. Then the bone is broken internally and moved apart one millimetre a day via remote control sensor. With the "Fit Bone" procedure, many of the disadvantages and

complications of the old Ilizarov procedure can be avoided or minimized, and therefore it can be tolerated better in patients and improve the quality of life while the process of limb lengthening is being undertaken. However, since this procedure will potentially disrupt growth plate, this "Fit Bone" procedure can only be carried out in adults who have limb length discrepancies (Singh *et al.*, 2006).

Another approach to the treatment of growth plate injury, which prevented partial growth plate closure, was first reported by Langenskiöld (1967). This procedure involves the removal of the defective bony area followed by placement of inert material such as fat to prevent bony bar reformation (Langenskiöld procedure) (Langenskiold *et al.*, 1986). Since its establishment, numerous clinical and experimental studies have shown that it can successfully inhibit bony bridging to various extent using different biological or synthetic interposition materials such as: autologous fat (Foster *et al.*, 2000; Hasler and Foster, 2002; Kim *et al.*, 2000; Osterman, 1994), hyaline cartilage (Lennox *et al.*, 1983; Wirth *et al.*, 1994), growth plate chondrocyte discs (Foster *et al.*, 1990; Hansen *et al.*, 1990), collagen-II gel (Foster *et al.*, 1990), and silicon or methyl-methacrylte (Peterson, 1984; Vickers, 1980). The principle of graft interposition is to prevent formation of vascular channel and bony bridging crossing the physis by inhibiting the penetration of epiphyseal and metaphyseal vessels and their accompanying osteoprogenitor cells to the growth plate defect, thus retaining an avascular environment. However this physeal bar resection technique has its limitations, as it is not suitable for patients with bony bars larger than 40% of the total physeal surface and less than two year of growth period left (Hasler and Foster, 2002). In addition, this procedure may also fail due to incomplete bony bridge resection and recurrence of the bar (Hasler and Foster, 2002).

These treatments are often not effective as they only address late symptoms, and do not usually lead to growth plate cartilage regeneration. Thus these treatments often need to be repeated in the case of distraction osteogenesis method, if the child still has a significant

growth period remaining, or in the case of Langenskiöld procedure if the physeal bar reforms at the site of defect.

### 1.6.4 Regeneration of Injured Cartilage

Although a very small amount of disorganised fibrocartilage has been occasionally identified along with a major of bony tissue formed at the growth plate injury site (Arasapam *et al.*, 2006; Chung *et al.*, 2006), natural regeneration of entire damaged growth plate does not occur as the avascular environment of the growth plate is disrupted allowing vascular invasion and osteoprogenitor migration to the injury site. In a study using interposition of fat at the central growth plate defect where an avascular environment was retained, Osterman (1994) demonstrated the regeneration of the remaining part of the growth plate, reduction of the size of the defect, and prevention of growth plate deformity. However, this observation in the capacity of growth plate to regenerate remains controversial as this result has not been reproduced by other researchers; in fact, many have shown that, besides preventing bony bridge formation with the fat interposition method, no cartilage regeneration was identified (Hasler and Foster, 2002; Kim *et al.*, 2000).

In an attempt to regenerate growth plate after injuries, there have been numerous investigations testing therapeutic efficacy of transplanting cells or tissues and delivering growth factors to the injured growth plate based on articular cartilage regeneration studies (Bruns and Steinhagen, 1999; Imhoff *et al.*, 1999; Martinek *et al.*, 2003; Mason *et al.*, 2000; O'Driscoll and Fitzsimmons, 2001). The use of hyaline cartilage as an interposition material to prevent bone bridge formation has been shown to be more effective than fat due to the inhibitory effect of hyaline cartilage on bone formation (Kuettner *et al.*, 1976; Lennox *et al.*, 1983; Wirth *et al.*, 1994). However this method does not lead to growth plate regeneration. Although reimplantation of a cultured growth plate chondrocyte disk

into a sheep tibial growth plate injury model inhibited bone bridge formation and adapted a growth plate column structure that was associated with endochondral calcification, no obvious growth plate regeneration was observed (Foster *et al.*, 1990). In this sheep model, the host immune response resulted in rapid implant necrosis as the proteoglycan rich cartilage matrix of the implant slowly eroded and exposed chondrocytes were lost (Foster *et al.*, 1990). Although similar work in rabbit tibia demonstrated that implantation of cultured chondrocytes embedded in agarose into growth plate defects resulted in a partial correction of angular deformity and a significant reduction in growth arrest, the cartilaginous implant did not induce growth plate regeneration as it was eventually replaced by endochondral bone (Lee *et al.*, 1998).

In contrast to the positive results obtained from treatment of articular cartilage defects, many similar treatment strategies produced unsatisfactory results in attempts to induce regeneration of injured growth plate cartilage. Previously, periosteal grafts induced production of new cartilage tissue (O'Driscoll and Fitzsimmons, 2001) and reduced symptoms of patients with osteochondral lesions for a short period, and delivery of growth factors such as BMPs, FGF-2, TGF-β and IGF-I in a biodegradable matrix scaffold (autogenous fibrin, collagen sponge or agarose gel) or via intra-articular injection to the defects improved healing of cartilage in *in vivo* animal models reviewed in Martinek *et al.* (2003). However, treatment of the sheep growth plate partial defects with periosteum implants (Wirth *et al.*, 1994) or the growth factor bone morphogenic protein (BMP-7) (Johnstone *et al.*, 2002) failed in prevention of bone bridge formation. Similarly, in a rat tibial growth plate fracture model, enhanced healing of the fracture growth plate was not induced by interposed periosteum in the injury site (Gruber *et al.*, 2002). Similarly, in contrast to the results obtained with osteochondral defects of rabbit knees where almost complete regeneration of articular cartilage was achieved with implantation of polymer scaffolds of mesenchymal stem cells transduced with retroviral vectors encoded for BMP-7

(Mason *et al.*, 2000), the muscle interposition injected with BMP-2 adenoviral vectors implanted at the rabbit tibial growth plate injury site increased osteogenic activity rather than chondrogenic activity (Lee *et al.*, 2002). These results suggest that cellular and molecular mechanisms for repair and regeneration of growth plate defects perhaps are not the same as those for permanent cartilage.

More recently there have been advances made with cell-based therapy with mesenchymal stem cells, where direct transfer of periosteum-derived mesenchymal stem cells embedded in agarose into the rabbit tibial growth plate defect resulted in regeneration of the physis, preventing growth arrest or angular deformity of the tibia (Chen *et al.*, 2003; Li *et al.*, 2004). In addition, the potential of growth factors in the treatment of growth plate defects has been suggested since muscle-based gene therapy with adenoviral vectors encoding for IGF-I restored the injured physis of rabbit tibia (Lee *et al.*, 2002). However these therapies can still be sporadic and temporary; to obtain the optimal clinical treatment for full prevention of angular deformity and limb length discrepancy using the technologies described above, further research is needed to investigate suitable biodegradable matrix scaffolds in combination with appropriate growth factor stimuli (Martinek *et al.*, 2003; Xian and Foster, 2006).

Recently, studies in the reconstruction of damaged proximal tibial growth plates of adolescent rabbits using three dimensional chondrocyte pellets synthesized *in vitro* that resembled the normal *in vivo* physis demonstrated a potential clinical application of this "artificial physis" in the treatment of growth plate injuries in children as this treatment strategy maintained bone longitudinal growth and reduced angularity of the affected tibia (Lee *et al.*, 2003; Yin *et al.*, 2004). These studies also showed that the implanted three-dimensional pellets were incorporated well into the host tissue, re-established the physis characteristic zonal structure, maintained an active proteoglycan metabolism and eventually resumed endochondral ossification. However the problem that still remains is

the short term sustainability of this implant structure. Unlike progress made towards regeneration of articular cartilage, biological regeneration of growth plate cartilage remains a fundamental challenge to researchers. Thus, it is important to understand the roles of molecules that are involved in regulating the growth plate repair before instigating biological treatments to prevent bony repair and initiate proper cartilage regeneration at the injured growth plate.

## 1.7 Expression and Roles of Regulating Factors in Fracture Repair

The injury responses that occur as the result of bone fracture are regulated and controlled by many different molecules. Since injured growth plate is often repaired by bony tissue, some of these cytokines, growth factors, and transcription factors that play regulatory roles in stimulating bone fracture repair described below in this section may also serve possible functions in signalling the cellular processes in bony bridge repair of injured growth plate (described in section 1.6.2).

### 1.7.1 Inflammatory Factors

#### *1.7.1.1 Pro-inflammatory Cytokines*

The first stage of cellular response to fracture trauma is the inflammatory phase where macrophages and other immune cells recruited to the fracture site release proinflammatory cytokines such as tumour necrosis factor (TNF-α) and interleukin (IL-1) in response to tissue injury or microbial challenge (Einhorn, 1998; Einhorn *et al.*, 1995; Kon *et al.*, 2001). The expression of pro-inflammatory cytokines TNF-α and IL-1β mRNA peaked on day 1 during early inflammatory phase of bone fracture (Gerstenfeld *et al.*, 2003; Kon *et al.*, 2001). Many studies have implicated IL-1β and TNF-α in regulating bone remodelling and homeostasis during bone formation (Bertolini *et al.*, 1986; Kumar *et*

*al.*, 2001). Although only TNF-α expression was up regulated on days 21 and 28 during the period of secondary bone formation when the establishment of marrow and bone remodelling occurs in a simple transverse fracture in mice (Kon *et al.*, 2001), in the remodelling event of a rat femoral marrow ablation model for intramembranous bone formation expressions in TNF-α and IL-1β were elevated (Kuroda *et al.*, 2005). In addition, TNF-α and IL-1α were immunolocalised not only in inflammatory cells, but also in mesenchymal cells in the periosteum, in hypertrophic chondrocytes of the fracture callus and later in bone lining cells on the newly formed trabecular bone surface in fracture injury site (Kon *et al.*, 2001). So far, the spatial expression of IL-1β during bone fracture repair has not been reported, however we predict a similar pattern to IL-1α as they share similar cellular functions. Furthermore TNF-α and IL-1β are important chemotactic factors for mesenchymal/fibroblast cell recruitment during connective tissue repair (Kovacs and DiPietro, 1994). Therefore, these pro-inflammatory cytokines may carry out multiple functions in subsequent healing responses such as in mesenchymal cell recruitment, proliferation and differentiation and remodelling of callus and bone at fracture injury sites.

Although TNF-α inhibits osteoblast differentiation *in vitro* (Abbas *et al.*, 2003; Gilbert *et al.*, 2000; Gilbert *et al.*, 2002), studies with mutant mice showed that the absence of TNF-α signalling leads to a delay in both intramembranous and endochondral bone formation during fracture repair, which suggests that TNF-α signalling may be involved in mesenchymal stem cell recruitment, differentiation, and resorption of mineralised cartilage, events necessary for proper bone fracture healing (Bertolini *et al.*, 1986; Gerstenfeld *et al.*, 2001; Gerstenfeld *et al.*, 2003). The participation of TNF-α in regulating the cartilage resorption event during endochondral ossification of fracture callus is further demonstrated by studies using TNF-α receptor-deficient mice showing its stimulatory effect on production of key proteolytic collagenase MMPs (9 and 14) that are crucial for vascularization and turnover of mineralised cartilage matrix during the endochondral

ossification process at fracture callus (Lehmann *et al.*, 2005). TNF-α has also been shown to be important to induce apoptosis of chondrocytes using avian chondrocyte and murine articular chondrocyte cultures *in vitro* (Aizawa *et al.*, 2001; Cho *et al.*, 2003), and fracture callus of TNF-α receptor-deficient mice (Gerstenfeld *et al.*, 2003). Furthermore it is also known that both TNF-α and IL-1β strongly inhibit the expression of cartilage extracellular matrix (ECM) protein Col-2 by decreasing chondrogenic transcription factor Sox9 mRNA levels (Murakami *et al.*, 2000). Sox9 is important for cartilage formation and expression of chondrocyte specific genes such as collagens Col-2 and Col-11 (see section 1.7.3.1). Cytokine TNF-α was found to stimulate or inhibit proliferation of isolated human osteoblasts depending on its concentration level, as low cytokine levels induced DNA synthesis in osteoblasts and high concentration cause reduction in cell growth (Frost *et al.*, 1997). The reduction in cell growth may be due to the apoptosis affect of TNF-α on cells, where TNF-α signals activation of specific protease cascades (caspases) (Nagata, 1996). On the other hand exogenous IL-1β treatment in vivo induced increased osteoblast numbers and decreased normal apoptosis of osteoblasts during fracture repair (Olmedo *et al.*, 1999). Therefore, these cytokines are tightly regulated to maintain cell homeostasis by mediating the appearance and disappearance of cells through two primary responses: cell survival and growth, and cell apoptosis (Aizawa *et al.*, 2001; Frost *et al.*, 1997; Olmedo *et al.*, 1999).

### *1.7.1.2 p38 MAP Kinase*

Mitogen activated protein kinases (MAPK) particularly the p38 MAPK has been identified to play an important signalling role in orchestrating injury or stress-induced responses and in bone formation. Although observation of transient p38 activation during bone or cartilage fracture repair has not been examined, induction of p38 activation has been reported in other tissue injuries such as the heart and spinal nerve (Luss *et al.*, 2000;

Schafers *et al.*, 2003). TNF-α not only induces p38 activation in various types of cells *in vitro* including chondrocytes, osteoblasts, endothelial cells, and peridontal ligament fibroblasts (Grethe *et al.*, 2004; Kumar *et al.*, 2001; Rossa *et al.*, 2005), it is also required for p38 activation in primary sensory neurons induced by spinal nerve ligation *in vivo* (Schafers *et al.*, 2003). Although pro-inflammatory cytokines (such as TNF-α and IL-1β) and environmental stress have been reported to activate p38 (Kumar *et al.*, 2001; Obata *et al.*, 2000), the activation and signalling of p38 also leads to production and signal transduction of these inflammatory cytokines (Lee *et al.*, 1994). The interaction between p38 and pro-inflammatory cytokines is important in controlling life and death signalling cascades in chondrocytes and osteoblasts (Aizawa *et al.*, 2001; Ichijo, 1999; Kumar *et al.*, 2001; Tsuboi *et al.*, 1999). Therefore, p38 activity may coincide with up-regulation of TNF-α and IL-1β expressions, during the inflammatory phase and later in the remodelling phase of bone repair.

