CELLULAR AND MOLECULAR MECHANISMS INVOLVED IN THE REPAIR OF THE INJURED GROWTH PLATE IN YOUNG RATS

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THESIS SUMMARY

The growth plate cartilage, which is located at the ends of children's long bones, is responsible for longitudinal growth of the skeleton. However, due to its cartilaginous composition and its location, the growth plate is commonly injured, mostly through fractures. An undesirable outcome to growth plate fracture is the bony repair of the injured cartilage at the fractured area. Consequently, children often incur skeletal angular deformities and growth arrest. Current corrective surgical treatments for these outcomes are highly invasive, and therapeutic interventions are not possible as little is known about the mechanisms and pathways that lead to bone bridge formation.

Using a rat model, previous studies have shown sequential inflammatory, fibrogenic, osteogenic and bone maturation responses involved in the bony repair of the injured growth plate. However, structural changes in the growth plate, at both the injury site and at the non-injured area, have not been closely examined previously, and little is known about the molecular mechanisms underlying the bony repair. Therefore, this PhD study, using a rat tibial growth plate injury model, aimed to examine the effects of growth plate injury on the structure and composition of the injured growth plate in a longitudinal study using micro-CT and histology. Microarray analysis of the injury site only, collected using laser capture microdissection was used to identify potential cellular and molecular mechanisms involved in bone bridge formation. In addition, Real Time RT-PCR on adjacent uninjured growth plate was used to examine potential cellular/molecular changes at the uninjured area and on whole growth plate scrapes to examine the potential involvement of Wnt signalling in bone bridge formation.

Micro-CT analysis revealed a significant increase in bone material within the injury site (when compared to normal) at 14 and 60 days post-injury, where 12% and 40% of the injury site was replaced by bone, respectively. Interestingly, although there were no changes in growth plate thickness between injured and normal rats at either day 14 or 60, at day 60, many small bone tethers formed at the adjacent growth plate outside the injury site but none were found in normal aged-matched control rats. Histological studies revealed dereased proliferation but increased apoptosis of chondrocytes at the adjacent growth plate cartilage, and RT-PCR analysis revealed differential expression of apoptosis-regulatory genes Bcl-2 and FasL (compared to normal), confirming the increase in apoptosis in the adjacent uninjured growth plate. Down-regulation of Sox-9 and IGF-1 on days 7 and 14 suggests that growth plate injury may slow down the rate of longitudinal growth by decreasing chondrocyte proliferation and/or differentiaiton soon after injury. Lastly, bone matrix protein osteocalcin was increased on day 60, suggesting degeneration and bone formation at the adjacent uninjured area.

Microarray analysis identified changes in several key BMP and Wnt signalling components across the time-course of bone bridge formation, including BMP-2, BMP-6, BMP-7, chordin, chordin-like 2, and Id-1, and β-catenin, Csnk2a1, SFRP-1 and SFRP-4, respectively, in early stages of bone bridge formation (day 4 and day 8). Osteocalcin expression was also prominent at day 8, supportive of osteoblast development and bone formation. During later stages (day 14), active bone formation and remodelling was prominent and was largely regulated by the BMP signalling pathway (increased BMP-1 and BMP-6 but decreased inhibitor chordin), as well as by Traf6, Fgfr1, osteopontin, Mmp9 and

Wnt signalling, where several genes were up and down-regulated. Expression levels of Wnt signalling inhibitors (SFRP-1, SFRP-4 and Wisp1) were increased at days 8 and 14 and may be negatively regulating bone formation during the osteogenic phases of the repair of the growth plate injury site. Findings were also suggestive of an overall increase in the canonical Wnt signalling pathway at days 4 and14, supported by increased expression of β-catenin and drecreased expression of Wnt inhibitors, and decreased expression of Fzd1 and Fzd2 and increased Lef1 expression, respectively. Overall, this study found a complex balance between the canonical and non-canonical Wnt pathways as well as an association with BMP signalling over the time-course of bone bridge formation.

Lastly, Real-Time PCR on Wnt signalling components revealed significant changes in gene expression of Wnt genes, receptors and inhibitors, but were inconclusive as the method of tissue isolation was not specific enough to represent true changes in gene expression.

DECLARATION

This work contains no material which has been accepted for the award of any other degrees or diplomas in any university or other tertiary institution to Carmen Elizabeth Macsai and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ABBREVIATIONS

Abbreviation	Full name
ALP	Alkaline phosphatase
APC	Adenomatous polyposis coli
ATF4	Activating transcription factor 4
Bglap2	Bone gamma-carboxyglutamate protein 2
BMPs	Bone morphogenic proteins
BMUs	Basic multicellular units
BSA	Bovine serum albumin
BSP	Bone sialoprotein
Camk2	Calmodulin dependent protein kinase 2
Cbfa1	Core-binding factor alpha 1
CBP	Cyclic AMP response element-binding protein
cDNA	complementary DNA
Ck1A1	Casein kinase 1, alpha 1
COX-2	Cyclo-oxygenase 2
cRNA	complementary RiboNucleic Acid
DMSO	Dimethyl sulfoxide
DNA	DeoxyriboNucleic Acid
DSH	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast growth factor
FHL2	Four and half LIM domain 2

Fzd	Frizzled
GH	Growth hormone
GSK-3	Glycogen synthase kinase-3
H&E	Haematoxylin & Eosin
HGF	Hepatocyte growth factor
HSCs	Hematopoietic stem cells
5-HT	Serotonin/5-HydroxyTryptamine
IGFBPs	IGF binding proteins
Ihh	Indian hedgehog
iNos	inducible Nitric oxide synthase
Id-1	Inhibitor of DNA binding-1
LCM `	Laser capture microdissection
LEF1	Lymphoid enhancer-binding factor 1
LRP-5/-6	Low density lipoprotein receptor-related protein-5/6
Ltbp2	Latent transforming growth factor beta binding protein 2
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MES	2-(*N*-Morpholino) EthaneSulfonic Acid
mm, µm	millimetre, micrometre
Mmps	Matrix metalloproteases
MSCs	Mesenchymal stem cells
NFAT	Nuclear factor of activated T cells
°C	Degrees Celcius

OCT	Optimal cutting temperature
OPG	Osteoprotegrin
Osx	Osterix
PCP	Planar cell polarity pathway
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
Ptc	Patched
PTHrP	Parathyroid hormone (PTH)-related peptide
%	Percent
RANK	Receptor activator of NF-κβ
RANKL	Receptor activator of nuclear factor-kappaB ligand
RNA	RiboNucleic Acid
rpm	revolutions per minute
RT	Reverse transcription
Runx2	Runt-related transcription factor 2
SFRP	Secreted frizzled related protein
Shh	Sonic hedgehog
Sox	SRY (sex determining region Y)-box
TCF	T-cell factor
TGF-β	Transforming growth factor-β
TH	Thyroid hormone
TNF-α	Transforming nuclear factor-α
Tph1	Tryptophan hydroxylase 1

Traf6	Tumour necrosis factor receptor-associated factor 6
TRAP	Tartrate resistant acid phosphatase
g, mg, µg, ng,	gram, milligram, microgram, nanogram
ml, ul	millilitre, microlitre,
M, mM	Mole, milliMolar
VEGF	Vascular endothelial growth factor
Wif-1	Wnt inhibitory factor-1
Wnt	Wingless-Int
Wisp	Wnt1 inducible signalling pathway protein

CHAPTER 1

LITERATURE REVIEW & PROJECT AIMS

1.1 Introduction to Literature Review

The growth plate, located at the ends of children's long bones, is responsible for longitudinal growth of the skeleton. The growth plate is a cartilaginous tissue made up of specialized cells called chondrocytes. Longitudinal bone growth occurs via a process called endochondral ossification, whereby synthesis of an intermediate cartilaginous template is gradually replaced by bone.

The growth plate is the weakest structure in the developing long bone, and is therefore a common place for injury. The most common injury to the growth plate occurs as a result of fracture. A common undesirable outcome to growth plate fracture is the formation of bone across the growth plate (bone bridge formation) that can lead to skeletal angulation and growth arrest in the involved children. Current corrective surgical treatments for these outcomes are highly invasive, and biological therapeutic interventions are currently not available as little is known about the molecular and cellular mechanisms and pathways that lead to bone bridge formation.

1.2 Immature long bone structure

The bone has several functions, which include mechanical support, protection of the body's internal organs (e.g. ribs), and to permit movement (e.g. long bones) and provide protection (e.g. skull) [1, 2]. Bones also provide a site for haematopoiesis (blood cell production) and act as a metabolic reserve of mineral salts, factors critical for the maintenance of normal cell and tissue function [2]. The long bones of the immature skeleton are divided into four main areas, including the epiphysis, growth plate, metaphysis and diaphysis **(Figure 1.1)**. Around

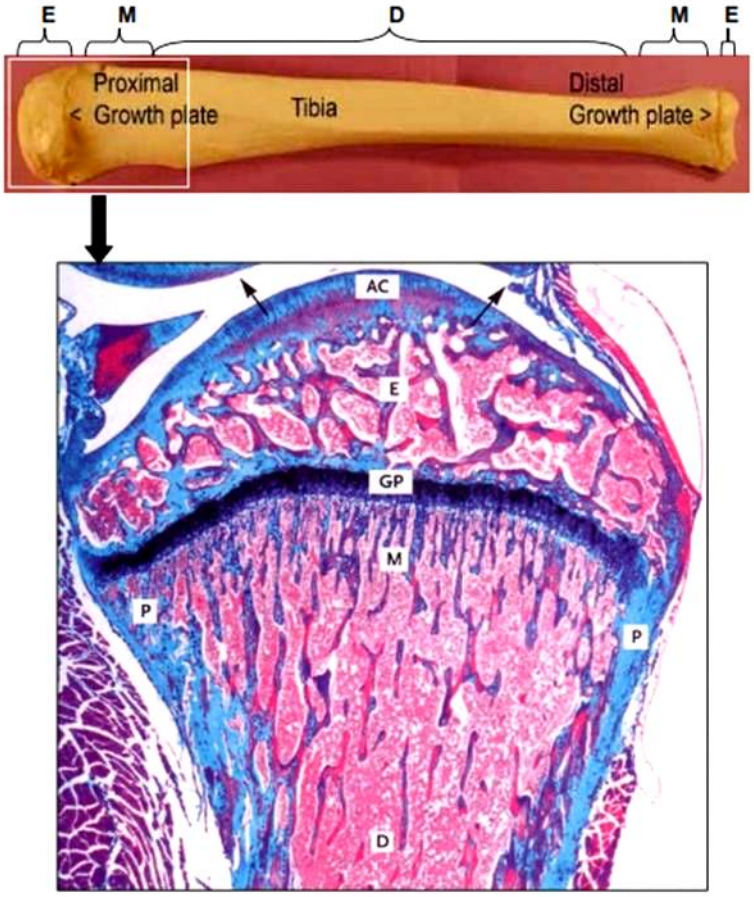

Figure 1.1 Immature long bone structure.