Currently it is unclear how the fate of cell lineages for intramembranous and endochondral pathways are selected and determined in the formation of bone structure during skeletal development and fracture repair. However, previous studies by Sun and colleagues (2002) have demonstrated that p38 MAPK plays an important role in the selection and determination of cell lineages during bone formation (Sun *et al.*, 2002). The transfection of a constitutively active form of MKK6 (CA MKK6) viral vector constructs, which is a MAP kinase kinase of the p38 cascade, in cells isolated from embryonic chicken membranous calvarium bone, resulted in the continuous elevation of p38 MAPK activity. Elevation of p38 activity in turn stimulated osteoblast differentiation with up regulation of mRNA expression of osteocalcin, and inhibited chondrogenesis with a reduction in Col-10 mRNA expression. On the other hand, suppression of p38 activation with a dominant negative form of p38 MAPK (DN p38) viral vector constructs, resulted in opposite effects with status of chondrogenesis rather than osteogenesis. This work is also supported by

previous studies using human bone marrow-derived mesenchymal stem cells (hMSCs) in examining the contribution of MAPK's including p38 in regulating cell differentiation of hMSCs into osteogenic or adipogenic lineage (Jaiswal *et al.*, 2000). It was found that the inhibition of MAP kinase activity with inhibitors also blocked the osteogenic differentiation pathway of the stem cells. Apart from the important roles of the p38 pathway in selection and determination of cell lineages, as well as in stimulating bone cell differentiation *in vitro* by inducing cbfa1 expression, and mineral deposition (Guicheux *et al.*, 2003; Hu *et al.*, 2003; Lee *et al.*, 2002; Suzuki *et al.*, 2002), p38 activity has been found to be important in regulating cell migration (Bakin *et al.*, 2002; Obata *et al.*, 2000; Ray *et al.*, 2003). Therefore, p38 MAP kinase activity may be important in regulating the recruitment of progenitor cells to the injury site and their subsequent osteoblast differentiation during bone repair.

### 1.7.2 Growth Factors

Many growth factors such as transforming growth factor (TGF–β1), fibroblast growth factor (FGF-2), platelet derived growth factor (PDGF-B), insulin-like growth factor (IGF-1), and bone morphogenic proteins (BMPs) not only play a regulatory role in cartilage and bone formation, but are also important in regulating bone fracture repair (Canalis *et al.*, 1988; Szczesny, 2002). These growth factors have been found to be produced by inflammatory and mesenchymal infiltrates, chondrocytes and osteoblasts at the injury site, and have been implicated in regulating the subsequent bone formation, restoration and remodelling responses (Bolander, 1992; Sakou, 1998; Tatsuyama *et al.*, 2000).

*1.7.2.1 TGF-β1*

TGF-β1, one member of the TGF-β superfamily, is present in all tissues, but with bone and blood platelets being the most abundant source for this factor (Bolander, 1992; Horner *et al.*, 1998; Seyedin *et al.*, 1986). In addition, receptors for TGF-β were also found at the highest concentration on osteoblasts compared to other cell populations, which demonstrates the importance of TGF-β in bone maintenance (Szczesny, 2002). In bone, TGF-β is synthesized mostly by osteoblasts but also by osteocytes, osteoclasts and chondrocytes as well as some populations of bone marrow and activated macrophages (Barnes *et al.*, 1999; Bolander, 1992; Horner *et al.*, 1998). Upon synthesis, TGF-β is released into extracellular space and stored in an inactive form bound by a latent complex (Centrella *et al.*, 1988). For TGF-β activity, the active form of TGF-β is cleaved from its latent complex by various proteolytic enzymes, low pH environments, and other physical factors. For example in bone remodelling, latent TGF-β stored in bone matrix is activated during proteolytic degradation of the bone matrix by osteoclasts to induce osteoblast formation and bone regeneration (Szczesny, 2002; Xian and Foster, 2006).

The expression and role of TGF-β1 during bone fracture repair has been extensively examined. During the immediate injury response of fracture repair TGF-β1 has been reported to be synthesised by platelets granules, and inflammatory cells (monocytes and macrophage) during the platelet degranulation event of hematoma formation, and released at the fracture gap (Assoian and Sporn, 1986; Bolander, 1992; Steinbrech *et al.*, 2000), where extracellular (latent) TGF-β1 is immuno-localised in the hematoma along the periosteum. Immunolocalisation of intracellular active TGF-β1 on some mesenchymal cells lining the cortex of the proliferating periosteum during the initial inflammatory event, may suggest a role of TGF-β1 in stimulating mesenchymal cell proliferation (Joyce *et al.*, 1990; Joyce *et al.*, 1990).The potential role of TGF-β1 in early injury response is further supported by the strong up-regulation in TGF-β1 mRNA expression during early

inflammatory phase on day 1 of bone fracture healing in a murine model (Cho *et al.*, 2002).

The regulatory role of TGF-β1 in fracture healing and remodelling is evident due to the constitutive expression of TGF-β1 during the subsequent osteogenic, chondrogenic and remodelling events in bone repair models with/without endochondral bone formation (Cho *et al.*, 2002; Joyce *et al.*, 1990; Kuroda *et al.*, 2005). Consistently, its spatial protein expression has been localised in osteoblasts lining the intramembranous bone adjacent to the injury site, in proliferating mesenchymal cells and chondrocytes in fracture callus, and in the matrix of new bone and in hypertrophic cartilage matrix during intramembranous and endochondral bone formation at the fracture (Barnes *et al.*, 1999; Joyce *et al.*, 1990; Steinbrech *et al.*, 2000). In membranous bone healing of rat mandibulae on day 37 when complete bony healing was observed, TGF-β1 was localized to the newly formed bone matrix and areas of remodelling and its receptor TGF-βRII was further observed in osteocytes, osteoblasts, and the newly formed periosteum in the remodelling bone (Steinbrech *et al.*, 2000).

When introduced into periosteum of normal rat femur, exogenous TGF-β1 has been shown to initiate intramembranous and endochondral ossification events by stimulating proliferation and differentiation of mesenchymal cells into chondrocytes/osteoblasts followed by production of extracellular matrix (Joyce *et al.*, 1990). In addition, exogenous TGF-β1 also stimulated the recruitment and proliferation of osteoblasts at a rabbit skull defect, resulting in a rapid deposition of bony matrix and bone repair (Beck *et al.*, 1993). It is also well documented by *in vitro* experiments that active TGF-β stimulates chondrocyte differentiation (Iwasaki *et al.*, 1995) and synthesis of Type II collagen and cartilage-specific proteoglycans (Seyedin *et al.*, 1985; Seyedin *et al.*, 1986) by mesenchymal progenitors, and promotes osteoblast division (Robey *et al.*, 1987). Furthermore during remodelling of the fractured bone, the resorption of the bone matrix by osteoclasts

activates the latent TGF-β stored in the matrix, resulting in activation of the osteoblastic bone regeneration (Szczesny, 2002; Xian and Foster, 2006).

*1.7.2.2 FGF-2*

FGF-2, also known as basic FGF, is an 18kDa protein expressed by most cells of mesoderm or neuroectoderm origin. FGF-2 is a potent mitogen for growth plate chondrocytes and calvarial derived mesenchymal cells (including periosteal fibroblasts) (Canalis and Raisz, 1980; Trippel *et al.*, 1993), but is also best known for its effects on endothelial cell replication, and neovascularization (Bolander, 1992; Canalis *et al.*, 1988). In bone, FGF-2 is also stored in the matrix bound to heparin sulphate proteoglycan (Bolander, 1992). During endochondral bone formation, this growth factor not only regulates chondrocyte proliferation and maturation, it may also be involved in the cartilage/bone transitional event, as direct infusion of FGF-2 into rabbit tibial growth plate accelerates vascular invasion, cartilage remodelling (indicated by stimulation of collagenase-IV), and ossification of growth plate (Baron *et al.*, 1994; De Luca and Baron, 1999), suggesting its angiogenic function in endochondral ossification. The stimulatory affect of FGF-2 on cell proliferation was also demonstrated in bone marrow-derived mesenchymal progenitors (Tsutsumi *et al.*, 2001). Although FGF-2 promoted an increase in DNA synthesis of calvarial osteoblasts *in vitro*, thus increasing the number of collagen and non-collagen synthesizing cells, FGF-2 had direct inhibitory effect on collagen I synthesis, a marker for osteoblast differentiation (Canalis *et al.*, 1988). Therefore, FGF-2 has no direct stimulatory effect on cell differentiation, and under some conditions, FGF-2 can directly inhibit osteoblast function. In addition, FGF-2 was also shown to stimulate human osteoblast proliferation and migration in a dose dependent manner (Mayr-Wohlfart *et al.*, 2002). Since FGF-2 is localised in various cells at different healing stage of bone

repair (Tatsuyama *et al.*, 2000), the effects of FGF-2 on cartilage/bone cells as described above may give insight to the functional role of FGF-2 in fracture healing.

During the initial inflammatory event of bone repair in a closed fracture rat model, FGF-2 was present in macrophages and platelets of granulation tissue in the developing fracture callus. FGF-2 and its receptor FGFR were also immunolocalised in periosteal mesenchymal cells and osteoblasts during intramembranous bone formation, and in mesenchymal cells, proliferative chondrocytes and osteoblasts in fracture callus during the endochondral ossification stage (Joyce *et al.*, 1991; Tatsuyama *et al.*, 2000). These findings coincided with the constant up-regulation in FGF-2 and FGFR mRNA levels during the repair periods of mesenchymal cell recruitment; osteoblast differentiation and bone formation after rat femoral marrow ablation. In this temporal gene expression study, up-regulation of FGF-2 and FGFR expression were further detected during osteoclast resorption and remodelling, and normal marrow regeneration (Kuroda *et al.*, 2005). Since FGF-2 is a potent mitogenic and chemotactic factor (Mayr-Wohlfart *et al.*, 2002), this may suggest its possible role in stimulating the recruitment and proliferation of mesenchymal progenitor cells, chondrocytes, and osteoblast at the injury site during bone regeneration and remodelling events

Consistent with FGF-2 and FGFR expression patterns, cell proliferation detected at the bone injury site peaked firstly in some inflammatory and mesenchymal cells on day 2, then in some periosteal cells and osteoblasts on day 3, and in chondroprogenitor cells and chondrocytes on day 4 (Tatsuyama *et al.*, 2000). Therefore, co-expression of FGF-2 and its receptor on various cells during bone repair, and its known function in stimulating cell proliferation suggest that FGF-2 may play important roles in stimulating cell proliferation during bone healing. In addition, FGF-2 accumulated in macrophages and platelets during the immediate injury response is released into the fracture site to induce additional FGF production by, and the proliferation of mesenchymal progenitor cells, inflammatory cells,

osteoblasts and chondrocytes, which are the cells required to orchestrate the subsequent intramembranous and endochondral ossification responses.

Although FGF-2 may be involved in angiogenic events of the growth plate (Baron *et al.*, 1994), and FGF-2 was present in hypertrophic chondrocytes during the cartilage maturation stage of fracture healing (Bolander, 1992), experiments by Tatsuyama *et al.* (2000) showed FGF-2 plus its receptor FGFR were immunohistochemically undetectable in mature hypertrophic chondrocytes, which suggests that the chondrocyte hypertrophic and neovascularization events of endochondral ossification might not require FGF-2 during fracture healing. Consistently, in vitro studies showed that FGF-2 inhibits terminal differentiation, hypertrophy and calcification of growth plate chondrocytes (Kato and Iwamoto, 1990). Furthermore, injection of exogenous FGF-2 into the fracture gap of rat enhanced the proliferation of chondroprogenitors cells in fracture callus, thus contributing to the formation of more cartilage and an increase in Col-2 expression (Nakajima *et al.*, 2001). However, this FGF-2 treatment did not induce rapid bone healing through promoting maturation of chondrocytes and replacement of cartilage by osseous tissue, which resulted in the prolonged cartilaginous callus phase.

#### *1.7.2.3 PDGF-B*

PDGF-B is one of the homodimeric proteins of the PDGF family. This growth factor was originally detected and described in granules of blood platelets (hence the name platelet-derived growth factor), but it is also synthesised by most cells of the mesoderm origin including monocytes, activated tissue macrophage, endothelial cells, and osteoblasts (Bolander, 1992). Although there are two different PDGF receptors (α and β), the β receptor is known to bind to the homodimer PDGF-B protein with highest affinity (Hart and Bowen-Pope, 1990). PDGF-B is a potent mitogen of cells from mesenchymal origin, as demonstrated in periosteal fibroblasts, osteoprogenitors and osteoblasts in fetal rat

calvariae (Canalis *et al.*, 1989; Hock and Canalis, 1994), and in human osteoblasts (Zhang *et al.*, 1991) where addition of PDGF-B induced an increase in H3 thymidine incorporation indicative of DNA synthesis. In addition, PDGF-B has also been shown to stimulate proliferation and growth of mesenchymal stromal cells in serum free conditions (Gronthos and Simmons, 1995). Besides the stimulatory effect of PDGF on cell proliferation to promote bone formation of fetal rat calvariae *in vitro* it was also found to induce cell chemotaxis and bone matrix synthesis of osteoblasts (Pfeilschifter *et al.*, 1990). This is supported by *in vivo* experiments involving injections of PDGF-B in the fracture gap of rabbit (Nash *et al.*, 1994) or in the periosteum of normal rat femur (Bolander, 1992). Nash *et al.* (1994) demonstrated its stimulatory effect on callus formation and acceleration of fracture healing, and Bolander (1992) described that the exogenous PDGF-B stimulated mesenchymal cell proliferation in the cambial layer of periosteum and induced new bone formation through intramembranous ossification. The above *in vivo* and *in vitro* studies suggest that PDGF has potent mitogenic and chemotactic abilities in mesenchymal and osteoblastic cells and plays an important role in bone healing. The chemotaxis-stimulating effect of PDGF has also been reported in monocytes, granulocytes and fibroblasts (Siegbahn *et al.*, 1990), also in osteoblasts (Tsukamoto *et al.*, 1991) and periodontal cells (Ray *et al.*, 2003).

The co-localization of PDGF-B and receptor β on various cells in the injury site at different stages during fracture repair of a closed transverse fracture mouse model implies a paracrine and autocrine mechanism of PDGF (Fujii *et al.*, 1999). In the early inflammatory phase of this fracture model, the signals for PDGF-B protein and its receptor β were weak but nevertheless detected on both inflammatory cells and mesenchymal cells. Since receptor β has also been reported to mediate cell migration and proliferation (Kundra *et al.*, 1994), the interaction between PDGF-B and its receptor may be a prerequisite to mediate signals required for the recruitment and proliferation of inflammatory and

mesenchymal cells at the early stage. During the intramembranous and endochondral ossification events of tibial fracture repair, PDGF-B and its receptor were strongly expressed on proliferating and hypertrophic chondrocytes at soft fracture callus, and on active osteoblasts in the newly formed woven bone of the adjacent periosteum and of the hard fracture callus. This indicates that PDGF-B may be involved in stimulating cartilage/bone formation at the injury site by promoting chondrogenic and osteogenic differentiation followed by its effect on their proliferation.

Furthermore PDGF-B has been shown to increase osteoclast formation (Franchimont and Canalis, 1995; Hock and Canalis, 1994)], and stimulate bone resorption through mediating prostaglandin PGE2 expression in neonatal mouse calvaria in organ culture (Tashjian *et al.*, 1982). Since PDGF-B and its receptor β were predominantly expressed on the osteoclasts (TRAP positive multinucleated cells) and osteoblasts during the remodelling phase of fracture repair, PDGF receptor β linked functions of cell chemotaxis and proliferation have been suggested to be required for regulating the osteoblast-osteoclast interaction for the maintenance of bone remodelling (Fujii *et al.*, 1999).

*1.7.2.4 IGF-I*

IGF-I is a 7.6 kDa polypeptide growth factor responsible for normal bone formation and remodelling (Yakar *et al.*, 2003). Its Type I receptor is a potent signalling system for cell proliferation, which stimulates growth while blocking apoptosis in various cell types. The critical role of IGF-I signalling in normal bone growth is confirmed by the severe growth retardation in IGF-I and its receptor knockout mice (Baker *et al.*, 1993; Liu *et al.*, 1993; Wang *et al.*, 2006). IGF-I synthesised by various cells of mesenchymal origin including chondrocytes and osteoblasts is released and stored in the extracellular matrix in latent form. With a biological half-life of only a few minutes, IGF-I is easily degraded.

However when bound with one of the seven known IGF binding proteins (IGF-BPs), its resistance to degradation is increased which further extended its half-life (Canalis *et al.*, 1988). Latent form of IGF-I is a result of IGF-BP3 binding to IGF-I together with a stable protein complex (acid labile subunit) (Szczesny, 2002).

During long bone development IGF-I has a potent effect on the growth plate, where it regulates the proliferation, differentiation and maturation of the chondrocytes undergoing endochondral ossification; hence mice null for IGF-I showed reduced chondrocyte proliferation and differentiation, and increased cell apoptosis (Wang *et al.*, 2006). In addition, IGF-I enhanced the spontaneous differentiation process of mandibular condyle-derived primary chondrocytes in a monolayer tissue culture model of hyaline cartilage, through accelerating the proliferation and differentiation of cartilage progenitor cells (Maor *et al.*, 1999; Reiter *et al.*, 2002).

Besides its effect on cartilage metabolism, IGF-I also has an anabolic effect on cell proliferation and synthesis of bone matrix protein in osteoblasts and their precursor cells, thus stimulating bone formation. This was shown in fetal rat calvariae culture, where IGF-I stimulated the replication of osteoprogenitor cells, osteoblasts and periosteal fibroblasts, and their rate of bone matrix apposition and collagen synthesis (Canalis, 1980; Hock *et al.*, 1988). Furthermore, localised delivery of IGF-I protein into a fracture injury site resulted in accelerated cartilage formation, and subsequent bone healing events (Wildemann *et al.*, 2003). There is also *in vivo* evidence indicating the essential role of IGF-I signalling in bone matrix mineralization, where transgenic mice with knockout of IGF-I receptor in their osteoblasts not only displayed a decrease in cancellous bone volume and trabecular numbers, but also showed a striking reduction in the rate of mineralization of the osteoid (Zhang *et al.*, 2002). This is further supported by the delay in initiation of matrix mineralization also seen in IGF-I null mice (Wang *et al.*, 2006).

Expression of IGF-I mRNA by in situ hybridization and localization of IGF receptors by immuno-staining were detected in periosteal cells, preosteoblasts, differentiated osteoblasts and the osteocytes in the developing bony callus formed during the intramembranous ossification stage of a transverse closed fracture repair rat model (Okazaki *et al.*, 2003). During the subsequent endochondral ossification event, IGF-I mRNA and IGF receptor were co-expressed by mesenchymal cells, proliferating chondrocytes, and early hypertrophic chondrocytes in the cartilaginous callus, and osteoblasts at the cartilage/bone transition. These co-expression results of IGF-I and it receptor suggest that IGF-I regulates bone repair in an autocrine and paracrine manner. Since IGF-I is strongly associated with induction of cartilage and bone metabolism, the above results further imply that IGF-I is involved in regulating proliferation and differentiation of the various cells at the fracture site during bony and cartilage callus formation.

#### *1.7.2.5 BMPs*

BMPs, members of the TGF-β super family, are a large group of secretory polypeptides that share common structural features. Some BMPs including BMP 2 to 7, where their expressions have been localised in cartilage and bone of developing long bone (Anderson *et al.*, 2000), have shown to be important in skeletal development by acting on mesenchymal cells to induce cartilage and bone formation (Zoricic *et al.*, 2003). BMPs have been shown to stimulate proliferation, migration and differentiation of mesenchymal cells into either osteogenic or chondrogenic lineages as reviewed by Sakou (1998) (Sakou, 1998). It has been reported that BMP-2, 3, 6, and 7 function to control the rate of proliferation and chondrocyte maturation, and stimulate chondrocyte differentiation and collagen production *in vitro* (Carrington *et al.*, 1991; De Luca *et al.*, 2001; Grimsrud *et al.*, 1999; Shea *et al.*, 2003), while BMP-2 and 7 can also induce osteoblast differentiation of

mesenchymal cells and stimulate mineralised bone matrix formation (Canalis *et al.*, 2003; Cheng *et al.*, 2003; Shea *et al.*, 2003). BMPs are strong regulators of bone formation as a result of their potential roles in mediating the migration of progenitor cells, proliferation of mesenchymal cells, differentiation of osteogenic cells, and bone remodelling (Mayr-Wohlfart *et al.*, 2002; Sakou, 1998; ten Dijke *et al.*, 2003).

Furthermore, previous bone fracture healing studies have suggested that migration and proliferation of primitive mesenchymal cells to the site of injury, their osteogenic differentiation and bone formation may be regulated by the signaling pathways of BMPs and their receptors (Cho *et al.*, 2002; Onishi *et al.*, 1998; ten Dijke *et al.*, 2003). Expression of BMPs analysed during fracture repair indicated the up-regulation of BMP-2, 3 and 4 during the stages of initial inflammatory phase, intramembranous bone formation, and endochondral formation (cartilage formation) (Bostrom *et al.*, 1995; Nakase *et al.*, 1994), and followed by bone formation and remodelling where up-regulation in BMP 7 was observed (Cho *et al.*, 2002). In addition, BMP 5 and 6 displayed constitutive expression throughout the reparative phase (Cho *et al.*, 2002). BMPs elicit their responses through binding and activating their membrane bound cell surface receptors, type-I (BMP-R1a, BMP-R1b) and type-II (BMP-R2, ACVR-2a, and ACVR-2b) receptors (Onishi *et al.*, 1998; Sakou, 1998), which activate downstream signalling by transducing Smad molecules that translocate to the nucleus and activate gene transcription (Kloen *et al.*, 2003). Therefore BMP receptors were also constitutively expressed by osteogenic and chondrogenic cells involved in intramembranous and endochondral ossification processes of bone repair. Recently, similar expression patterns of BMPs and their receptors were detected at the inflammatory, followed by the osteogenic and remodelling events during growth plate repair (Ngo *et al.*, 2006).

### 1.7.3 Transcription factors

The two major transcription factors controlling chondrocyte and osteoblast differentiation for cartilage and bone formation are Sox9 and cbfa1 (**Fig 1.7.1**). The activities of these factors are required for skeletal development and growth, as mutations in these genes lead to severe abnormalities in skeletal structures. During bone repair, these transcription factors are expressed on various cells of mesoderm origin to possibly regulate the subsequent new cartilage and bone formation in the injury site.

#### *1.7.3.1 Sox9*

Sox9 is a member of Sox (sex reversal Y-related high-mobility group box protein) family of transcription factors. Besides its pivotal role in the vertebrate sex determination pathway (which will not be discussed here), Sox9 is a major role player in chondrogenesis. Sox9 mRNA expression in skeletal tissues is present in all chondrocyte progenitors during mesenchymal condensation and mature chondrocytes, but absent in hypertrophic chondrocytes and osteoblasts (Ng *et al.*, 1997; Wright *et al.*, 1995; Zhao *et al.*, 1997). Co-expression of the Sox9 gene with chondrogenic specific genes such as Col-2, an early marker of chondrocyte differentiation, may imply the role of Sox9 in specifying chondrocytic lineage selection. In addition, Sox9 not only binds directly to its binding site present on Col-2 chondrocyte-specific enhancers and controls Col-2 expression in chondrocytic cell lineage (Bell *et al.*, 1997; Lefebvre *et al.*, 1997), it also regulates Col-11 expression by binding to its chondrocyte specific enhancers (Bridgewater *et al.*, 1998). This provides strong evidence of Sox9 as a regulator of chondrogenesis through directly activating the expression of these chondrogenic genes.

Using chimeric mice where Sox9 gene is removed, Bi *et al.* (1999) were able to further demonstrate that Sox9 is completely required for the formation of normal mesenchymal condensations, the conversion of mesenchymal cells to chondrocytes,

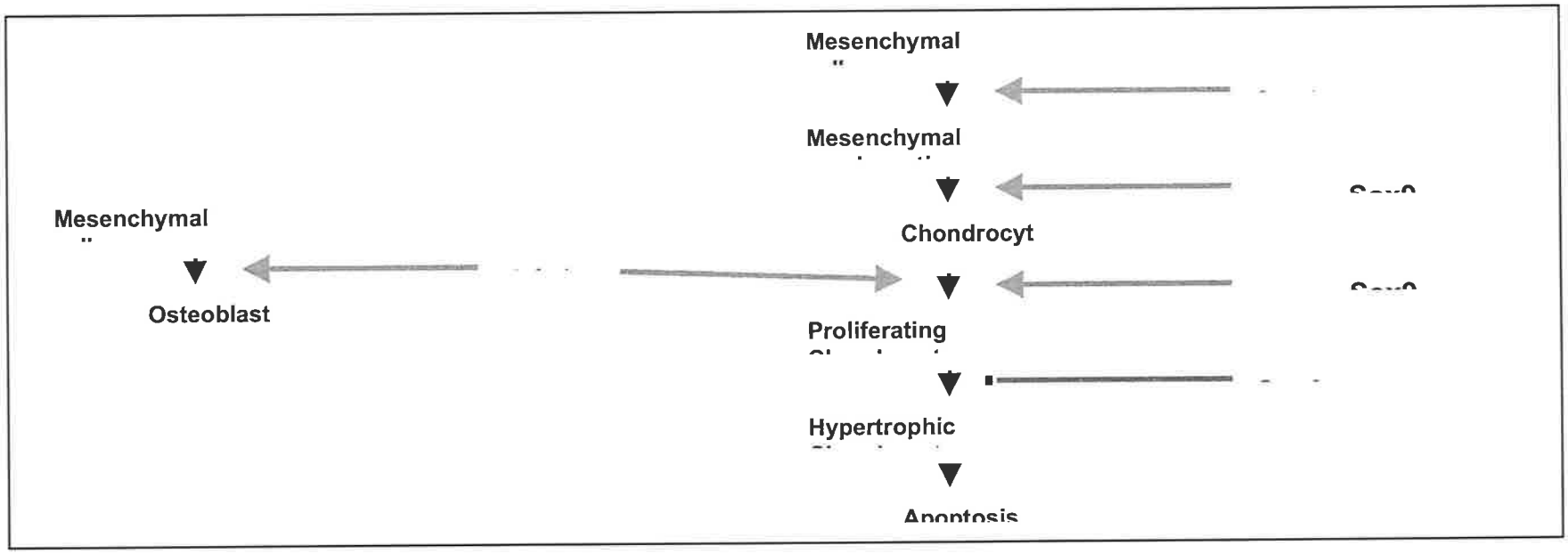


**Fig 1.7.1 Transcription factors that control chondrocyte and osteoblast differentiation.**

Sox9 is essential for mesenchymal condensation and mediates the chondrocyte differentiation and proliferation process induced by L-Sox5/Sox6, but Sox9 inhibits hypertrophic chondrocyte differentiation. Cbfa1 is essential of osteoblast differentiation. Illustration is based on reviews: De Crombrugghe B *et al.* (1997), Curr Opin Cell Biol (13: 721-727); and Akiyama H *et al.* (2002), Genes Dev (16: 2813-2828).

the proliferation of chondrocytes and the synthesis of chondrocyte marker genes such as Col-2a1, Col-9a2 and Col-11a2 (Bi *et al.*, 1999). Therefore, the absence of Sox9 gene in the chimeric mice resulted in the failure of mesenchymal cells to undergo condensation and subsequent chondrogenic differentiation and cartilage ECM protein production. Interestingly, the absence of Sox9 gene in the chimeric mice also resulted in the suppression of two other Sox proteins L-Sox5 and Sox6, which are essential for cartilage formation, suggesting that Sox9 mediates the function of these Sox proteins. On the other hand, although chondrocytes differentiated poorly and expressed cartilage ECM protein genes at very low or undetectable levels in L-Sox5 and Sox6 double gene knockout mice, Sox9 expression and the mesenchymal condensation process were not effected (Smits *et al.*, 2001). This indicates that L-Sox5 and Sox6 do not regulate Sox9 and are necessary for chondrogenesis only after mesenchymal condensation.