A human tibia showing the major anatomical regions of an immature long bone, including the epiphysis (E), metaphysis (M), diaphysis (D) and the growth plate. The growth plate is located at both the proximal and distal ends of the long bone. The morphology of these regions is further illustrated in the histological image of the proximal tibia (boxed area) and identifies the location of the periosteum (P) adjacent to the metaphysis and diaphysis. Above the growth plate (GP), the epiphysis (E) is covered by articular cartilage (AC), which is responsible for its enlargement in the direction of the arrows. The bone image was provided by Dr. CJ Xian and the histological image was adapted from Kerr JB (1999) Atlas of Functional Histology.

and within these four main areas lie the periosteum, cortical bone, compact bone, inner trabecular or spongy zone and articular cartilage.

1.2.1 Epiphysis, growth plate, metaphysis and diaphysis

The epiphysis is located at the ends of each long bone. Initially, at the beginning of development, it consists of a completely cartilaginous structure, composed of hyaline cartilage, called the chondroepiphysis [3]. During postnatal skeletal development, the chondroepiphysis begins to form bone, known as the secondary ossification centre. The ossification centre is important for the shape and form of the bone and continues to enlarge until the cartilage area has been almost completely replaced by bone at the time of skeletal maturity. Another important constituent of the epiphysis is the articular cartilage that covers the surface of the epiphysis. Articular cartilage is divided up into four zones, including the superficial zone, the transitional zone, the radial/deep zone and the calcified zone [4]. A distinct line called the tidemark separates the radial from the calcified zone [4]. Each zone has a distinct matrix region that is composed primarily of type II collagen, aggrecan and water [5]. Articular cartilage functions to absorb mechanical shock by distributing the applied load onto the bony supporting structures below [5]. In addition, it provides the joints with an excellent friction-free surface, lubrication and wear-characteristics required for continuous gliding motion [6].

The growth plate is an organised cartilaginous structure composed of specialised cells called chondrocytes which are divided into three distinct zones based on their morphology and state of maturity. The growth plate is responsible for longitudinal growth of the skeleton, through

a tightly controlled process called endochondral ossification, which involves the synthesis of a calcified cartilage scaffold and its subsequent conversion into bone [7]. A detailed description of chondrocytes and the constituents of the cartilage matrix are provided in sections 1.2.2 and 1.2.3, respectively. Growth plate structure and function is described in more detail in section 1.3 and the regulation of endochondral ossification is described in section 1.3.5.

The metaphysis is located below the growth plate and is the site of new bone formation. Bone forming and resorbing cells are recruited from the metaphysis to the chondro-osseous junction to remove the mineralized cartilaginous matrix of the hypertrophic zone and to form woven bone, also referred to as primary trabecular bone or primary spongiosa **(Figure 1.2)**. This bone is resorbed by osteoclasts and is replaced by lamellar bone to form secondary spongiosa **(Figure 1.2)**. Therefore, there is a very high level of bone modelling and remodelling occurring within the metaphysis, involving a very tight regulation of osteoblastic and osteoclastic activity.

Bone consists of two forms, including woven and lamellar bone, which are produced after osteoblasts synthesise and secrete osteoid, the organic component of the extracellular matrix which undergoes mineralisation to form bone [2]. Woven bone is produced when osteoblasts produce osteoid rapidly, characterised by the haphazard arrangement of collagen fibres. In contrast, lamellar bone is highly organised where collagen is in a regular parallel alignment [2, 8]. Lamellar bone is strong and efficient whereas woven bone is comparatively weak, bulky and inefficient. During skeletal development and bone fracture repair, woven bone is

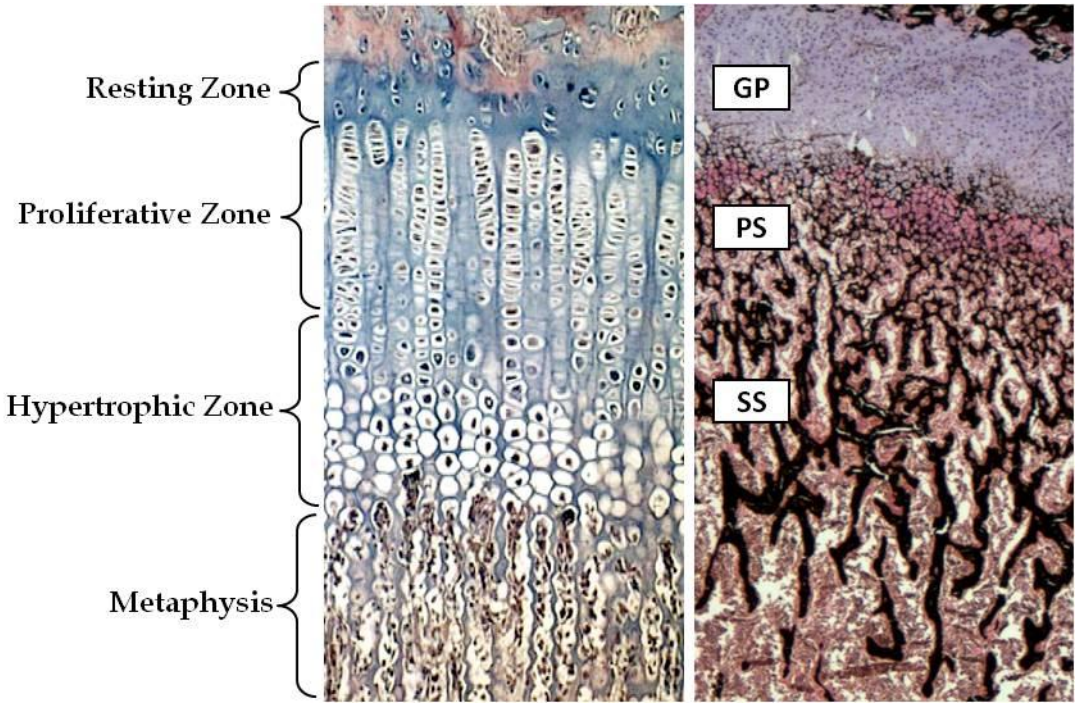

Figure 1.2 Structure of the growth plate and metaphysis.

The growth plate is made up of chondrocytes and is divided into three distinct zones including the resting, proliferative and hypertrophic zones. The metaphysis is located below the growth plate (GP) and is made up of the primary spongiosa (PS) and secondary spongiosa (SS). The images are adopted from Xian CJ et al (2006) *J Cell Biochem*.

the first type of bone to be produced and under the influence of functional stresses, is gradually remodelled to form mature lamellar bone. In some pathological conditions e.g. in Paget's disease, woven bone persists, causing skeletal fragility [9].

Finally, the diaphysis is the main shaft or mid section of a long bone and consists of a compact bone wall (cortical bone), the periosteum which is loosely connected to the cortical bone wall, and a medullary central cavity filled with bone marrow. The cortical bone of the diaphysis provides protection to the shaft when pressure is applied and flares out at both ends of the bone to form the metaphysis. At birth, the medullary cavity of long bones is comprised of active bone marrow, abundant with dividing multipotential stem cells, termed hematopoietic stem cells (HSCs). Through a process termed haemopoiesis, HSCs provide a continuous source of progenitor cells which give rise to a variety of mature blood cell types, including erythrocytes, granulocytes, lymphocytes, monocytes and platelets [10]. Haematopoiesis is essential in the renewal of circulating blood cells every few days to months and an important source of haematopoietic-derived bone cells, including bone resorbing osteoclasts [10]. With increasing age, bone marrow becomes less active, accruing increased numbers of fat cells and adipose tissue.

1.2.2 Skeletal cells

There are several cell types that are responsible for the formation and remodelling of bone. These cells are derived from either a mesenchymal cell lineage (chondrocytes, osteoblasts and osteocytes) or a haematopoietic stem cell lineage (osteoclasts). Undifferentiated mesenchymal cells reside within the bone canals, endosteum, periosteum and bone marrow,

and haematopoietic stem cells are found within the bone marrow or circulating blood [11]. There are many factors that regulate the recruitment, proliferation, differentiation and function of these cell types, which is discussed in later sections.

1.2.2.1 Chondrocytes

Chondrocytes are the primary cell type of cartilage and originate from embryonic mesenchymal condensations that are responsible for the patterning of the skeleton, including the head, trunk and limbs. After mesenchymal cells have been recruited to the future sites of skeletal development and have condensed, they are differentiated down the chondrogenic lineage, through a process called chondrogenesis. After commitment to the chondrogenic lineage, chondrocytes either differentiate to form hypertrophic chondrocytes or remain as chondrocytes. The chondrocytes that differentiate to hypertrophic cartilage eventually gives rise to the formation of the growth plate, the region of the bone that is responsible for the longitudinal growth of the skeleton. As mentioned, chondrocytes of the growth plate are divided up into three distinct zones, based on their different morphological and biochemical properties (reviewed in section 1.3). Chondrocytes that do not differentiate to the hypertrophic state become persistent cartilage, including articular cartilage that is located on joint surfaces [4, 12].