Furthermore, heterozygous Sox9+/- mouse mutants with skeletal abnormalities and Sox9 haploinsufficiency (50% of Sox9 being insufficient to fulfil its physiological functions) displayed an abnormal increase in the length of hypertrophic zones and premature mineralization in the bones of mutant embryos. This indicates that sufficient expression of Sox9 is required in the pre-hypertrophic zone during normal skeletal development, in order to prevent premature differentiation of hypertrophic chondrocytes and cartilage mineralization (Bi *et al.*, 2001). In humans, heterozygous mutations in the Sox9 gene results in campomelic dysplasia, a rare genetic disease, where most patients die in the perinatal period from respiratory complications. Campomelic dysplasia patients with similar disease phenotype as the Sox9+/- mice display hypoplasia of most skeletal elements derived by endochondral ossification and are associated with XY sex reversal and sometimesd heart or kidney abnormalities.

A functional Sox9 transcription factor demonstrates extreme importance in normal neonatal development, which includes appropriate sex determination and cartilage and

long bone development, as well as during fracture repair. The regulatory role of transcription factor Sox9 at different stages during fracture repair was examined (Sakano *et al.*, 1999). During the early inflammatory phase of fracture healing in mouse tibia with closed transverse fracture on day 2-3, along with chondrocyte specific gene Col-2, Sox9 protein was not immuno-stained in inflammatory cells or mesenchymal cells of adjacent periosteum. This was consistent with the low Sox9 and the lack of Col-2 mRNA expressions as detected by Northern blot, which implies that Sox9 is not involved during the early injury phase. As cartilage callus started to develop and mature at the injury site with up-regulation in Col-2 production on day 5-9, Sox9 mRNA was also up-regulated along with intense immunolocalization of Sox9 in immature and mature chondrocytes undergoing proliferation. However, as chondrocytes differentiated into hypertrophic chondrocytes during endochondral ossification on day 14-21, Sox9 signalling declined and its expression was turned off in the hypertrophic cells. The lack of Sox9 immunostaining in fibroblasts, osteoblasts, and osteocytes, and the dramatic decline in Sox9 and Col2a mRNA levels during intramembranous and endochondral ossification at injury site, further suggest that Sox9 signalling is not required for the recruitment, differentiation and proliferation of these cells. Since Sox9 transcription is essential for chondrogenesis and cartilage formation during embryonic development (Bi *et al.*, 1999) and adult fracture repair which recapitulates embryonic skeletal formation (Ferguson *et al.*, 1999), Sox9 signalling is required to promote chondrogenic differentiation and proliferation, but is suppressed later on to initiate hypertrophic chondrocyte differentiation.

#### *1.7.3.2 cbfa1*

Core binding factor (cbfa1), as known as OSF2 or RUNX2, is a member of a family of osteoblast-specific transcription factors with a DNA binding alpha subunit. They also share a conserved 128 amino acid DNA-binding domain called the runt domain

because of its homology to the Drosophila Runt gene (Ducy *et al.*, 1997; Otto *et al.*, 1997). Bone matrix proteins osteocalcin and Col-1 are co-expressed with transcription factor cbfa1 in various cell types and are target proteins of cbfa1 transcription (Ducy *et al.*, 1997). During embryonic skeletal development, cbfa1 expression was detected in all progenitor cells of mesenchymal cell condensations formed to model the future skeleton of mouse embryo at 12.5dpc (day post-conception) (Ducy *et al.*, 1997). Cbfa1 was also expressed in osteoblasts of every bone tissue type examined but was absent from other tissues including the permanent cartilage of 16 dpc mouse embryo (Ducy *et al.*, 1997). In another experiment, cbfa1 mRNA expression was localised in the perichondrium and periosteum lining the shaft of the developing long bone, and in mature and hypertrophic chondrocytes undergoing endochondral ossification in 18dpc mouse embryo (Ferguson *et al.*, 1999). In addition, cbfa1 was weakly expressed in mature and hypertrophic chondrocytes of postnatal mouse growth plate but strongly expressed in the new bone of the metaphysis. In all adult mouse tissues, cbfa1 expression is strictly restricted to osteoblasts, calvariae and bone (Ducy *et al.*, 1997).

Furthermore, there are reports indicating that, the cbfa1 mRNA pattern expressed during fetal skeletal development is again re-induced during fracture repair, a process where skeletal regeneration occurs. In a rib fracture mice model (Kawahata *et al.*, 2003), temporal gene expression of cbfa1 using RT-PCR and Northern analysis showed up regulation in cbfa1 expression during intramembranous ossification of periosteum adjacent to injury site on day 5-8 and endochondral ossification of the cartilaginous callus formed to bridge to fracture site on day 10-12. More specifically, spatial localisation of Cbfa1 mRNA during fracture repair using in-situ hybridization has been determined in rib and tibial fracture mice models (Ferguson *et al.*, 1999; Kawahata *et al.*, 2003). Both experiments detected cbfa1 expression in mesenchymal cells in the fracture hematoma and in periosteal cells on day 2-5, in osteoprogenitor cells and osteoblasts surrounding the adjacent

periosteum and newly formed woven bone on day 5-8, in mature chondrocytes of soft callus on day 6-10, and in hypertrophic chondrocytes and osteoblasts of hard callus on day 10-14 at the fracture site.

Although the expression patterns of cbfa1 from embryonic development through to adulthood, and during fracture repair may give insights to its role on skeletal development and bone maintenance/repair, the two major independent functions of cbfa1 on bone formation were determined using genetic experiments. Firstly, mutant cbfa1-/- null mice generated by gene targeting had lack of bone formation were much smaller and died at birth of respiratory distress as the soft cartilaginous rib cage that developed due to ossification failure provided inadequate support for the lungs (Komori *et al.*, 1997; Otto *et al.*, 1997). Heterozygous mutation in cbfa1 causes cleidocranial dysplasia, a genetic disease characterized in humans and mice by a delay in osteoblast differentiation in membranous bones, which results in hypoplastic clavicles and large open spaces in the skull (Lee *et al.*, 1997; Mundlos *et al.*, 1997; Otto *et al.*, 1997). This indicated that cbfa1 is a necessary transcription factor for osteoblast differentiation from mesenchymal progenitors in both endochondral and intramembranous bones. Secondly, transgenic over expression of cbfa1 in pre-hypertrophic chondrocytes of cbfa1 deficient mice partially rescued endochondral ossification process defects resulting from cbfa1 deficiency, including lack of hypertrophic chondrocyte differentiation, vascular invasion and expression of VEGF at the hypertrophic zone, and the absence of multinucleated osteoclasts for the resorption of cartilage matrix; however, the over expression did not rescue the lack of osteoblast differentiation and bone formation in the cbfa1 deficient mice (Takeda *et al.*, 2001; Zelzer *et al.*, 2001). Therefore, besides bone formation, cbfa1 plays another separate role in stimulating hypertrophic chondrocyte differentiation and the subsequence process of endochondral ossification. These expression patterns of cbfa1 along with many genetic experiments indicate that lack of or reduction in cbfa1 expression

inhibits osteoblast differentiation and hypertrophic chondrocyte differentiation, which strongly suggests the roles of this transcription factor in regulating bone formation by inducing osteoblast differentiation, and in endochondral ossification of transitional cartilage.

## 1.8 Project Rationale, Hypothesis and Aims

### 1.8.1 Project Rationale and Overview

Although some of the cellular mechanism for bone bridge formation and the injury responses to growth plate damage have been previously identified and characterized (as described in section 1.6.2), it is currently unclear what molecules are potentially involved in regulating the cellular responses following growth plate injury. Based on the possible roles of pro-inflammatory cytokines and growth factors, transcription factors and matrix proteins in regulating bone fracture repair described in sections 1.7 and 1.4.7, and summarized in **Figure 1.8.1**, the first part of this research project will involve examining whether cytokines IL-1β and TNF-α, and growth factors TGF-β1, FGF-2, PDGF-B and IGF-I are involved in regulating the growth plate injury responses described in section 1.6.2 by determining their temporal changes in mRNA expression and spatial cellular localisation of their proteins during a 35 day growth plate injury repair time course period in young rats. In addition, in order to verify that the bone bridge repair of growth plate defect is through intramembranous ossification and not endochondral bone formation process, we further examined the effects of growth plate injury on mRNA expression of cartilage specific genes Sox-9 and Col-2, and bone matrix protein osteocalcin.

The previously well documented links between TNF-α signalling and p38 activation to regulate injury-induced inflammatory responses, cell migration, differentiation

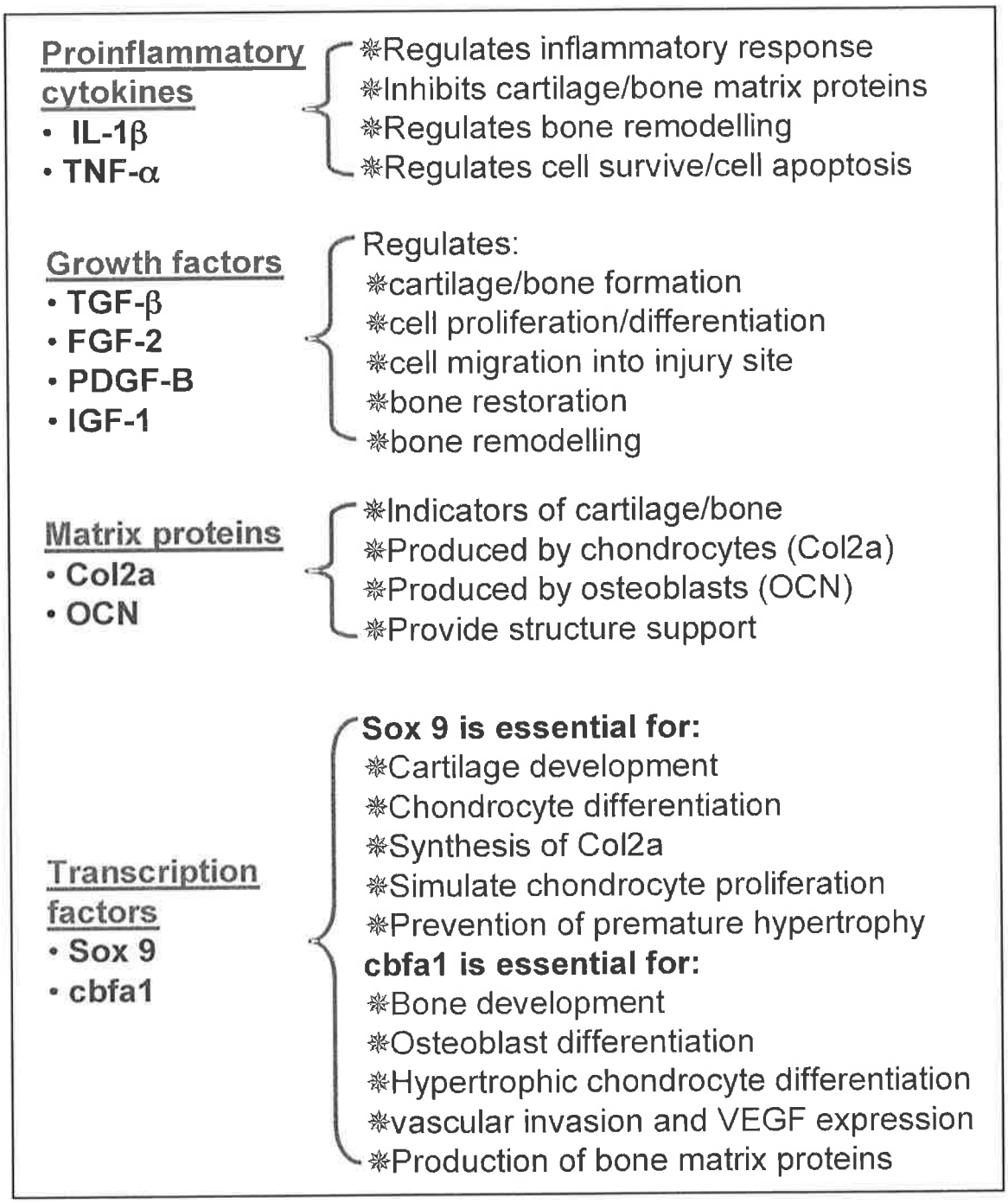


**Figure 1.8.1. Possible roles of regulatory factors in fracture repair**

A chart summarising the possible roles of proinflammatory cytokines, growth factors, transcription factors and matrix proteins in regulating the response to bone fracture healing described in section 1.7 and 1.4.7.

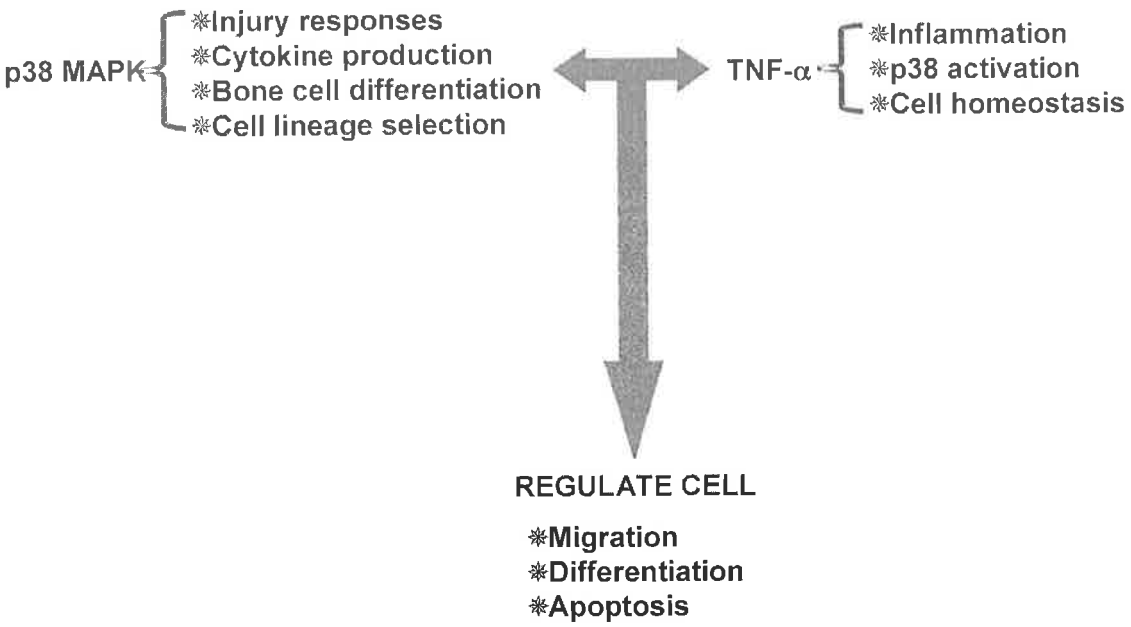


**Figure 1.8.2. Possible roles of p38 activation and TNF-α signalling.**

We propose here the interaction between p38 activation and TNF-α signalling is involved in regulating cell migration, differentiation and homeostasis responses, as well as the bony repair of injured growth plate.

and homeostasis events described in section 1.7.1 is summarised in **Figure 1.8.2**. However, it remains unknown whether there may be a potential interaction between TNF-α and p38 during *in vivo* growth plate injury, and whether p38 activation is important in the bony repair of the injured growth plate. In this project, following gene profiling experiments, the temporal p38 activation pattern after growth plate injury will be investigated using Western blotting. To further determine whether p38 activation requires TNF-α signalling, rats with growth plate injuries will be treated with a TNF inhibitor. Although TNF-α is known to play an important role in regulating the events of bone formation in bone fracture repair (section 1.7.1), its actions in the bony repair of injured growth plate cartilage remain unclear. Therefore, the potential roles of TNF-α signalling in regulating the injury-induced inflammatory response, mesenchymal and osteoprogenitor cell recruitment, proliferation, apoptosis and bone cell differentiation at injured growth plate prior to bony repair will be examined by inhibiting TNF signalling in rat growth plate injury model with TNF antagonist. Finally, as a means to examine the possible roles TNF-α – p38 signalling may have in regulating the mitogenic and chemotactic cellular events during bony repair following growth plate injury, we will investigate the potential importance of interaction between TNF-α signalling and p38 activation in proliferation, migration and apoptosis of cultured rat bone marrow-derived mesenchymal cells (rBMMC) treated with exogenous TNF-α, as well as TNF-α and p38 inhibitors.

### 1.8.2 Project Hypothesis

The activation of p38 MAP kinase signal pathway after growth plate injury is mediated by TNF-α, which is induced during growth plate injury inflammatory response. In addition, TNF-α – p38 signalling pathway is important in regulating mesenchymal progenitor cell migration and proliferation processes and the subsequent bony repair of injured growth plate.

### 1.8.3 Project Aims

***Chapter 2 Aims:***

1. To characterise gene expression profiles of inflammatory cytokines and growth factors at the injured growth plate in young rats.
2. To confirm the bone bridge formation at growth plate injury site is not through endochondral bone formation but mainly an intramembranous process.

***Chapter 3 Aims:***

1. To characterise the temporal and spatial patterns of p38 MAP kinase activation at the injured growth plate during the early inflammatory and fibrogenic injury responses.
2. To examine the roles of TNF-α signalling in p38 activation and in the subsequent bony repair of injured growth plate.
3. To examine whether mesenchymal progenitor cell proliferation and migration induced by TNF-α signalling is through p38 MAP kinase pathway *in vitro*. To examine whether TNF signalling is required for rBMMC apoptosis *in vitro*.

# END OF CHAPTER 1

# CHAPTER 2

# EXPRESSION OF PROINFLAMMATORY CYTOKINES AND GROWTH FACTORS AT THE INJURED GROWTH PLATE CARTILAGE IN YOUNG RATS

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**Expression of proinflammatory cytokines and growth factors at the injured growth plate cartilage in young rats**

*Bone 2004, Vol 35: 1307-1315*

**Zhou F.H.** (Ph.D. Candidate)

Designed the research questions and planned relevant experiments to test these questions. Performed analysis on all samples, obtained and interpreted all result data, and wrote this manuscript. Finally, carried out all additional experiments, and manuscript adjustments and corrections required for publication acceptance by Bone.

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**Foster B.K.** (co-supervisor)

Supervised development of research idea and evaluated the manuscript.

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**Sander G.** (Research collaborator)

Assisted in the design of primers for real-time RT-PCR.

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**Xian C.J.** (Principal Supervisor)

Supervised in the design and development of work, performed most of the animal surgery, helped in data interpretation and manuscript evaluation, and acted as corresponding author.

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# CHAPTER 3

# TNF-α MEDIATIES p38 MAP KINASE ACTIVATION AND NEGATIVELY REGULATES BONE FORMATION AT THE INJURED GROWTH PLATE IN RATS

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***Journal of Bone and Mineral Research 2006, Volume 21 (7): Page 1075-1088***

# STATEMENT OF AUTHORSHIP

**TNF-α mediates p38 MAP kinase activation and negatively regulates bone formation at the injured growth plate in rats**

*Journal of Bone and Mineral Research (JBMR) 2006, Vol 21(7): 1075-1088*

**Zhou F.H.** (Ph.D. Candidate)

Designed the research questions and planned relevant experiments to test these questions. Carried out all the rat growth plate injury trials, collected all tissue samples, and conducted all the primary cell culture experiments. Performed analysis on all samples, obtained and interpreted all result data. Wrote and submitted this manuscript to JBMR for peer review. Finally, carried out all additional experiments, and manuscript adjustments and corrections required for publication acceptance by JBMR.

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**Foster B.K.** (co-supervisor)

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**Zhou X.F.** (Research collaborator)

Gave technical assistance in Western blotting experiments and evaluated this manuscript.

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Supervised the development of cell culture experiments and helped in data interpretation.

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Supervised in the design and development of this work, did the animal surgery, assisted in data interpretation, evaluated this manuscript, and acted as corresponding author.

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# CHAPTER 4

# GENERAL DISCUSSION & CONCLUSIONS

## 4.1 General Summary

In this PhD project, before the hypothesis "activation of p38 MAP at the injured growth plate is mediated through TNF-α signalling during the initial inflammatory response" was tested, the expression patterns of various cytokines and growth factors, in particular TNF-α, were firstly analysed at the sequential and overlapping inflammatory, fibrogenic, osteogenic, and remodelling events previously identified in a proximal tibial growth plate injury model in young rats (Xian *et al.*, 2004). Increased levels of gene expression of IL-1β, TNF-α, TGF-β1, FGF-2, PDGF and IGF-I at the injured growth plate were observed (reported in chapter 2), suggesting that these factors may play important roles in regulating the cellular events before, during and after bony bridge formation. In addition, the expression patterns of cartilage specific genes Sox9 and Col-2 and bone specific protein osteocalcin at the reparative stage, were consistent with previous histological and immunohistochemical observations showing direct differentiation of mesenchymal progenitors to osteoblasts, osteocalcin production and direct bone formation, as well as the lack of new cartilage formation and endochondral ossification at the growth plate injury site prior to the bone bridging event (Xian *et al.*, 2004).

Furthermore, the direct relationship between p38 activation and TNF-α signalling, induced by the initial inflammatory response after growth plate injury, and roles of this signalling pathway in positively regulating migration and proliferation of mesenchymal progenitor cells following the inflammatory event and prior to bony bridge formation were established using *in vivo* and *in vitro* analysis in chapter 3. Although excess TNF-α protein was shown to induce apoptosis in mesenchymal cell culture, the level of endogenous TNF-α signalling did not induce obvious cell apoptosis at the growth plate injury site. Finally TNF-α negatively regulates the bone bridge formation process at growth plate injury site by inducing mesenchymal cell recruitment and proliferation but inhibiting their osteoblastic differentiation (by suppressing cbfa1 transcription) and delaying osteoblastic

maturation and their subsequent bone matrix synthesis events. The direct inverse relationship between TNF-α and cbfa1 is also reflected by their strong negative correlation in gene expression levels throughout the entire growth plate injury repair process as reported in chapter 3.

## 4.2 The Pathway to Understanding Bone Bridge Formation

Unlike repair of bone fractures, which leads to bone regeneration, full regeneration of growth plate does not occur at the growth plate injury site (as discussed in section 1.6). Often, in response to growth plate injuries particularly the Salter & Harris Type III and IV, a large enough bone bridge is formed at the defect area, which can disturb the function of the growth plate in longitudinal bone growth and lead to shortening or angular deformities of the affected limb (Foster and Johnstone, 2002; Ogden, 2000; Salter and Harris, 1963). Current treatments for growth plate injury-induced growth deformities described in section 1.6.3 are mainly surgical corrective procedures, involving Ilizarov surgery (limb lengthening method through distraction osteogenesis) (Ilizarov and Frankel, 1988) and Langenskiold procedure (replacement of bone bridge with interposition fat materials) (Hasler and Foster, 2002; Langenskiold *et al.*, 1986). Both procedures are extremely painful and invasive, and they often do not effectively induce growth plate regeneration. Therefore, understanding the cellular and molecular events and regulatory mechanisms leading to the osteogenic and calcification event at the growth plate wounding site is an important step for developing biological and preventative strategies to initiate desirable growth plate cartilage regeneration and prevent bone bridge formation.

As a means to understand the cellular processes leading to the bony repair at growth plate injury site, firstly Lee *et al.* (2000) described lack of expression in markers specific for cartilage formation (Col-2) and endochondral ossification, Ihh, and VEGF at

the injury site using a growth plate drill-hole injury model in young mice (Lee *et al.*, 2000). This may imply that the bone bridge formation process is not through endochondral ossification. Later, using a similar drill-hole injury model in young rats, we also showed lack of cartilage formation by the absence of cartilage proteoglycan metochromatic staining and Col-10 immuno-localisation at the injury site prior to bone bridge formation (Xian *et al.*, 2004). In addition, immuno-localisation results of osteogenic markers cbfa1, osteocalcin and alkaline phosphatase on mesenchymal progenitors and differentiated osteoblasts at the growth plate injury site suggest that the formation of trabecular bone bridge occurs directly via intramembranous ossification or direct bone formation (Xian *et al.*, 2004). Since the initial identification of three cellular responses prior to bony repair and remodelling at the rat growth plate injury site, namely the initial acute inflammatory phase, the intermediate fibrogenic phase, and the subsequent osteogenic phase, a series of studies have been performed in our laboratory characterising the potential molecules involved in regulating these cellular events (Arasapam *et al.*, 2006; Chung *et al.*, 2006; Ngo *et al.*, 2006; Zhou *et al.*, 2004; Zhou *et al.*, 2006).

Based on previously identified regulatory factors that are induced during bone fracture repair (described in section 1.7) (Gerstenfeld *et al.*, 2003), in this study, the levels of gene expression of pro-inflammatory cytokines (IL-1β, TNF-α), growth factors (TGF-β1, FGF-2, PDGF, IGF-1), cartilage/bone transcription factors (Sox9, cbfa1) and matrix proteins (Col-2, osteocalcin) were also found to be affected during the inflammatory, fibrogenic, osteogenic and remodelling cellular events during growth plate injury repair which involved no obvious intermediate cartilage formation as reported in chapter 2-Zhou *et al.* (2004) as well as in chapter 3-Zhou *et al.* (2006). Similar gene expression patterns for IL-1β, TNF-α, FGF-2, and osteocalcin during the growth plate injury were obtained in separate rat growth plate injury experiments described in chapter 2 and chapter 3, suggesting that the 2mm drill-hole growth plate injury model at rat proximal tibia used

here provides reproducible results. In addition, the endochondral ossification process was not detected in our molecular characterisation of events leading bone bridge formation in chapters 2 &3, which further supports the direct osteogenic cellular mechanism for bone bridging (Lee *et al.*, 2000; Xian *et al.*, 2004).