1.2.2.2 Osteoblasts

Undifferentiated mesenchymal cells commit and differentiate into bone forming osteoblasts through a process known as osteogenesis [13, 14]. Osteoblasts are responsible for bone formation during longitudinal bone growth by depositing and mineralising the extracellular

bone matrix at the chondro-osseous junction and metaphysis. During bone remodelling, in growing and adult bones, osteoblasts not only synthesise new bone at sites of bone resorption, but also play an essential role in regulating recruitment [15, 16], development and function [17, 18] of osteoclasts.

Cells committed to the osteoblast lineage are termed pre-osteoblasts and express alkaline phosphatase enzyme, an early marker of osteoblast differentiation [19, 20]. Mature osteoblasts express osteocalcin and osteopontin (bone sialoprotein), both late markers of osteoblast differentiation [19, 20]. At the conclusion of bone formation, osteoblasts can take one of three routes, either transforming into bone-lining cells, becoming entrapped within the bone matrix differentiating into osteocytes and lastly, becoming lost from the bone surface [2].

1.2.2.3 Osteocytes

Osteocytes are the most abundant cell type in bone, making up 90% of all bone cells [21, 22]. Osteocytes are terminally differentiated osteoblasts, embedded within mineralized matrix residing in individual spaces called lacunae. Osteocytes are satellite in shape and also comprise of a vast network of long branching cytoplasmic processes which they use to communicate to each other in the bone matrix as well as to osteoblasts and bone lining cells on the bone surface [23] and finally, to osteoclasts and some bone marrow cells [24]. Osteocytes use these cellular processes that network through the skeleton to communicate strain and stress signals, exchange nutrients and regulate the overall metabolism of the tissue [25, 26].

More recently, osteocytes have been referred to as the mechanosensor in bone, as they are able to sense and respond to load-induced strains [27], translating this information into biochemical signals to cells on the bone surface [28, 29]. These signals affect the levels of bone formation and/or bone resorption and are dependent upon osteocyte viability. For example, mechanical stimulation of human bone reduced osteocyte apoptosis, increasing bone formation rates, whereas unloading decreased osteocyte viability, resulting in bone loss [30]. Furthermore, osteocytes can target bone remodelling, where apoptotic osteocytes co-localise with regions of local osteoclast recruitment and bone resorption *in vivo* [31, 32]. Hence, osteocytes have a crucial role in controlling bone homeostasis and bone remodelling.

1.2.2.4 Osteoclasts

Osteoclasts are bone resorbing cells which function in conjunction with osteoblasts to remodel bone. Osteoclasts are derived from hematopoietic stem cells (HSCs) and differentiate into mature osteoclasts through a process called osteoclastogenesis [33]. HSCs give rise to monocytes/macrophage lineage progenitor cells that can then differentiate into mononuclear osteoclasts. Mononuclear osteoclasts have a resorptive capacity, however they then fuse with other mononuclear osteoclasts to form multinuclear osteoclasts [11]. Following multinucleation, the structure of the osteoclast is reorganized and includes new features such as the sealing zone, ruffled borders, and a transcytosis system, developed to discharge the resorbed bone debris [33]. Osteoclast formation and bone resorption are largely dependent on the interaction between osteoclast precursors and cells of the osteoblast lineage which produce osteoclastogenic molecules, as discussed in section 1.4.4.

1.2.3 Matrix molecules in cartilage

The cartilage matrix is synthesised by chondrocytes and consists mainly of collagens and proteoglycans, particularly type II collagen and aggrecan, respectively. Type II collagen and aggrecan are both specific phenotypic markers for chondrocytes [5, 34]. Type II collagen is found almost exclusively within cartilage and is present within all regions of the growth plate, although it is down regulated in the hypertrophic zone where there is a switch in expression from type II collagen to type X collagen [35]. Mutations in the Col2a1 gene responsible for type II collagen results in a group of disorders known as chondrodysplasias, characterised by the malformation of cartilaginous structures and related defects (cleft palate, deafness and disproportionate dwarfism) [36, 37]. Transgenic mice to model this disease were generated by the inactivation of the Col2a1 gene [38], and morphological and histological examination revealed that many of the organs and bones of the affected mice developed normally, including cranial bones and ribs. However, there were no signs of endochondral bone or functional growth plates in the long bones. The cartilage tissues showed disorganised columnar arrangements and there was no marrow within the marrow cavities. These findings indicate that type II collagen is essential for the formation, organisation and growth of the growth plate of long bones, but a well organised cartilage is not essential for the mineralisation of long bones or synthesis of periosteal bone. It is also not important for the formation of marrow cavities but is essential for capillary invasion of the cavities to form marrow [38].

Type II collagen has an important interaction with the proteoglycan aggrecan, which imparts additional strength and hydration of the cartilage [39]. Aggrecan is an aggregating

proteoglycan and has a specialised hyaluronic acid binding domain that interacts with hyaluronic acid via a link protein, forming a proteoglycan aggregate structure [40, 41]. This structure provides a stable source for attracting water molecules to hydrate the cartilage matrix and provides mechanical strength to the cartilage [39]. The attraction of water molecules results in the swelling of the tissue and is regulated and restricted by the type II collagen fibre network [39].

Hypertrophic chondrocytes secrete a matrix containing type X collagen, fibronectin, osteocalcin, osteopontin and alkaline phosphatase [42, 43]. Type X collagen is a short chain non-fibrillar collagen expressed exclusively in the hypertrophic zone of the growth plate and is a marker for cartilage undergoing endochondral ossification [44, 45]. Type X collagen is a homotrimer of alpha 1 (X) chains encoded by the NC1 domain of the COL10A1 gene. Mutations clustered in the NC1 domain can disrupt growth plate function leading to the development of Schmid metaphyseal chondrodysplasia, a disease characterised by short statue, metaphyseal widening and sclerosis [46]. As type X collagen is only present within the hypertrophic zone of the growth plate it is believed that it plays a role in regulating matrix mineralisation [1, 47-49]. Another proposed function is to provide an easily resorbed foundation for the deposition of bone matrix by providing support as the cartilage matrix is degraded, facilitating in the removal of type II collagen fibrils and influencing the vascular invasion of the cartilage matrix [47-50].

1.2.4 Matrix molecules in bone

The bone matrix is a composite material almost entirely synthesised by osteoblasts. This matrix is composed of an inorganic (mineral) portion and an organic portion, and provides physical strength and resilience to fracture [3]. The inorganic material constitutes 60-70% of bone and is mainly hydroxyapatite, with some carbonate and acid phosphate groups. Apatite is a small plate-like crystal which imparts bone its physical strength. The organic portion constitutes approximately 22-35% of the bone and is osteoid collagen, a specialised form of type I collagen synthesised by osteoblasts. Osteoblasts orchestrate the deposition of inorganic mineral salts within the osteoid matrix giving bones its characteristic rigidity and functional strength. The remaining composition of bone is made up of water (5-8%), and bone cells (osteoblasts, osteocytes and osteoclasts).

Collagen type I is the major extracellular matrix component of bone, accounting for 90% of the organic matrix [51]. It is a specialized form of collagen embedded in a glycosaminoglycan gel. Collagen I is important in imparting strength in bone and provides a scaffold for the mineralisation process [1]. Collagen type I is deposited in bone in parallel layers to produce mature bone (lamellar bone) or in a weave array to form woven or immature bone [2, 8]. Woven bone is formed quickly during fetal development, fracture repair and at the metaphysis of growing bones.

Collagen I is a heterotrimer made of two alpha 1(I) chains and one alpha 2(I) chain, products of the COL1A1 and COL1A2 genes, respectively and is expressed highly in fibroblasts and osteoblasts [51-53]. Osteoprogenitor transcription factor Runx2 is one of the positive

regulators of the osteoblast-specific expression of both type I collagen genes [54]. Mutations in either the COL1A1 or COL1A2 genes can lead to osteogenesis imperfecta (OI), where >90% of cases possess mutations in one of these genes [55]. OI is a heterogenous disorder characterised by bone fragility and fractures [56].

The glycosaminoglycan supporting gel also contains non-collagenous proteins including osteocalcin, osteopontin and osteonectin, which have an affinity for calcium. Osteocalcin constitutes 10-20% of all non-collagenous proteins in bone. Osteocalcin is secreted by osteoblasts and recruits osteoclasts and/or osteoclast precursors for bone resorption [57]. Osteocalcin also binds calcium and hydoxyapatite and regulates the rate of mineralisation [58, 59]. Furthermore, recent evidence supports a role for osteocalcin in the regulation of glucose metabolism and fat mass [60, 61]. Mice lacking osteocalcin displayed decreased insulin secretion, β-cell proliferation, glucose tolerance, insulin resistance and increased adiposity. Therefore, osteocalcin appears to behave like a hormone and favours insulin secretion [61]. Osteonectin constitutes 5-10% of non-collagenous proteins and is also synthesised and secreted by osteoblasts. Osteonectin regulates the formation and growth of hydroxyapatite crystals, appearing with mineralisation, linking collagen to hydroxyapatite [62-64]. Osteopontin, secreted by osteoblasts and osteocytes, is found at active sites of bone metabolism, enhancing cell survival, migration, and anchoring osteoclasts to resorptive sites [65, 66]. Osteopontin also negatively regulates hydroxyapatite formation [58, 67].