Interestingly the cellular responses to this 2mm drill-hole growth plate injury model in rats used here closely resemble those of intramembranous ossification bone fracture healing events particularly in the marrow ablation bone repair model and rat diaphyseal drill-hole model. Similar expression patterns of various cartilage/bone proteins such as Col-2, osteocalcin and cbfa1 analysed during growth plate injury in chapter 2 were recently reported in a gene expression study during intramembranous bone regeneration in the rat femoral marrow ablation model (Kuroda *et al.*, 2005), which demonstrated initial suppression of Col-2, cbfa1 and osteocalcin expression and later up-regulation of osteocalcin and cbfa1 expression during osteogenic event. As reported in chapter 2, the down-regulation of Col-2 at the injured growth plate during the immediate inflammatory response and later during the remodelling phase may be due to the up-regulation in expression of IL-1β and TNF-α , which are potent inhibitors of Col-2 (Murakami *et al.*, 2000). Since TNF-α is a negative regulator of bone formation (Gilbert *et al.*, 2000; Gilbert *et al.*, 2002; Hashimoto *et al.*, 1989), the suppression of cbfa1 and osteocalcin mRNA levels during the initial inflammatory event could be regulated by TNF-α.

Very recently, up-regulated levels of gene expression of additional regulatory factors during injury responses have been reported with the same drill-hole injured growth plate model in rats, including BMPs of the TGF-β superfamily, inflammatory mediators cyclo-oxygenase (COX-2), inducible nitric oxide synthase (iNOS), and rodent specific cytokine-induced neutrophil chemoattractant (CINC-1) (Arasapam *et al.*, 2006; Chung *et al.*, 2006; Ngo *et al.*, 2006).

From the BMP expression study using the same growth plate injury model, Ngo *et al.* (2006) showed the up regulation in both BMP-3 & 4 and their BMP receptors from day 1-7, and their immuno-localisation on various inflammatory cells, mesenchymal progenitors and osteoblasts. This may suggest that BMP-3 and 4 signalling is important in regulating the initial inflammatory response and in mediating the downstream fibrogenic, and osteogenic events during growth plate repair. In addition, while the up-regulation of BMP-2 occurred on day 7 and was localised in mesenchymal cells and osteoblasts during the osteogenic event, BMP-7 expression increased after the osteogenic event during bone bridge remodelling at the growth plate injury site. These observations may imply the involvement of BMP-2 and BMP-7 in regulating the osteogenic and remodelling event of bone bridge formation, respectively. Consistently, the expression of BMPs is also induced by the various inflammatory, reparative and remodelling responses during bone fracture healing (Cho *et al.*, 2002; Nakase *et al.*, 1994). The similarity between the BMP gene expression profiles during growth plate repair and bone fracture repair may indicate that they share similar regulatory roles in mediating the injury responses in different tissue types.

While the current study has focused on expression and potential roles of IL-1β and TNF-α expressions and p38 activity at the injured growth plate (as reported in chapters 2 & 3), recent studies have reported up-regulated expression levels of other potential inflammatory mediators COX-2, iNOS and CINC at the injured growth plate in rats (Arasapam *et al.*, 2006, Chung, 2006). In these new growth plate injury experiments, the up-regulation in expression of inflammatory mediators COX-2, iNOS and CINC-1 along with the proinflammatory cytokines occurred during the initial inflammatory response on day 1. Similarly, the inflammatory mediator COX-2, induced by inflammatory response to fracture repair, has been shown to be essential for regulating both intramembranous and endochondral bone formation and the subsequent remodelling event (Gerstenfeld *et al.*,

2003; Zhang *et al.*, 2002). Although up-regulation of CINC-1, the rodent gene equivalent to IL-8 in humans, has not been previously described in bone fracture repair studies, it has been shown to play a vital role in neutrophil infiltration during inflammation and wound healing, since it is a potent chemoattractant expressed by inflammatory cells including neutrophils, mast cells, macrophages and T cells (Baggiolini *et al.*, 1995; Lin *et al.*, 2004; Ramos *et al.*, 2003; Shibata, 2002). These inflammatory mediators CoX-2, iNOS, and CINC-1 may be important in regulating the cellular events leading to bone bridge formation and maturation at the growth plate injury site.

In these two new experiments using a smaller 1.5mm drill hole growth plate injury model in rats (Arasapam *et al.*, 2006, Chung, 2006), however, the subsequent cellular and molecular processes for bony repair of injured growth plate were slightly different from those previously reported by Xian *et al.* (2004) and in chapter 2. These recent reports showed that bone bridge formation occurred through direct intramembranous ossification as well as endochondral bone formation, as indicated by areas of disorganised cartilaginous tissues localised with alcian blue metachromatic staining, up-regulation in mRNA expression of cartilage specific genes Sox 9, Col-2 and Col-10 from day 4-10, and presence of Col-2 and Col-10 immuno-staining at the growth plate injury site on day 4, 8 & 10 (Arasapam *et al.*, 2006, Chung, 2006). These new reports suggest involvement of the cartilaginous tissue formation event during and prior to bony formation in this model. Therefore, whilst the bony repair process of injured growth plate occurs mainly through intramembranous ossification, we can not completely exclude the possibility of endochondral ossification, to a small extent, occurring at the injury site during the fibrogenic and osteogenic phases from day 4-10, particularly when the drill hole injury site created in these experiments was smaller in area (Arasapam *et al.*, 2006; Chung *et al.*, 2006).

Since the inflammatory event is the first cellular event at the injured growth plate, our lab has proposed that these various inflammatory mediators induced immediately after growth plate injury, including TNF-α–p38 signalling, COX-2, iNOS, and CINC-1 could play similar as well as differential regulatory roles in regulating the subsequent events leading to bony repair. To address this question, three separate growth plate injury experiments have been carried out to examine potential roles of the TNF-α signalling (chapter 3), roles of COX-2 and iNOS enzymes (Arasapam *et al.*, 2006), and roles of neutrophils (Chung *et al.*, 2006), in mediating the initial inflammatory response and in influencing subsequent bony tissue repair. Although inhibition of TNF-α signalling using a TNF-α inhibitory drug reported here suppressed the increase in p38 activation induced by inflammation, the inhibition did not change numbers of inflammatory cells and expression of pro-inflammatory cytokine (IL-1β and TNF-α), indicating that while TNF-α is the mediator of p38 activation, it can not solely influence the cellular events of inflammation after growth plate injury. In recent experiments, inhibition of COX-2 and iNOS using specific inhibitors (Arasapam *et al.*, 2006), and depletion of neutrophils using antiserum (Chung *et al.*, 2006) resulted in the significant reductions in inflammatory infiltrate (particularly neutrophils), and only COX-2 and iNOS inhibition reduced pro-inflammatory cytokine expression. Therefore, these inflammatory mediators COX-2 and iNOS mediate the growth plate injury induced inflammatory response by recruiting neutrophils, which are required to initiate the downstream healing responses. Since TNF-α inhibition did not effect neutrophil infiltration, and depletion of neutrophils did not change gene expression of TNF-α either, this may indicate that the signalling pathways of TNF-α and neutrophils in the growth plate injury responses are independent from each other.

During the reparative fibrogenic and osteogenic phases of the bony repair of injured growth plate, although inhibition of TNF-α signalling reported in chapter 3 reduced migration and proliferation of mesenchymal cells, it increased trabecular bone formation,

which coincided with increased expression of the osteogenic specific genes cbfa1 and osteocalcin. Furthermore, the positive effect of TNF-α on mesenchymal cell proliferation and migration was shown *in vitro* to be mediated through p38 MAP kinase activation. Therefore, while TNF-α signalling is required to promote mesenchymal cell migration and proliferation at the injury site through the p38 pathway, TNF-α signalling negatively regulates cbfa1 and osteocalcin expression and formation of trabecular bone at the injured growth plate during the bone bridge formation and maturation events. This current in vivo study is consistent with previous *in vitro* experiments showing the negative effect of TNF-α on bone formation by suppressing cbfa1/RUNX2 expression (Abbas *et al.*, 2003; Gilbert *et al.*, 2002). Similar observations were made in the study conducted by Chung *et al.* (2006), where depletion of neutrophils during the early growth plate injury response resulted in less mesenchymal cell recruitment and cartilaginous tissue formation at the growth plate injury site, which were accompanied by increased osteoblast differentiation, trabecular bone and marrow formation on day 10 during bony repair. This further suggests that neutrophils function by recruiting mesenchymal cells, and enhancing chondrogenic differentiation from mesenchymal cells and delaying osteogenic differentiation and marrow formation. On the other hand Arasapam *et al.* (2006) indicated COX-2 and iNOS inhibition mainly delayed the differentiation of mesenchymal cells to cartilage cells and did not affect bone formation on day 8 during bony repair of injured growth plate. Furthermore during the remodelling phase only COX-2 inhibition resulted in reduction in osteoclast activity as indicated by the significant reduction in RANK-L/OPG ratio. These findings suggest that COX-2 and iNOS may play a role in stimulating cartilage tissue formation from mesenchymal cells on day 8 whereas COX-2 may be involved in the remodelling of bone bridge of injured growth plate. Taken together, the current and recent studies suggest that the injury-induced inflammatory response may play a role in regulating subsequent cellular events leading bony repair at the injured growth plate.

## 4.3 Thesis Conclusion

This PhD project has demonstrated that, during the initial inflammatory response after growth plate injury in a rat model, there was an up-regulated expression of TNF-α, a pro-inflammatory cytokine which is known to be essential for inducing bone fracture healing and in p38 MAP kinase activation but which negatively regulate osteoblast differentiation *in vitro*. In this growth plate injury model, we have discovered that there was also a transient activation of p38, which was mediated through TNF-α signalling during the inflammatory phase. In addition, we also revealed that, through activation of p38 pathway, TNF-α signalling may stimulate mesenchymal cell mobility, and mesenchymal and osteoprogenitor cell proliferation at the injury site during the intermediate fibrogenic and subsequent osteogenic phase of injured growth plate. Finally we identified the direct negative relationship between TNF-α activity and cbfa1 transcription and bone matrix formation during bony repair in this *in vivo* model. Therefore, TNF-α - p38 signalling pathway is involved in influencing the initial inflammatory response and subsequent fibrogenic and osteogenic events, and it negatively regulates bone bridge formation at the injured growth plate.

## 4.4 Future Directions

Although blocking the various inflammatory mediators of the inflammatory response led to a slight alteration in the subsequent bony repair process, none of which resulted in any growth plate regeneration. This indicates that there must be other molecular as well as cellular and mechanical contributors involved in bony repair rather than cartilage regeneration of this drill hole growth plate model in rats. After the avascular environment of growth plate, which is essential for cartilage maintenance, is disrupted by Salter Type III/IV fracture or experimentally induced by dental drill to a large enough extent, and if

movement does not interrupt vascularization and angiogenesis events at the injury site, bone bridging will occur mostly through intramembranous ossification (Wattenbarger *et al.*, 2002; Xian *et al.*, 2004). However recently at growth plate injury site of smaller size, bony repair also occurs through a fibrocartilage intermediate (Arasapam *et al.*, 2006; Chung *et al.*, 2006). In addition, previous experiments using interposition material such as fat or cartilage material at growth plate defect interrupted angiogenesis event and prevented bone bridge formation (Foster *et al.*, 1990; Kim *et al.*, 2000; Lennox *et al.*, 1983). Therefore, examining various angiogenic factors (particularly VEGFs) by inhibiting their functions during growth plate injury may provide additional understanding in the importance of angiogenesis in the bony repair.

Recently due to the technological improvements developed in the efficiency of using small amount of RNA for micro-array analysis for gene expression studies (Wang *et al.*, 2004), along with the availability of microdissection facility, rather than using RNA isolated from entire injured growth plate as described here, we should be now able to target the growth plate injury site and examine gene expression specifically and globally using microdissection and gene-array technologies. This will allow us to simultaneously characterise up-regulation of other potential regulatory genes during the injury responses leading to bone bridge formation via intramembranous ossification specifically at the injury site.

Since a significant increase in TNF-α-mediated p38 MAP kinase activation was only detected during the growth plate injury-induced inflammatory response prior to bony repair, p38 MAP kinase may also play a significant role in regulating inflammation as well as the downstream healing events. Although p38 activity has been shown to be required to stimulate mesenchymal cell proliferation and migration as demonstrated in chapter 3 and it is involved in inducing osteoblast differentiation of mesenchymal progenitors as shown in previous in vitro experiments (Hu *et al.*, 2003; Jaiswal *et al.*, 2000; Lee *et al.*, 2002; Suzuki

*et al.*, 2002), the regulatory properties of p38 during growth plate injury induced repair responses remain unclear. Future studies could include examination of effects of blocking p38 activity at the injury growth plate using a p38 inhibitor, and the direct effect of p38 activation induced by the initial injury response, in regulating various inflammatory cytokines (particularly IL-1β and TNF-α) expression and on bony tissue repair of injured growth plate. These studies will provide further insights to understanding the bone bridge formation process and mechanisms.

# APPENDICES

# Appendix 1. Experimental Protocols Used in Chapter 2 and 3

## Appendix 1.1 Total RNA Isolation from Growth Plate Cartilage

***Solutions required*:**

- RNAwiz™ (Ambion, USA)
- Chloroform (without IAA)
- RNase free water (DEPC treated MilliQ $H_2O$)*
- Isopropanol
- 75% cold ethanol
- RNeasy® mini column kit (Qiagen, Australia)
- RNase-Free DNase Set (Qiagen, Australia)

* Add 1ml DEPC (diethyl pyrocarbonate) to treat 1L of Milli Q $H_2O$, and shake vigorously to bring DEPC into solution. Incubate for over night (12 hours) and autoclave.

***Disruption and Homogenization of Tissues:***

Each Growth plate tissue sample was ground to a powder form in liquid nitrogen with a mini mortar and pestle. Then 500 µl of RNAwiz was added to the sample and ground until the sample was uniformly homogeneous in RNAwiz. The homogenant was decanted into 1.5 mL screw top tube. Another 500 µl of RNAwiz was added to the mortar and then transfered to the rest of the homogenant. The tissue samples homogenated in RNAwiz were stored at -20 °C.

***RNA isolation from rat growth plate using RNAwiz™***

Growth plate homogenants were brought to room temperature (RT) and incubated at RT for 5 min. Chloroform was added to the RNAwiz sample at 0.2× of RNAwiz used to homogenise the tissues. Then the sample was mixed vigorously for 20 sec, and incubated at RT for 10 min. The mixture was centrifuged at 13,200 rpm for 16 min at 4°C. The aqueous RNA phase was carefully transferred and halved into two clean RNase free tubes. Then 250 µl of RNase-free water was added to each tube and mixed well. Followed by addition of 500 µl of isopropanol to each tube. Sample in tubes were mixed well and incubated at -20 °C for 30 min. The tubes were centrifuged at 13,200 rpm for 16 min at 4°C. RNA pellets were washed with 1 ml 75% ethanol by vortexing. The tubes were centrifuged at 13,200 rpm for 5 min at 4°C.The RNA pellets were air dried in the fume hood. The pellets were dissolved in 50 µl of RNase free water at 37°C for 10min. The two

50 µl aqueous RNA samples from the same animal were pooled together for further purification on RNeasy mini column.

***RNA clean up with RNeasy® mini column kit***

**(Adapted from RNeasy® Mini Handbook, Qiagen, Cat# 74104)**

***Solutions required*:**

- Buffer RLT (Qiagen, Australia) plus βME (10µl βME in 1ml RLT buffer)
- Buffer RW1 (Qiagen, Australia)
- Buffer RPE (Qiagen, Australia)
- RNase-free DNase set (Qiagen Australia)
- Buffer RDD (Qiagen, Australia)
- RNase-free $H_2O$ (Qiagen, Australia)
- 100% Ethanol
- 10mM Tris-Cl, pH = 7.5

***Methods:***

To the 100 µl reconstituted RNA, 350 µl of RLT buffer plus βME was added and mixed well. Then 250 µl of 100% ethanol was add to the RNA sample and mixed well. The mixture was transferred into an RNeasy mini column and centrifuged at 13,000 rpm for 15 sec. The mini column was washed with 350 µl bufferRW1 by centrifuging at 13,000 rpm for 15 sec. For on-column DNase digestion of the RNA, 70 µl of buffer RDD was added to10 µl of DNase stock. Then the total volume was added directly to the column and incubated at RT for 15 min. The mini column was washed again with 350 µl bufferRW1 by centrifuging at 13,000 rpm for 15 sec., followed by washes with 500 µl buffer RPE and 13,000 rpm spin for 15 sec. The buffer RPE wash was repeated but 2 min spin. In a clean 2ml tube the column was centrifuged for 1 min to remove excess buffer RPE. RNA was eluted with 30 µl of RNase free water by centrifuging for 1 min.

Purity of RNA was measured with a GeneQuant RNA/DNA calculator. RNA was diluted (1:100) in 10 mM Tris-Cl, pH = 7.5 before the absorbance value at 260nm and 280nm of the sample was obtained. Pure RNA has an $A_{260}/A_{280}$ ratio of 1.9-2.1.

Determination of RNA concentration was determined with Gene Quant RNA/DNA calculator (Amersham). RNA was diluted (1:100) in RNase –free water before absorbance value at 260nm of the sample was obtained. Since $A_{260}$ reading of 1 = 40 μg/ml RNA, then the formulas are: concentration of RNA sample (μg/ml) = 40 x $A_{260}$ x dilution factor;
Total yield (μg)= Concentration x Volume of sample (in mls)

***1% denaturing agarose gel electrophoresis to check RNA integrity***

***Solutions***

- 10× MOPs buffer in DEPC $H_2O$ (200mM MOPs, 50mM Na Acetate, 10mM EDTA, pH=7 in 1L).
- Formaldehyde
- Agarose
- Loading buffer*

*Loading buffer per sample consist of:

| | |
|---|---|
| 2.5μl | 10× MOPs, |
| 4.4μl | 12M Formaldehyde |
| 12.5 μl | 18M Formamide |
| 2.5 μl | Coomassie blue dye |
| 1 μl | 10mg/ml Ethidium Bromide |

***Methods:***
Firstly, 5 μL of RNA extract samples (1-2 μg) were mixed with 20 μL of loading buffer and were denatured at 65 °C for 5 mins. The samples were loaded into the wells of a 1% denaturing agarose gel (1% (w/v) agarose, 1X MOPS, 13.2% formaldehyde in DEPC $H_2O$) immersed in 1X MOPS running buffer in a Bio-Rad mini gel tank. The samples were electrophoresed at ~80 V until the loading dye band had migrated approximately 5 cm from the wells. The gel was placed in an ImageMaster® (Pharmacia Biotech) and the image of the gel was photographed using Fuji Polaroid film. All samples with two rRNA bands at 28 & 18s indicate good RNA integrity. Therefore, are used for cDNA transcription.

**Appendix 1.2 Reverse transcription of total RNA to cDNA using SuperScript™ II (Adapted from Invitrogen, Cat # 18064-014 instructions)**

***Solution Required:***

- SuperScript II RNase H⁻ reverse transcriptase (Invitrogen, Australia)
- 5× First strand buffer (Invitrogen, Australia)
- 0.1 DTT (Invitrogen, Australia)
- Random Decamers (GeneWorks, Australia)*
- dNTPs mix (Geneworks, Australia)
- RNase OUT (Invitrogen, Australia)

* Reconstitute 100 μg of random decamer in 1 ml of water to obtain concentration of 100ng/μl. Prepare batches of 10 μl and 100 μl aliquot

***Methods:***

To prepare total RNA for cDNA transcription, 2 μl of 200ng random decamers and 1μl of dNTPs mix was added to separate 2μg growth plate RNA samples, then RNase-free water was added to make each sample up to 12 μl. The sample mixtures were heated to 65 °C for 5min and quick chilled on ice. Tubes were centrifuge briefly to collect the contents (remember to set heat block to 70 °C). 4μl of 5X first-strand buffer, 2 μl of 0.1M DTT and 1 μl of RNase OUT were added to the sample mixtures, mixed gently and incubated at 25 °C for 10min. Mixtures were incubate at 42 °C for 2 min, then 1 μl of SuperScript were added and mixed by pipetting gently up and down, and mixtures were further incubated at 42 °C for 50 min. Finally reactions were inactivated by heating the mixtures at 70 °C for 15 min. The 20 μl cDNA samples, which will be used as templates for amplication in real time RT-PCR, were stored cDNA at -20 °C.

**Appendix 1.3 Immunohistochemistry using rat proximal tibia sections.**

***Reagents required:***

- Xylene
- 100% Ethanol
- $H_2O_2$
- 10× PBS (0.58M $Na_2HPO_4$, 0.17M $NaH_2PO_4$, 0.68M NaCl, pH =7.4)
- 1× DAKO retrieval solution (pH 6) (Dako Cytomation, Australia)
- 1× DAKO retrieval solution (pH 10) (Dako Cytomation, Australia)
- Glycine
- Skim milk powder
- Normal Pig Serum
- 10% BSA in 1× PBS
- ABC-complex reagents (Dako Cytomation, Australia)
- Liquid DAB and DAB substrate (Dako Cytomation, Australia)
- Primary antibodies ( anti-IL-1β, TNF-α, FGF-2 from Santa Cruz, USA, and IGF-I from GroPep, Australia)
- Secondary biotinylated Multilink antibody (swine anti goat, mouse, and rabbit from Dako Cytomation, Australia)
- 10% Normal rabbit serum in 1% DAKO block (Dako Cytomation)
- Normal rat serum
- Primary antibody (anti-pp38 from Cell Signalling Technology, Australia)
- Secondary biotinylated antibody (rabbit anti- mouse from Dako Cytomation , Australia)

***Methods:***

1. Heat sections of overnight at 60°C for better attachment of sections on slides.
2. Deparaffinise:
   - 2× 7 mins in xylene to remove wax.
   - 2× 3 mins in 100% ethanol to remove xylene.
3. Quench sections with 0.5% $H_2O_2$ (0.833 of 30% $H_2O_2$ in methanol to 50 ml) at room temperature (RT) for 1 hour.

   Wash in $H_2O$ 5 mins 2× and once in PBS 5 mins.

4. Incubate in 1× DAKO retrieval solution (pH 6 or pH 10 depending on the antibody type) at 75°C for 1 hr and cool on bench for 20 mins.
5. Wash in water 5 mins, PBS 5 mins.
6. Soak sections in 0.2% glycine in PBS (0.1g in 50ml PBS) for 30 mins to block the remaining reactive site of fixative. Wash in water 5 mins.
7. Blocking step:
   - For cytokine (IL-1β and TNF-α) and growth factor (FGF-2 and IGF-I) staining use10% skim milk powder and10% pig serum in 1× PBS as blocking buffer.
   - For pp38 staining, use 10% Normal rabbit serum in 1% DAKO block as blocking buffer.

   Block all sections for 1.5 hr at RT. Flick off and briefly rinse in PBS 5 mins.
8. Dilute primary antibody in 1% BSA in 1× PBS.
   - anti-IL-1β (1:500) on sections treated in DAKO retrieval solution, pH 6
   - anti-FGF-2 (1:300) on sections treated in DAKO retrieval solution, pH 6
   - anti-IGF-I (1:50) on sections treated in DAKO retrieval solution, pH 10
   - anti-TNF-α (1:100) on sections treated in DAKO retrieval solution, pH 10
   - anti-pp38 (1:50) on sections treated in DAKO retrieval solution, pH 10

   Add primary antibodies to sections in a moist incubation box O/N at 4°C*. Wash sections in 1× PBS 5 mins.
9. Biotinylated secondary antibody incubation:
   - For cytokine (IL-1β and TNF-α) and growth factor (FGF-2 and IGF-I) staining, use biotinylated DAKO multi link antibody (1:500) in 1% BSA in 1× PBS.
   - For pp38 staining, use biotinylated rabbit anti-mouse (1:500) in 10% Normal rabbit serum plus1% Rat serum in 1× PBS.

   Incubate sections for 1 hr at RT. Wash sections in PBS for 5 mins 3×.
10. Incubate sections with ABC complex (1:600: e.g. 600µl PBS + 1µl sol A + 1µl sol B) incubate for at least 30 mins prior to use) for 1 hr. Wash sections in 1× PBS for 5 mins 3×.
11. DAB incubation: liquid DAB (1:200: e.g. 4 µl DAB + 400µl DAB substrate buffer + 400µl 1× PBS). Incubate till brown colour.
12. Counter stain in Hematotoxylin (10s), rinse in water, in $LiCO_2$ (10s), rinse in water, tip in 70% ethanol, 100% ethanol and in xylene 2×.
13. Place xylene based mounting media on the coverslip and mount sections.

* Add 1%BSA/PBS solution to negative control sections instead of primary antibodies

## Appendix 1.4 Western Blotting Analysis

***Western Buffer Preparation – Solutions***

Most solutions were adapted from:

- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A laboratory manual Second edition. Cold Spring Harbour Laboratory Press, USA. Page 18.51-18.65.
- Protocol manuals for pp38 monoclonal and p38 polyclonal antibodies from Cell Signalling Technology, Australia.

**2× SDS gel-loading buffer (Cell Signalling Technology, Australia)**

125 mM Tris-Cl (pH 6.8 at 25°C)

100 mM dithiothreitol (DTT)

2% SDS (electrophoresis grade)

0.1% bromophenol blue

10% glycerol

2× SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. DTT should be added just before the loading buffer is used, from a 1 M stock*.

* Prepare 1M DTT stock in Sodium Acetate (0.01M) at pH=5.2. store at –20°C.

**Tris-glycine electrophoresis buffer (Sambrook et al., 1989)**

25 mM Tris

250 mM glycine (electrophoresis grade) (pH 8.3)

0.1% SDS

A 5× stock can be made by dissolving 15.1 g of Tris base and 94 g of glycine in 900 ml of MilliQ $H_2O$. Then 50 ml of a 10% (w/v) SDS stock solution is added, and the volume is adjusted to 1L with $H_2O$.

**Transfer buffer (1×) (Sambrook et al., 1989)**

39mM glycine

48mM Tris base

0.037% SDS

20% methanol

Make a 10× glycine and Tris base stock requiring:

390mM glycine → 29.28g

480mM Tris base → 58.08g in 1L, pH 8.3

Prepare 1L of transfer buffer (pH 8.3) by adding 100ml of 10 × glycine and Tris base (pH=8.3) to 200ml of methanol and 3.7ml of 10%SDS, then make solution up to 1L with MilliQ $H_2O$.

**Preparation of 30% acrylamide (Sambrook et al., 1989)**

Dissolve 29 g of acrylamide and 1 g of Bis-acrylamide in 100 ml of $H_2O$.
Heat to dissolve and stir with a magnetic stirrer.

**10× TBS (Tris-buffered saline) (Cell Signalling Technology)**

To prepare 1 litre of 10× TBS: add 24.2 g Tris base, 80 g NaCl into MilliQ $H_2O$, adjust pH to 7.6 with HCl (always use at 1×).

**1× TBS, 0.1% Tween-20 (TBST)**

Add 100ml of 10× TBS, and dissolve 1ml of Tween-20 in 800ml MilliQ H2O, then make solution to 1L.

**Blocking Buffer (5% skim milk block)**

1× TBST with 5% w/v skim milk powder; for 50 ml, add 2.5g of milk powder into 50ml of 1× TBST.

**Primary antibody dilutions**

- For monoclonals, anti-pp38 (1:1000) (Cell Signalling) and anti-β-actin (1:5000) (Sigma, Australia), 5% skim milk block is used.
- For polyclonal anti-p38 (1:500) (Cell Signalling), 1%BSA in 1× PBS is used.

**HRP conjugated Secondary antibody dilutions**

- For pp38 detection, rabbit anti mouse HRP pre-absorbed in 2% rat serum (1:10,000) from Sigma, Australia is used
- For p38 detection, goat anti rabbit HRP (1:2000) from Sigma, Australia is used.