1.3 Growth plate structure and function

The growth plate, which is located at the ends of children's long bones, is responsible for longitudinal growth of the skeleton which occurs via a process called endochondral ossification, whereby an intermediate cartilaginous template is made and then replaced by woven bone. The growth plate, made up of specialized cells called chondrocytes, consists of three distinct histological zones beginning with the resting zone and extending through the proliferative and hypertrophic zones **(Figure 1.2)**. Briefly, endochondral ossification is initiated when pre-chondrocytes in the reserve zone are stimulated to proliferate and then these chondrocytes proceed through stages of maturation and hypertrophy [68]. Hypertrophic chondrocytes produce a matrix that undergoes calcification, forming a scaffold for new bone. Hypertrophic chondrocytes also undergo apoptosis as new blood vessels start to invade the calcified cartilage, bringing in bone forming cells (osteoblasts) and bone resorbing cells (osteoclasts). New bone is formed after osteoblasts, derived from bone marrow osteoprogenitor cells, invade the partially resorbed cartilage and lay down new bone at the adjacent metaphyseal bone region **(Figure 1.2)**.

1.3.1 Chondrogenesis

Chondrogenesis is the process whereby growth plate and articular cartilage is formed from mesenchymal condensations, during embryonic development [69, 70], bone growth and fracture healing [34, 71]. During embryonic development, mesenchymal cells derived from the lateral plate mesoderm, are recruited to future sites of skeletal development and express adhesion molecules (N-cadherin and neural cell adhesion molecule) forming mesenchymal condensations. Mesenchymal condensations become committed to the chondrogenic lineage

after expressing the chondrogenic transcription factor Sox-9, which initiates the expression of chondrocyte-specific extracellular proteoglycans and collagens, including collagen II [72]. Subsequently, chondrocytes differentiate into prehypertrophic chondrocytes followed by hypertrophic chondrocytes, which express Indian hedgehog (Ihh) [73], and Runx2 and Runx3 [74], respectively. Eventually, hypertrophic chondrocytes become part of the growth plate cartilage, which in humans is formed at approximately 8 weeks gestation [7]. Hypertrophic chondrocytes produce a matrix made up of collagen X [41] and express angiogenic factors to induce blood vessels to invade the calcified cartilage bringing in bone cells to lay down new bone [75, 76] (discussed in section 1.3.4). Finally, chondrocytes die at the junction between the growth plate and metaphysis by apoptosis autophagic cell death [77] (discussed in section 1.3.4). Chondrocytes that do not undergo hypertrophy form articular cartilage, the permanent cartilage located on the outer edge of the epiphysis [4]. Lastly, the chondrogenic events that occur during embryonic development are similar to cartilage repair which occurs during normal bone fracture healing, where mesenchymal progenitor cells are recruited to the injury site and differentiate to chondrocytes forming a scaffold for new bone [78].

1.3.2 Resting Zone

Chondrocytes of the growth plate resting zone are subdivided into two distinct cell types, being the reserve and epiphyseal chondrocytes [79], and reside in an abundant matrix consisting of collagen II and aggrecan [80]. Epiphyseal chondrocytes are rounded in shape, sparsely distributed, and usually scattered singularly within the cartilage matrix [79, 81]. Conversely, reserve chondrocytes are flattened and often grouped in pairs, aligned parallel to

the long axis of the bone [79]. The precise role of the resting zone remains unknown, however it has been shown that these chondrocytes act as a source of stem cells or progenitor cells for the production of chondrocyte columns in the proliferative zone [80], therefore determining the rate and extent of linear bone growth [79]. The proliferative capacity of resting zone chondrocytes diminishes with age, increasing the rate of growth plate senescence and thus slowing and eventually ceasing linear growth [79].

1.3.3 Proliferative Zone

The growth plate proliferative zone is located immediately below the resting zone. Proliferative chondrocytes are sourced from the resting zone after chondrocytes enter the cell cycle and are stimulated to proliferate. Chondrocytes are organized in longitudinal columns of flattened cells which undergo rapid division to produce two daughter cells that line up along the long axis of the bone [82, 83]. The orientation of proliferative chondrocytes is responsible for the longitudinal direction of bone growth achieved during endochondral ossification. Chondrocytes in the proliferative zone produce a matrix of predominantly collagen type II, and various proteoglycans. Non-collagenous proteins are also produced that organize the matrix and regulate the mineralization process [84].

Cells in the proliferative zone eventually stop dividing, and enter a pre-hypertrophic stage where they are committed to become hypertrophic chondrocytes [82, 85]. The parathyroid hormone-related peptide (PTHrP)/Indian hedgehog feedback (Ihh) loop (discussed in section 1.3.5.1) tightly regulates the transition of proliferative chondrocytes into hypertrophic chondrocytes [73, 86]. Furthermore, the rate of cellular proliferation is regulated (discussed

in section 1.3.5) such that it will offset cellular loss of hypertrophic chondrocytes at the chondro-osseous junction [87, 88].

1.3.4 Hypertrophic Zone

Hypertrophic chondrocytes in the growth plate are still organized in columns but have increased their volume by five to ten times. Hypertrophic chondrocytes produce a matrix made up of collagen X and also express Runx2, bone sialoprotein (BSP) and secreted factors including VEGF and matrix metalloproteases 9 and 13 (Mmp9 and Mmp13). There is a high level of alkaline phosphatise activity which is involved in the mineralisation of the growth plate. The hypertrophic chondrocytes calcify the extracellular matrix, providing a scaffold for the formation of bone matrix. Hypertrophic chondrocytes also release angiogenic factors such as VEGF bringing in osteoclasts to resorb the matrix and osteoblasts to deposit bone [75, 89].

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 [7]. The mechanism by which hypertrophic chondrocytes die is currently under review. The long-standing opinion is that hypertrophic chondrocytes die by apoptosis [90], but several studies have opposed this view, describing the process to be morphologically distinct from apoptosis. It is likely that hypertrophic chondrocytes die by autophagic cell death [77], or other non-apoptotic processes [91].

1.3.5 Regulation of Growth Plate Function

There are a number of systemic factors, locally secreted factors, and transcription factors involved in regulating endochondral ossification and longitudinal bone growth. A number of the major players discussed below regulate endochondral ossification by controlling the pace and onset of chondrocyte proliferation and differentiation.

Two major systemic (or endocrine) factors regulating longitudinal bone growth include growth hormone (GH) and thyroid hormone (TH). GH is a potent stimulator of bone growth through promoting the expression of insulin-like growth factors (IGF), known as the GH/IGF axis [83, 92]. While excess GH can result in gigantism, the effects of GH deficiency are closely associated with growth retardation [93].

Thyroid hormone also has a critical role in the regulation of endochondral ossification [94]. In children, hypothyroidism causes growth arrest and short stature [95], while an increase in thyroid hormone leads to advanced bone maturity, premature growth plate closure and eventual short stature [96]. The underlying mechanisms of thyroid hormone action are less clear but thyroid hormone has been shown to regulate chondrocyte proliferation and differentiation, as well as mineralization and angiogenesis [97-99]. Furthermore, thyroid hormone has been shown to regulate several known pathways involved in the regulation of growth plate function including the Wnt signalling pathway [100], GH/IGF signalling [101], the parathyroid hormone-related protein (PTHrP)/Indian hedgehog (Ihh) feedback loop [94] and fibroblast growth factor (FGF) signalling [102].

1.3.5.1 Parathyroid hormone-related protein and Indian hedgehog

In fetal bone, parathyroid hormone-related protein (PTHrP) is produced in perichondral cells and periarticular chondrocytes. PTHrP diffuses away from these sites to mediate its actions on the growth plate by binding to PTH/PTHrP receptors. These receptors are expressed in the lower proliferative zone and in the higher prehypertrophic zone [73, 103]. Activation of PTH/PTHrP receptors by PTHrP binding retains chondrocytes in a proliferative state and delays their differentiation into hypertrophic chondrocytes. Mice lacking PTHrP die at birth from inadequate respiration as a result of improperly formed bones, particularly the rib cage [104]. Furthermore, mutant PTH/PTHrP receptors cause devastating chondrodysplasias, including Jansen's disease and Blomstrand's disease [105].

In the fetal growth plate, Indian hedgehog (Ihh) is expressed by prehypertrophic and hypertrophic chondrocytes and regulates the synthesis of PTHrP. The Ihh signal induces the expression of PTHrP by periarticular chondrocytes, inhibiting differentiation of chondrocytes in the proliferative pool from maturing into hypertrophic chondrocytes [73]. Chondrocytes that no longer receive PTHrP are committed to hypertrophy and transiently express Ihh until fully hypertrophic [73]. Therefore, together, Ihh and PTHrP regulate the proliferation and differentiation of fetal growth plate chondrocytes by a local negative feedback loop [105]. Fibroblast growth factor signalling and bone morphogenic protein signalling have been shown to interact with this pathway to regulate endochondral ossification [106, 107].

The existence of the PTHrP/Ihh feedback loop has also been examined in the postnatal growth plate. Localisation and expression of PTHrP, Ihh and their receptors (PTH/PTHrP receptor and patched (ptc), respectively) have been identified in all maturational zones of the postnatal growth plate of chicks [108], mice [109], rats [110] and humans [111]. Interestingly, differences in the pattern of expression have been observed across the different species. Nonetheless, these studies suggest that components of the feedback loop act to locally regulate chondrocyte proliferation and differentiation and no longer rely on signalling via the perichondrium and epiphysis.