- For β-actin detection, the rabbit anti mouse HRP (1:20,000) is used.

**Ponceau Red A Protein dye**

Add 0.2% w/v Ponceau S to 3% TCA (trichloroacetic acid) in MilliQ $H_2O$, then mix and filter dye through 1M Whatmann paper. Store at RT in the dark.

***SDS Polyacrylamide Gel Electrophoresis***

1. Set up polyacrylamide gel electrophoresis equipment form Amersham Biosciences, UK according to manufacture instructions.
2. Preparation of 10 % SDS PAGE (5 ml)

| | |
|---|---|
| $H_2O$ | 1.9 ml |
| 30% acrylamide mix | 1.7 ml |
| 1.5M Tris (pH 8.8) | 1.3 ml |
| 10% SDS | 0.05 ml |
| 10% ammonium persulfate | 0.05 ml |

   Mix all, then add 2μl TEMED , mix immediately
3. Load gel using syringe immediately and add a layer of water to prevent gel from drying. Allow 20-30min for gel to set.
4. Preparation of 5% stacking gel (1ml)

| | |
|---|---|
| $H_2O$ | 0.68 ml |
| 30% acrylamide mix | 0.17 ml |
| 1M Tris (pH = 6.8) | 0.13 ml |
| 10% SDS | 0.01 ml |
| 10% ammonium persulfate | 0.01 ml |

   Mix all, then add TEMED 1μl, mix immediately. Insert comb into position and load stacking gel using 1 ml tips.
5. Preparing the loading sample:

   Add 30 μg of growth plate protein in 1× loading buffer plus 50mM DTT.
6. Boil samples for 6 mins, and then place them immediately on ice.
7. Load samples:
   1) Remove the comb from stacking gel and briefly run under water to remove gel residue.
   2) Place the gel into gel tank and add 1× Tris-glycine electrophoresis buffer. Especially in the well.

3) Using a 20 µl pipette and fine pipette tip slowly load samples.
4) Lastly, load 5µl of protein marker (Bio RAD, Australia).
5) Place electrode lid on the tank and run gel at 50V and then increase to 200V when the loading dye has reached the resolving gel. Stop electrophoresis when dye front is 1cm away the bottom of resolving gel.

***Electrotransfer of proteins on to Nitrocellulose membrane using Amersham semi-dry transfer system.***

1. In a shallow container place 6 sponges and nitrocellulose membrane (cut to the right size) and add 1× transfer buffer
2. Firstly, wet the apparatus and then place 3 sponges plus the SDS gel on top, and then place the nitrocellulose membrane on top of the gel (make sure there are no air bubbles). Lastly, place the last 3 sponges and then the electrode lid.
3. Run transfer at 60mA for 1 hr and 15 mins.
4. After stopping the transfer, label makers clearly with colour pencils and place the membrane into water.
5. Pour the water out and add Ponceau red to check the quality of transfer (1 min).
6. Remove Ponceau red using water, for 5 mins.

***Western blotting detection of pp38, p38 and β-actin***

1. Wash in 1× TBST for 5 mins (3×)
2. Block membrane in 5% milk in 1× TBST blocking buffer
3. Wash with TBST for 5 mins 3×
4. Incubate membrane in 1ml primary antibody (anti-pp38, -p38 or -b-actin at the appropriate dilution describe in Western blotting solution section).
5. Add the primary antibody on a large plastic dish (petri-dish), and then slowly place the membrane with the protein side facing directly down onto the antibody solution (make sure no air bubble is formed). To prevent the membrane form drying out during ithe overnight ncubation at 4°C, parafilm is used to cover the membrane, and the plastic dish is further stealed with a lid or plastic wrap.
6. Wash 3× for 5 mins each with TBST.
7. Incubate membrane with HRP-conjugated secondary antibodies in 1 ml Blocking Buffer for 1 hr at RT.
8. Wash with 1× TBST for 5 mins (3×).

9. Prepare enhanced chemiluminescence solution A and B

Solution A: 5ml Tris, pH 8 (0.1M),
22µl Coumaric acid in DMSO (90mM) stock (stored at –20°C),
50µl Luminol in DMSO (250mM) stock (stored at –20°C).

Solution B: 5ml Tris, pH 8 (0.1M)
3ml 30% $H_2O_2$.

Finally, incubate membrane in Solution A and B for 1 min in dark room, and expose to X-ray film (for pp38 detection expose for 15mins; p38 detection expose for 30mins, and β-actin detection expose for 5mins). Develop and fix x-ray film using Kodak developer and fixer (Kodak, Australia).

# Appendix 2 Personal Publications and Conference Presentations

## Appendix 2.1 Personal Publications (2003-2006)

***Refereed Journal Articles***

1. Penttila IA, Flesch IE, McCue AL, Powell BC, **Zhou FH**, Read LC, Zola H. Maternal milk regulation of cell infiltration and interleukin 18 in the intestine of suckling rat pups. **Gut** 2003;52(11):1579-1586. *Impact Factor*: 7.692. Ranked second highest journal in the field of Intestinal research.

2. Xian CJ, **Zhou FH**, McCarty RC, Foster BK. Intramembranous ossification mechanism for bone bridge formation at the growth plate cartilage injury site. **Journal of Orthopaedic Research** 2004;22(2):417-426. *Impact Factor*: 2.916. Ranked third highest journal in the area of Bone biology research.

3. Zhou XF, Song XY, Zhong, JH, Barati S, **Zhou FH**, Johnson SM. Distribution and localization of pro-brain-derived neurotrophic factor- like immunoreactivity in the peripheral and central nervous system of the adult rat. **Journal of Neurochemistry** 2004;91(3):704-715. *Impact Factor*: 4.604. Highly ranked journal in the area of Neuroscience.

4. **Zhou FH**, Foster BK, Sander G, Xian CJ. Expression of proinflammatory cytokines and growth factors at the injured growth plate cartilage in young rats. **Bone** 2004;35(6): 1307-1315. *Impact Factor*: 3.939. Second highest ranked journal in area of Bone biology.

5. Zhou XF, Li WP, **Zhou FH**, Zhong JH, Mi JX, Wu LL, Xian CJ. Differential effects of endogenous brain-derived neurotrophic factor on the survival of axotomized sensory neurons in dorsal root ganglia: a possible role for the p75 neurotrophin receptor. **Neuroscience** 2005;32(3): 591-603. *Impact Factor*: 3.410. Highly ranked journal in the area of Neuroscience.

6. Song XY, **Zhou FH**, Zhong JH, Wu LL, Zhou XF. Knockout of p75NTR impairs re-myelination of injured sciatic nerve in mice. **Journal of Neurochemistry**

2006;96(3): 833-842. *Impact Factor*: 4.604. Highly ranked journal in the area of Neuroscience.

7. Ngo TQ, Scherer MA, **Zhou FH**, Foster BK, Xian CJ. Expression of Bone Morphogenic Proteins and Receptors at the Injured Growth Plate Cartilage in Young Rats. **Journal of Histochemistry and Cytochemistry** 2006;54(8): 945-954. *Impact Factor*: 2.208. Second highest ranked journal in area of histo/cyto-chemistry.

8. **Zhou FH**, Foster BK, Zhou XF, Cowin AJ, Xian CJ. TNF-α mediates p38 MAP kinase activation and negatively regulates bone formation at the growth plate injury site in rats. **Journal of Bone and Mineral Research** 2006;21(7): 1075-1088. *Impact Factor:* 6.527. Highest ranked journal in area of Bone biology research.

***Published Abstracts for conference presentations***

1. Symposium: **Zhou FH**, Foster BK, Pyragius T, Sanders G, Xian CJ. Biphasic up-regulated gene expression of pro-inflammatory cytokines in the injured growth plate. The Australia Society for Medical Research (SA Division) Annual Scientific Meeting, Adelaide, South Australia (May 2003); Abstract 30.

2. Symposium: **Zhou FH**, Foster BK, Moore L, Xian CJ. Molecular characterisation of injury Responses at growth plate cartilage. The Matrix Biology Society of Australia and New Zealand 27$^{th}$ Annual Scientific Meeting, Acheron, Victoria (September 2003); Abstract 54.

3. Poster: **Zhou FH**, Foster BK, Sanders G, Xian CJ. Expression of pro-inflammatory cytokines and growth factors after growth plate injury in rats. The 3$^{rd}$ International Clare Valley Bone meeting, Clare, South Australia (March 2004).

4. Symposium: **Zhou FH**, Foster BK, Zhou XF, Xian CJ. Activation of p38 MAP Kinase and up-regulation of cytokines TNF-α and IL-1β at injured growth plate. The Australian Society of Medical Research (South Australia Division) meeting, Adelaide, South Australia (June 2004); Abstract 12

5. Symposium/Poster: **Zhou FH**, Foster BK, Zhou XF, Xian CJ. Activation of p38 MAP Kinase and up-regulation of cytokines TNF-α and IL-1β at injured growth plate. The Matrix Biology Society of Australia and New Zealand 28th Annual Scientific Meeting, Rottnest Island, Western Australia (September 2004); Abstract 14

6. Poster: Covino MA, Cool JC, **Zhou FH**, Macsai CE, Foster BK, Xian CJ. Effects of methotrexate chemotherapy on the structure, matrix synthesis and bone formation at the growth plate of rat growing long bones. The Matrix Biology Society of Australia and New Zealand 28th Annual Scientific Meeting, Rottnest Island, Western Australia (September 2004); Abstract 33

7. Symposium: **Zhou FH**, Foster BK, Zhou XF, Xian CJ. Activation of p38 MAP Kinase and up-regulation of cytokines TNF-α and IL-1β at injured growth plate. Australian & New Zealand Orthopaedic Research Society 10th Annual Scientific Meeting, Sydney, NSW (October 2004); Abstract 44.

8. Symposium: Xian CJ, Cool JC, **Zhou FH**, McCarty RM, Covino MA, Macsai CE, Foster BK. Growth plate cartilage injury responses and bone growth arrest. Proc Australian Orthopaedic Association South Australian Scientific Meeting, Adelaide, South Australia, Oct 2004.

9. Poster: **Zhou FH**, Foster BK, Xian CJ. Expression of pro-inflammatory cytokines and growth factors after growth plate injury in rats. Orthopaedic Research Society 51st Annual Meeting, Washington D.C., USA (February 2005); Abstract 1053.

10. Symposium: **Zhou FH**, Foster BK, Zhou XF, Xian CJ. Roles of TNF-α in growth plate injury responses in young rats. The Matrix Biology Society of Australia and New Zealand 28th Annual Scientific Meeting, Victor Harbour, South Australia (October 2005); Abstract 75.

11. Poster: Ngo TQ, Scherer MA, **Zhou FH**, Foster BK, Xian CJ. Expression of Bone Morphogenic Proteins and Receptors at the Injured Growth Plate Cartilage in Young Rats. The Matrix Biology Society of Australia and New Zealand 28th

Annual Scientific Meeting, Victor Harbour, South Australia (October 2005); Abstract 46.

12. Symposium: Xian CJ, **Zhou FH**, Chung R, Arasapam G, Scherer M, Ngo T, Cool J, and Foster BK Roles of the injury-induced inflammatory response in the bony repair of the growth plate cartilage. European Calcified Tissue Society Scientific Meeting, Prague, Czech Republic (May, 2006).

**Appendix 2.2 Conference Presentations (Talks and Posters)**

1. Symposium talk: **Zhou FH**, Foster BK, Pyragius T, Sanders G, Xian CJ. Biphasic up-regulated gene expression of pro-inflammatory cytokines in the injured growth plate. The Australia Society for Medical Research (SA Division) Annual Scientific Meeting, Adelaide, South Australia (May 2003); Abstract 30.

2. Symposium talk **Zhou FH**, Foster BK, Moore L, Xian CJ. Molecular characterisation of injury Responses at growth plate cartilage. The Matrix Biology Society of Australia and New Zealand 27$^{th}$ Annual Scientific Meeting, Acheron, Victoria (September 2003); Abstract 54

3. Poster: **Zhou FH**, Foster BK, Sanders G, Xian CJ. Expression of pro-inflammatory cytokines and growth factors after growth plate injury in rats. The 3$^{rd}$ International Clare Valley Bone meeting, Clare, South Australia (March 2004).

4. Symposium talk: **Zhou FH**, Foster BK, Zhou XF, Xian CJ. Activation of p38 MAP Kinase and up-regulation of cytokines TNF-α and IL-1β at injured growth plate. The Australian Society of Medical Research (South Australia Division) meeting, Adelaide, South Australia (June 2004); Abstract 12

5. Symposium talk/Poster: **Zhou FH**, Foster BK, Zhou XF, Xian CJ. Activation of p38 MAP Kinase and up-regulation of cytokines TNF-α and IL-1β at injured growth plate. The Matrix Biology Society of Australia and New Zealand 28$^{th}$ Annual Scientific Meeting, Rottnest Island, Western Australia (September 2004); Abstract 14

6. Symposium talk: **Zhou FH**, Foster BK, Zhou XF, Xian CJ. Activation of p38 MAP Kinase and up-regulation of cytokines TNF-α and IL-1β at injured growth plate. Australian & New Zealand Orthopaedic Research Society 10th Annual Scientific Meeting, Sydney, NSW (October 2004); Abstract 44.

7. Poster: **Zhou FH**, Foster BK, Xian CJ. Expression of pro-inflammatory cytokines and growth factors after growth plate injury in rats. Orthopaedic Research Society 51st Annual Meeting, Washington D.C., USA (February 2005); Abstract 1053.

8. Symposium talk: **Zhou FH**, Foster BK, Zhou XF, Xian CJ. Roles of TNF-α in growth plate injury responses in young rats. The Matrix Biology Society of Australia and New Zealand 28th Annual Scientific Meeting, Victor Harbour, South Australia (October 2005); Abstract 75.

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