A critical role for Ihh in regulating endochondral bone formation was demonstrated when it was shown that Ihh could stimulate chondrocyte proliferation independently of PTHrP [112]. Ihh signalling also has an important role in osteoblast differentiation, both in embryonic and postnatal bone formation. Mice with a targeted deletion of Ihh in postnatal chondrocytes resulted in a loss of trabecular bone, accompanied by a failure in osteoblast maturation and reduction in osteoblast activity [113]. Furthermore there was a significant decrease in components of the Wnt signalling pathway suggesting that Ihh expression by postnatal chondrocytes activates the Wnt signalling pathway to regulate osteoblast differentiation [113]. The deletion of Ihh in postnatal chondrocytes also had a dramatic effect on the structure and composition of the growth plate and articular cartilage, leading to dwarfism [113]. Therefore, Ihh is essential for maintaining the growth plate and is required for sustaining trabecular bone and skeletal growth [113].

1.3.5.2 Fibroblast growth factors

Fibroblast growth factor (FGF) signalling plays an essential role during endochondral ossification, regulating chondrocyte proliferation and differentiation. FGF ligands are expressed in condensing mesenchyme during embryonic skeletogenesis and in the postnatal growth plate during endochondral ossification. FGFs bind to one of four fibroblast growth factor receptors (FGFRs) and heparan sulfate proteoglycans (HSPGs) [114, 115]. FGF ligands expressed in the perichondrium can also act on FGFRs on growth plate chondrocytes to regulate bone growth [116]. FGF components interact with other signalling pathways, namely BMP and Ihh pathways and the systemic factor thyroid hormone [102] to regulate chondrocytes.

Fibroblast growth factor receptor 3 (FGFR3) is expressed by proliferating and hypertrophic chondrocytes in the growth plate and has a critical role in endochondral bone formation [116]. Achondroplasia, the most common form of human dwarfism, results from a single point mutation in FGFR3, in most cases substituting an arginine for a glycine residue in the transmembrane domain of the tyrosine kinase-coupled transmembrane receptor that is expressed in the growth plate [117, 118]. This type of mutation results in enhanced activity of the receptor and when introduced into mice using gene targeting, these mice developed skeletal dysplasia closely mimicking that of achondroplasia in humans [119]. Conversely, FGFR3 knockout mice demonstrated limb overgrowth, suggesting that FGFR3 is a negative regulator of bone growth [120, 121]. Interestingly, FGF18-null mice exhibit a skeletal overgrowth more severe than FGFR3-null mice [122, 123].

It is clear that FGFR3-mediated dwarfism is mostly attributed to an inhibition of chondrocyte proliferation. Many studies have identified STAT transcription factors as the major regulators of FGF-mediated inhibition of chondrocyte proliferation. The role of STAT proteins is now delineated as being necessary, but not sufficient, and is one of several components that FGF signalling uses to mediate chondrocyte proliferation. Other players include transcription factors p107 and p130 [124, 125], p21 [126], Ink family Cdk inhibitors [127], ERK and p38 MAP kinases [128, 129]. Conversely, a recent study [130] claims a major role for ERK and p38 MAP kinases in FGF-mediated growth inhibition and postulates that STAT components are not involved.

1.3.5.3 Wnt signalling

Wnt signalling has a role in both embryonic cartilage development and postnatal endochondral bone formation. Wnt-5a together with Wnt-5b is shown to regulate the proliferation of chondrocytes and their maturation into hypertrophic chondrocytes in both the embryonic and postnatal growth plate [131, 132]. For a comprehensive coverage of the involvement of Wnt signalling in skeletal development and bone growth, please refer to my published review article [133] (section 1.4.6).

1.3.5.4 Bone morphogenic proteins

Bone morphogenic proteins (BMPs) are members of the transforming growth factor-ß (TGF-ß) superfamily. Expression and localisation studies of BMPs in the growth plate have shown that BMP-2 and BMP–6 are expressed at highest levels in the hypertrophic zone, BMP-7 is expressed at highest levels in the proliferative zone [134, 135], and BMP-4 is weakly

expressed in the prehypertrophic zone [134]. Another study revealed BMP-1 through to 7 was expressed at highest levels in the hypertrophic region [136].

Microdissection and microarray analysis has revealed novel information on the expression of BMP agonists and antagonists in the growth plate. A strong BMP signalling gradient across the growth plate was identified, where antagonists of BMP signalling are highly expressed in the resting zone (gremlin, chordin, and BMP-3), and agonists (BMP-2 and –6) in the hypertrophic zone. This gradient may be an important mechanism by which BMP-related genes regulate chondrocyte proliferation and differentiation. BMP receptors, BMPR1a, -1b and –2, were expressed in all zones of the growth plate [135].

BMPs signal through serine/threonine kinase receptors, type 1 and 2 [137], and downstream signalling molecules Smad1, 5 and 8 [138, 139]. These proteins have a role in the development of skeletal tissue during embryogenesis, including the formation of mesenchymal condensations and cartilage differentiation [140, 141]. BMPs also have a major role in the regulation of postnatal longitudinal bone growth, including regulating osteoblast differentiation and function [142-144], regulation of chondrocyte proliferation [145] and hypertrophic differentiation in the growth plate [146, 147]. Studies demonstrate that BMP regulation of chondrocyte differentiation is variable; some identifying BMPs to delay hypertrophic differentiation [107], but the majority support a role for BMPs in stimulating chondrocyte differentiation [145].

1.3.5.5 Growth hormone and insulin-like growth factors

Growth hormone (GH) and Insulin-like growth factors (IGFs) are critical regulators of longitudinal bone growth. The role of the GH/IGF system in the growth plate and in postnatal longitudinal bone growth has brought about considerable debate. The somatomedin hypothesis [148] initially proposed that GH regulated skeletal growth by stimulating the production of IGF-1 in the liver. As an endocrine factor, IGF-1 was transported to skeletal tissue to promote the expansion of the growth plate. It is now understood that both circulating and locally produced IGF-1 have an important role in stimulating chondrocyte proliferation [149-152]. Furthermore, GH may have an IGF-dependent and independent role in maintaining longitudinal bone growth [153]. IGF-1 is expressed at much higher levels in the perichondrium than in the growth plate in 1 week and 12 week old mice, suggesting that IGF-1 produced in the perichondrium also acts on receptors in the growth plate to regulate endochondral ossification [92].

IGF ligands can bind to two receptors, IGF1R and IGF2R, and to IGF binding proteins (IGFBP 1-6) [154-157]. IGF-1 is transported in the circulation and extracellular fluid bound to IGFBPs and a liver-derived glycoprotein, protecting and prolonging its half-life. IGFBPs can also inhibit or potentiate the actions of IGF-1 [155, 158]or have IGFBP independent effects [158, 159]. IGF-2 is also expressed in the growth plate and may play a role in chondrocyte proliferation [153]. IGF-1 is essential in embryonic longitudinal bone growth as IGF-$1^{-/-}$ mice display dwarfism only days post coitus [160]. Chondrocytes in the growth plates of long bones of the mutant mice also show decreased proliferation, increased apoptosis, abnormal differentiation and delayed mineralisation [160].

IGF-2 is important in early development where disruption in IGF-2 function resulted in severe growth retardation in rat embryo's aged 16-19 days post coitum, persisting at birth [161]. Furthermore, in the growth plate of early postnatal rats, IGF-2 is expressed at high levels in the resting and proliferative zone and is the predominant IGF expressed at 1 week of age [92]. However, with increasing age (1-12 weeks), there is an increase in IGF-1 expression and a dramatic decrease in IGF-2 expression in the growth plate [92]. This decline with age may be responsible for the decline in the rate of bone growth with increasing age [92, 162].

1.3.5.6 Sox9 and Runx2 in regulation of endochondral ossification

Sox9 is a key transcription factor controlling chondrogenesis [163]. During embryogenesis, Sox9 is required for prechondrogenic mesenchymal cell condensations and prechondrocyte and chondroblast differentiation, which is essential in the patterning of the skeleton. Sox9 activates and interacts with the transcription factors, Sox5 and Sox6, to induce the expression of the essential cartilage matrix genes Col2a1, Col2a2 and aggrecan [72, 164]. Sox9 is expressed in all chondroprogenitors and all differentiated chondrocytes, but not in hypertrophic chondrocytes. Sox9 is expressed by proliferating chondrocytes in the growth plate and is essential in controlling the rate of chondrocyte proliferation, as well as delaying chondrocyte hypertrophy [165]. Mutations in the human Sox9 gene cause campomelic syndrome, a lethal skeletal malformation syndrome and autosomal sex reversal [166-168]. Sox5 and Sox6 also regulate chondrocyte differentiation, delaying the onset of terminal hypertrophic differentiation [169, 170].

Runt-related transcription factor 2 (Runx2) is essential in osteoblast differentiation and plays a major role in regulating chondrocyte maturation, Ihh expression, and subsequently, chondrocyte proliferation. Nonetheless, a deficiency in Runx2 is only sufficient to delay chondrocyte maturation [171, 172]. However $Runx2^{-/-}3^{-/-}$ mice demonstrate a complete absence of chondrocyte maturation [74], demonstrating that Runx3 is important in assisting Runx2 in chondrocyte maturation. Runx2 regulates Ihh expression, by directly activating the Ihh promoter enhancing chondrocyte proliferation [74]. PTHrP can also inhibit Runx2 expression, which strongly suggests that Runx2 coordinates with the Ihh/PTHrP negative feedback loop to regulate chondrocyte proliferation and differentiation [173]. Sox-9 and co-repressor histone deacetylase-4 have also been shown to inhibit Runx2 expression [174, 175].

1.3.6 Vascularisation and mineralisation of the growth plate

Three major vascular supplies associate with the growth plate, including the epiphyseal artery, perichondral artery and metaphyseal arteries. The epiphyseal artery passes through the reserve zone and terminates in the uppermost cells of the proliferative zone. The perichondral artery supplies the perichondral ring of LaCroix. Lastly, the metaphyseal artery supplies the periphery of the growth plate, and is the main nutrient artery that enters the metaphysis. This is of particular importance, providing access for a number of highly specialized cells involved in bone formation [3]. The vessels turn back on themselves to form a venous return. Importantly, these vessels do not penetrate the hypertrophic zone, which results in an avascular lower proliferative and hypertrophic zone [68].

The vascular invasion of the growth plate is a critical process in endochondral ossification, providing a passage for the recruitment of the cell types involved in cartilage resorption and bone deposition, and providing the signals necessary for bone formation [176]. Vascular endothelial growth factor-A (VEGF-A) is expressed at high levels by hypertrophic chondrocytes and in mineralised regions of the growth plate, and is the most crucial stimulator of angiogenesis [89]. Inhibition of VEGF-A expression almost completely blocked the invasion of blood vessels, leading to significantly decreased trabecular bone formation and a thickened hypertrophic zone [75]. VEGF has several other important roles during endochondral ossification, including regulating the differentiation of progenitor cells into chondrocytes, osteoblasts, endothelial cells, and osteoclasts [177, 178].

Matrix metalloproteinases (Mmps) also play a critical role due to their ability to cleave ECM components, an essential requirement for angiogenesis to succeed. Mmp9, expressed by several cell types at the base of the growth plate, is the most important Mmp in ECM degradation, where Mmp9 inactivation yielded phenotypes comparable to VEGF-A inactivation [76]. Mmp13 is expressed by hypertrophic chondrocytes and osteoblasts, at the site of vascular invasion, and supports the actions of Mmp9 [179-181].

Mineralisation occurs between the chondrocyte columns at the lower hypertrophic zone [182]. The matrix surrounding late hypertrophic chondrocytes is mineralised through the deposition of hydroxyapatite, the inorganic mineral of bone composed of calcium and phosphate [7, 182]. Membrane-bound matrix vesicles are thought to provide the nucleation site for mineralisation [183]. Mineralisation of matrix vesicles and cartilage matrix is

dependent on alkaline phosphatase activity, which functions to remove extracellular pyrophosphate [183].

1.4 Bone Formation

The bony skeleton is formed by two distinct processes, which are intramembranous and endochondral ossification. Endochondral ossification is essential during bone development, requiring a cartilage scaffold prior to the formation of new bone. Intramembranous ossification is direct bone formation and is essential throughout life, particularly during skeletal remodelling and fracture repair. Skeletal cells critical during bone formation and remodelling include osteoblasts, osteocytes, and osteoclasts, derived from mesenchymal stem cells and hematopoietic stem cells, respectively. These cells are responsible for synthesising new bone (osteoblasts), resorbing bone (osteoclasts) and detecting bone damage and activating sites of bone remodelling (osteocytes). The intimate association between these cell types in maintaining skeletal bone mass is becoming widely recognised, where an imbalance in their function can lead to the debilitating low bone mass disease osteoporosis or alternatively, increased bone formation, causing osteopetrosis [184].

1.4.1 Endochondral ossification and intramembranous ossification

Endochondral ossification occurs at the growth plate during normal bone growth and within fracture callus when osteoblasts form osteoid on a cartilaginous template. Endochondral ossification permits elongation and thickening of the bone during fetal development and throughout childhood until bone growth ceases [2, 68]. As discussed in section 1.3, vascularisation of the calcified cartilage brings in bone forming cells (osteoblasts) and

bone/cartilage resorbing cells (osteoclasts), which are responsible for modelling and converting the calcified cartilage into trabecular bone. New bone is made on the surface of the calcified cartilage scaffold by osteoblasts, which are derived from bone marrow mesenchymal stem cell osteoprogenitor cells [68, 185].

Intramembranous ossification does not require a cartilaginous scaffold to be formed, prior to the laying down of new bone. Instead, cells of mesenchymal condensations differentiate directly into bone-forming osteoblasts. Bones most notably formed through this process include the flat bones of the skull, and parts of the pelvis, scapula and clavicles, and cortex (cortical bone) of the long bones [68, 186].

Cortical bone formation occurs at the periosteum, a thin layer of osteogenic and fibroblastic cells located along the periosteal cortex of cortical bone [187]. Cortical bone covers the shaft of all bones and is able to expand after mesenchymal stem cells located within the osteogenic layer of the periosteum differentiate into osteoblasts, laying down new bone [188]. Expansion occurs largely during postnatal development in response to increases in bone length (endochondral ossification) [188, 189] and works in coordination with osteoclasts which resorb bone on the inner (endosteal) surface to regulate cortical thickness [190]. Periosteal expansion slows significantly with the completion of longitudinal bone growth, however continues slowly throughout adult life [191, 192]. With increasing age, the amount of bone resorbed on the endocortical surface exceeds that of periosteal apposition, leading to a loss in cortical bone thickness [190]. Bone remodelling also occurs on the periosteal surface, but at a very low rate in comparison to the endosteal surface [191].

1.4.2 Mesenchymal stem cells and osteoblastogenesis

Friedenstein et al [193, 194] identified multipotential precursor cells in bone marrow stroma, termed colony forming unit fibroblasts, due to their ability to form colonies when plated on plastic. These non-hematopoietic, self-renewable, multipotent stem cells are now commonly referred to as mesenchymal stem cells (MSCs) and have the potential to differentiate into a range of skeletal tissue cells including chondrocytes, osteoblasts and adipocytes, forming cartilage, bone and fat, respectively [195, 196]. Other cell types derived from the MSC lineage include tenocytes, fibroblasts, skeletal myocytes, visceral stromal cells, cardiomyocytes, neurons and hepatocytes [197-199]. Bone marrow (BM) is the main source of MSCs, however they also reside in fat [200, 201], muscle [202], synovial membrane [203], periosteum [204], bone [205, 206], dental tissues [207, 208], blood [209], umbilical cord blood [210], pericytes [211] and skin [212]. The application of MSCs in a clinical setting is highly desirable, due to their ability to self-renew and proliferate, and their multi-potential ability to form various cell types and repair a large number of bone and musculoskeletal diseases; however these clinical applications are in early stages [213].

The maintenance and/or commitment of MSCs down the osteoblast lineage is reliant upon many genes and signalling pathways. The precise regulation remains to be clearly elucidated, however commitment of MSCs to the osteoblast lineage is regulated by several transcription factors, including Runx2, osterix, activating transcription factor 4, and the Wnt, BMP and Notch signalling pathways are known to be major players. The Wnt signalling pathway has a significant role, whereby β-catenin expression and activation in MSCs results in osteoblast differentiation and an absence stimulates chondrogenesis [214, 215]. Several

other components of this pathway play a role in the regulation of MSC differentiation down the osteogenic pathway, including casein kinase 2 [216], Frizzled 2 [217], Wnt-3a [218, 219], Wnt-5a [220, 221], Dkk-1 [222], Wnt-1- induced secreted protein 1 [223, 224] and T-cell factor-7 [218].

BMP2 has been shown to inhibit myogenic differentiation of MSCs and stimulate osteoblast differentiation indirectly via Wnt/β-catenin signalling [225, 226]. BMPs -4, -6, -7 and -9 have also been shown to regulate osteoblast differentiation of MSCs [227-230]. BMP antagonist, chordin, negatively regulates the differentiation of MSCs, where its knockdown in hMSCs results in increased ALP expression and calcium mineral deposition [231].

Notch signalling can maintain bone marrow MSCs by suppressing osteoblast differentiation, by means of Hes1, Hey1 and HeyL mediated inhibition of Runx2 expression [232]. Mice lacking components of the Notch signalling pathway had a massive accumulation of bone in the bone marrow cavities of long bones in 8-week old mice. This is attributed to a significant increase in the activity of osteoblasts and a severe reduction in the number of MSCs [232].

1.4.3 Regulation of osteoblast differentiation and bone formation

Critical stages in bone cell differentiation (osteoblastogenesis) include MSC commitment to the osteoblast lineage and generation of osteoprogenitor cells, which proliferate and then migrate to the bone surface. At the bone surface, osteoprogenitor cells are differentiated into preosteoblasts, and then mature bone-forming osteoblasts, which are characterised by the expression of the early bone marker alkaline phosphatase enzyme (ALP) [186, 233, 234],

and by the expression of late osteogenic markers including osteocalcin and osteopontin [235], respectively. Finally, osteoblasts can transform into bone-lining cells or terminally differentiate into osteocytes [235]. Mechanisms that control the process of osteoblastogenesis have until recently, remained largely unknown. Commitment of MSCs towards the osteoblast lineage is induced by the runt family transcription factor Runx2. Specific markers favouring the osteoblast lineage early in differentiation still remain to be elucidated; however, CD106 might be a useful surface marker in identifying BM-MSCs undergoing osteogenic differentiation [236]. Recent work has strengthened the role of Runx2, and has identified other critical transcriptional factors involved in osteoblastogenesis, including Osterix, β-catenin and activating transcription factor 4, as discussed below. Several growth factors, hormones and other signalling pathways have also demonstrated a crucial role in regulating osteoblastogenesis. Of significant importance is the Wnt signalling pathway and this is described in detail in my review paper [133] in section 1.4.6.

1.4.3.1 Runx2

Runx2 has been identified as the master regulator of osteoblast differentiation [237, 238]. Runx2, a transcription factor belonging to the Runx family is crucial for several aspects of bone formation, including the regulation of osteoblast differentiation [239]. Runx2 binds to the cis-acting element OSE2 in the promoter of the mouse Osteocalcin gene (OG2) and is an important component in regulating osteocalcin mRNA levels [239]. Runx2 is regulated by many factors including Twist1, Msx2, Bapx1, Stat1, Shn3, all of which inhibit Runx2 activity, and SATB2 which is a positive regulator [240-244]. Runx2-null mice die at birth

due to respiratory failure, and are small in stature, which is due to the maturational arrest of osteoblasts and a failure of the skeleton to undergo mineralisation [245, 246]. In the null mice, both intramembranous and endochondral bone formation were blocked; although cartilage development was almost normal [245, 246], the cartilage lacked both vascular and mesenchymal cell invasion [245]. These mice also lacked ossification centres and were unable to develop marrow [246]. Heterozygous mice were phenotypically similar to humans suffering from cleidocranial dysplasia [246].

The level of Runx2 expression regulates the level of bone maturity. Runx2 is expressed at high levels in earlier stages of osteoblast differentiation, in immature osteoblasts, and at low levels during osteoblast maturation [247]. Runx2 expression maintains a pool of immature osteoblasts in the formation of trabecular bone, whereas the suppression of Runx2 induces the expression of late stage differentiation, forming compact bone [247]. This was demonstrated in dominant negative-Runx2 mice, where the structure and composition of trabecular bone closely resembled compact bone, inducing late stage osteoblast differentiation, increasing bone volume and bone mineralisation [247]. However, the inhibition of Runx2 does not always induce bone formation, as suppression of Runx2 at the beginning of osteoblast differentiation suppressed bone formation and bone resorption, leading to osteopenia [248].

1.4.3.2 Osterix

Osterix (Osx) is a zinc finger-containing protein that belongs to the Sp/KLF family of transcription factors. Nakashima et al (2002) first identified Osx and demonstrated that Osx

is expressed in osteoblasts of all endochondral and membranous bones. Although both Runx2 and Osx deficient mice fail to undergo intramembranous and endochondral ossification, owing to a complete lack of osteoblast differentiation [245, 246, 249], osteogenic cells deficient of Osx still expressed Runx2 at levels comparable to that in wild-type osteoblasts [249]. Furthermore, Osx was not expressed in Runx2 null mice embryos, demonstrating that Osx acts downstream of Runx2 [249]. In chondrocyte differentiation, however, Runx2-independent regulation and function of Osx were shown, whereby Osx inhibits chondrocyte maturation in response to PTHrP [250]. Recently, Osx was shown to inhibit chondrogenesis, inhibiting the differentiation of immature chondrocytes into hypertrophic chondrocytes, but stimulating osteoblast differentiation [250].

1.4.3.3 β-catenin

β-catenin is a member of the catenin family and plays a pivotal role in the canonical Wnt signalling pathway [133]. β-catenin has been shown to induce early osteoblast differentiation but not later stages. Transfection of a mesenchymal progenitor cell line (C3H10T1/2) with β-catenin, cultured with or without BMP-2 revealed that expression of β-catenin alone induced 4-fold more ALP activity and ALP mRNA expression than with BMP-2 protein alone [214]. When combined, ALP activity levels were slightly less than that with β-catenin transfection alone. However, in contrast to the strongly up-regulated expression of osteocalcin after BMP-2 treatment, β-catenin transfection did not increase osteocalcin expression and this supports β-catenin not being needed for later stages of osteoblast differentiation [214]. Using reporter gene assays, Bain et al. (2003) also showed that β-catenin was activated during early stages of BMP-2 induced bone cell differentiation.

β-catenin is also critical in the determination of cell fate between osteoblasts and chondrocytes in embryonic skeletal development. Osteoblast precursors lacking β-catenin are arrested in osteoblast differentiation and develop instead into chondrocytes [251]. Consistently, another study demonstrated that β-catenin was essential in determining whether mesenchymal progenitors would become chondrocytes or osteoblasts regardless of regional locations or ossification mechanisms [252].

1.4.3.4 ATF4

Activating transcription factor 4 (ATF4) is a basic leucine-zipper transcription factor that is ubiquitously expressed, but has preferential protein accumulation within osteoblasts [253]. There is an abundance of literature investigating the role of ATF4 in late stage osteoblast differentiation and bone formation since it was first investigated *in vivo* by Yang et al (2004). The expression of bone sialoprotein and osteocalcin was markedly reduced in ATF4 deficient mice, both in embryos and at birth [253]. Embryos completely lacked trabecular bone and defects in bone formation persisted into adulthood where mice never achieved a normal bone mass [253]. This extreme bone loss could be attributed to the dramatic reduction in collagen I content observed in embryonic mice lacking ATF4. ATF4 does not affect the expression of collagen I in osteoblasts but specifically reduces the synthesis of collagen I by decreasing amino acid transport [253]. ATF4 also functions to regulate osteoblast proliferation and apoptosis, inhibiting proliferation and increasing apoptosis in ATF4 deficient cells [254].

ATF4 has a pivotal role in inducing the mRNA expression of osteocalcin through its interaction with Runx2 and their binding sites on the osteocalcin promoter, OSE1 and

OSE2s respectively [255]. Several factors interact with ATF4 and Runx2 to either inhibit or enhance the activities of these transcription factors on osteocalcin expression and thus, bone formation. General transcription factor IIA-γ binding to ATF4 increased levels of ATF4 protein in osteoblasts by preventing ATF4 protein degradation [256]. PTH increased ATF4 mRNA and protein expression and stimulated ATF4-dependent OSE1 activity in osteoblasts [257]. Factor-inhibiting ATF4-mediated transcription (FIAT) binds to ATF4 to inhibit ATF4 activity, thus inhibition of FIAT increased the bone forming activities of ATF4 in osteoblasts [258]. RSK2 is responsible for the phosphorylation of ATF4, and is heavily phosporhylated in osteoblasts [253]. It is suggested that ATF4 is a substrate of RSK2, particularly since the skeletal phenotypes of mice deficient in either ATF4 or RSK2 are very similar [253].

1.4.4 Osteoclasts: formation, regulation and bone resorption

Osteoclasts are derived from hematopoietic stem cells (HSCs) of the monocyte/macrophage lineage through a process called osteoclastogenesis [33]. The formation of osteoclasts is initiated during bone remodelling and is largely dependent on the interaction between osteoclast precursors and cells of the osteoblast lineage. This interaction/communication can be via direct contact, gap junctions, diffusible paracrine factors and/or the liberation of growth factors within the bone matrix during resorption. The OPG/RANKL/RANK system has been identified as the critical mediator of osteoclastogenesis [259-262].

Upon stimulation, osteoblasts express the osteoclastogenic ligand, receptor activator of NF-κβ ligand (RANKL) [263]. In addition, macrophage colony-stimulating factor (M-CSF) is secreted by osteoblasts and stimulates mononuclear hemopoietic osteoclast precursors to proliferate and express the RANKL receptor RANK. The binding of RANKL to RANK

stimulates osteoclast precursors to express a number of fusion proteins required for osteoclast progenitor fusion and activation [264]. On the other hand, osteoprotegerin (OPG) is also produced by cells of the osteoblast-lineage, which serves as a decoy receptor for RANKL enabling osteoblasts to inhibit and thus regulate osteoclast formation [265-267].

Osteoclastogenesis is regulated by many locally acting cytokines and systemic hormones which often act to induce and/or repress OPG, RANKL and RANK expression to inhibit or induce osteoclastogenesis. These include calcitonin [268], androgens [269], thyroid hormone [270], insulin [271], PTH [272], IGF-1 [273], interlukin-1 [274], CSF-1 [275], PDGF [276], BMPs [277, 278], TGF-β [279, 280], TNF-α [281], growth hormone [282] and glucocorticoids [283].

After osteoclasts attach to the bone matrix at sites which are referred to as podosomes, integrins on the multinucleated osteoclasts interact with matrix proteins, namely osteopontin and BSP, forming tight seals with the underlying bone matrix [284]. Within these seals, osteoclasts form ruffled bordered membranes, which secrete the proteolytic enzyme cathepsin K, and hydrochorlic acid. Acid and lysosomal enzymes that are released into the resorptive cavity degrade the matrix and dissolve the mineral bone (hydroxyapatite), components, which are then removed by the osteoclasts [285, 286].

Cathepsin K is a major bone proteinase, highly and selectively expressed by osteoclasts [287]. Cathepsin K can degrade a large number of bone matrix proteins; however it is only cathepsin K that can degrade native type-1-collagen in its triple-helix structure [288].

Inhibition of cathepsin K *in vitro* and *in vivo* inhibits bone resorption, where mice demonstrate increased trabecular number and thickness, characteristic of osteopetrosis [289, 290]. Conversely, overexpression of cathepsin K in mice resulted in thinning of the metaphyseal bone (osteopenia). Consequently, inhibitors of cathepsin K have been developed and trialled in rats and monkeys in the aim to treat postmenopausal osteoporosis in humans [287, 291].

1.4.5 Bone remodelling and regulation

Bone remodelling is the coupled resorption of bone, and formation of new bone at discrete foci called basic multicellular units (BMUs) [292]. In the adult skeleton, nearly all BMUs are undergoing remodelling. During skeletal growth and development, many BMUs are committed to a slightly different process called modelling. It is now known that, apart from osteoclasts and osteoblasts, osteocytes play important roles in regulating bone remodelling.

In modelling, the bone formation stage occurs without the preceding bone resorption phase, to increase the bone size during growth, particularly periosteal surfaces. However, the process of modelling and remodelling at the growth plate/metaphysis during longitudinal growth differs to the modelling occurring on periosteal surfaces [3]. During endochondral ossification, osteoclasts are recruited to the chondro-osseuous junction to resorb cartilage bars, followed by osteoblasts which form new woven bone to replace cartilage. With the coordinated action of osteoclasts and osteoblasts, the woven bone is then modelled and become the stronger and more mature lamellar bone [2, 3, 68]. Upon skeletal maturation, bone turns its focus to remodelling which is a process that is continued throughout an adult's lifespan.

Remodelling is important in maintaining a normal skeletal mass, repairing microdamage to the skeleton and regulating systemic calcium homeostasis [293]. Bone remodelling is initiated when lining cells on the surface of bone become activated and retract [293]. Osteoclasts are recruited to the active site and determine the extent (surface and depth) of bone resorption, excavating a resorption cavity [294]. After approximately 2-4 weeks, osteoclasts die by apoptosis and osteoblasts arrive and line the resorption cavity. The osteoblasts secrete and mineralise osteoid, determining the level of bone formation in the BMUs [293, 294]. Bone formation and mineralisation of a single BMU take approximately 4-6 months [293].

With aging and/or diseases, the volume of bone removed in a focal BMU may not equate the volume of bone replaced, whereby a net bone loss and/or structural damages can occur. For example, menopausal women sustain an increase in bone loss due to estrogen deficiency. Estrogen withdrawal is associated with increased remodelling intensity, whereby bone formation cannot keep up with the increasing activity of osteoclasts [295]. Consequently, up to 40% and 30% of postmenopausal women develop osteopenia and or osteoporosis, respectively [296, 297]. Osteoporosis is characterised by low bone mineral density and micro-architectural fragility and susceptibility to fracture [294, 296, 298]. Conversely, osteopetrosis is the result of a decrease in bone resorption and/or increase in bone formation, characterised by an overgrowth of bone [299, 300]. A large amount of research has been dedicated to developing anti-resorptive and bone stimulating drugs to treat osteoporosis, including strontium renelate [301, 302], which is the only compound able to simultaneously decrease bone resorption and stimulate bone formation [303]. Strontium renelate was shown

to significantly reduce vertebral and hip fracture risks in women with post-menopausal osteoporosis [303] and is regarded as critical in the management of osteoporosis [303]. Other treatments include the intermittent use of the anabolic agent, parathyroid hormone (PTH) [304, 305] and/or anti-resorptive agents particularly bisphosphonates including raloxifene [306, 307], alendronate [308, 309], and more recently zoledronate [310].

1.4.5.1 Osteocytes and bone remodelling

Osteocytes, terminally differentiated bone cells derived from osteoblasts and embedded within bone matrix, are the first to sense microcracks, fractures and loss of mechanical loading from their residence within the bone matrix [28, 311]. This occurs via their dendritic processes, by which osteocytes then communicate by signalling to osteoblasts or bone lining cells on the bone surface initiating bone remodelling [312]. For example, in response to microdamage, signals sensed and transmitted by osteocytes to bone lining cells degrade the bone matrix using collagenase and the bone lining cells lift off, forming a basic multicellular unit (BMU). Consequently, osteoblasts release chemoattractants such as monocyte chemoattractant protein-1 that direct the movement of osteoclast precursors, first together to allow fusion, then towards the bone surface of the BMU [15, 16]. Multinucleated osteoclasts then attach to the bone surface, via interactions with bone matrix proteins such as osteopontin, leading to the formation of mature bone resorbing osteoclasts [313, 314].

Several studies have implicated the Wnt signalling pathway in osteocyte mechanosensation [315-317], whereby the expression of the SOST gene product sclerostin, is exclusive to osteocytes and antagonizes the Wnt/β-catenin pathway by binding to the LRP-5/6 co-receptors [318, 319]. Recent studies have shown that parathyroid hormone down-regulates

the expression of sclerostin in osteocytes to increase bone formation [320, 321] and the rate of bone remodelling, via LRP-5-dependent and independent mechanisms, respectively [322]. Sclerostin has also been shown to inhibit BMP-stimulated bone formation both *in vitro* and *in vivo* [319, 323-325], however its mechanism of action differs from conventional BMP antagonists [319, 326]. Whereas sclerostin was shown not to inhibit stimulation of direct BMP target genes or to act as a direct BMP antagonist, BMP-induced osteoblastic differentiation was inhibited after Wnt signalling was antagonised by sclerostin in osteoblastic cells [319]. This view of action opposes that sclerostin can also inhibit BMP-induced bone formation by binding directly to BMPs [324]. Recent evidence has shown that osteocytes express OPG and RANKL at levels comparable to osteoblasts and this is dependent on Wnt/β-catenin signalling in osteocytes [327]. Conditional deletion of β-catenin in osteocytes resulted in severe skeletal defects indentifying a crucial role of osteocytes in bone homeostasis [327].

Furthermore, osteocyte apoptosis co-localises with sites of osteoclastic bone resorption *in vivo* [31, 32]. Mechanisms by which osteocyte apoptotic bodies control targeted bone resorption remains largely unknown, however it is thought that apoptotic bodies secrete cytokines affecting the number and the differentiation state of osteoclasts [31, 328]. Proposed mechanisms include a cease in the expression of the anti-resorptive inhibitor TGF-β by osteocytes [329] and/or osteocyte-mediated expression of TNF-α by osteoclast precursor cells [31], a cytokine shown to regulate osteoclast formation [330]. Lastly, in immature metaphyseal bone, there is a higher density of osteocytes than in the cortical bone,

which supports the need for greater amounts of bone remodelling occurring in the metaphyseal bone [2, 331], particularly during early postnatal development [332].

1.4.6 Roles of Wnt signalling in bone growth, remodelling, skeletal disorders and fracture repair (published review article; Appendix 2.1)

1.5 Growth plate injuries and repair outcomes

There are many factors that are capable of disrupting the growth plate and consequently the process of endochondral ossification. These include mutations for genes important in normal bone development (such as matrix proteins, hormones, growth factors and transcription factors). There is also the impact of environmental factors such as nutrition, infection and medical treatments (i.e. radiotherapy and chemotherapy). However, the most common insult to the growth plate is trauma injury or fracture [186, 333], constituting approximately 20–25% of all skeletal injuries in children [334]. Injury to the growth plate is common because the growth plate is cartilage and therefore is the most fragile structure located in the developing ends of long bones [68]. Fractures can result from either an acute injury, such as incurred during sporting activities or from overuse i.e. in endurance athletes.

The prognosis for growth plate fracture varies depending on the types and levels of fracture. Many fractures involving the growth plate heal without any impairment of longitudinal growth but some lead to clinical manifestations of shortening and/or angulations of the bone. The Salter-Harris classification system is the standard in diagnosing the fracture along with predicated outcomes. This system divides growth plate fractures into five categories (types I

to V) **(Figure 1.3)**. Outcomes generally worsen as the category number increases; for example, the prognosis for a type I and II fracture is excellent. However, when the fracture involves the entire width of the growth plate, as in Salter's Type III and IV injuries, bony repair or bone bridge formation often occurs, replacing the fractured area with mature trabecular bone, an area previously made up of only cartilage [335, 336]. Evidently, this bony bridge disturbs the process of endochondral ossification and often results in skeletal angular deformities and growth arrest.

1.5.1 Current treatments

Current surgical corrective treatments for growth plate injury-induced bone growth arrest and deformities are highly invasive and painful and are often not effective as they only address symptoms and do not induce cartilage healing. Two commonly used surgical treatments for limb lengthening includes the Ilizarov method [337] and the Langenskiold procedure [338]. More recently internal distraction (FitBone) has also been introduced. The Langenskiold procedure involves the removal of the bone bridge defect and interposition with autologous fat [338, 339]. However, the success rate for this procedure has been reviewed as poor with many patients having reformation of the bone bridge due to fat displacement or necrosis [340]. The Ilizarov is an extendable external fixation method which requires the bone to be broken, pins to be inserted through the bone and attached to external scaffolding [337, 341]. Screws for extending the frame are turned daily to pull the bone apart at the break site and as a result new bone is then encouraged to grow to bridge this gap. However, the procedure is lengthy (usually taking as long as 6-months), extremely painful, leaves bad scarring and has a very high incidence of infection [342, 343]. In

Figure 1.3 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).

addition, this procedure may be required to be repeated if the child still has many year's of growth potential left.

The fully internal distraction method using FitBone is an advantageous alternative to the Ilizarov method, with minimal risk of infection and minimal pain [344, 345]. To achieve lengthening, the bone is separated into two segments and stabilized through the insertion of the FitBone distraction device. The FitBone device is activated through a computerized control unit and moves the bone segments apart at a rate of approximately one millimetre per day and this is continued until the desired bone length is acheived [344].

Although internal distraction is highly successful, the avoidance of surgical intervention would be the best treatment. This could be achieved through a biological treatment that stimulates cartilage regeneration at the fracture site, avoiding bone bridge formation and thus skeletal abnormalities. Biological treatments are currently unavailable due to a lack of knowledge on the mechanisms and pathways involved in bone bridge formation.

1.5.2 Repair mechanisms of injured growth plate

To study the mechanisms for bony repair of the growth plate and investigate treatments, experimental growth plate injury (with some similarity to Salter's Type IV growth plate fractures) has been performed in several animal models including mice [346], rats [347-349], rabbits [350] and sheep [351-353]. Previously, a series of sheep studies primarily examined ways to prevent bone bridge formation and to induce cartilage regeneration through implantation of cultured chondrocytes [351] and periosteal tissues [353] or by filling the

injury site with recombinant human osteogenic protein-1 or BMP-7 [352]. Recently, ovine bone marrow derived MSCs were transplanted in the growth plate injury site of lambs using gelatine sponges [354]. At 5-weeks post-injury, the application of MSCs increased fibrous tissue formation and decreased osteogenic bone formation in the injury site, however it failed to restore growth plate cartilage [354]. Therefore, these therapeutic investigations and similar studies did not show proper growth plate cartilage regeneration, and bone bridge formation was not prevented at the injured growth plate. The lack of success in these studies can be attributed to the lack of knowledge on the mechanisms and pathways involved in bone bridge formation.

Studies by Xian et al (2004) and Zhou et al (2004) have examined cellular and molecular mechanisms involved in bone bridge formation in rats, where a drill-hole injury model in the proximal tibial growth plate was established in 6-week-old rats [347, 349]. While the drill-hole injury model is unable to simulate a traumatic growth plate injury in the clinical situation, it has more similarity to the Salter-Harris type IV fracture than other types of growth plate fractures **(Figure 1.4)**. Studies demonstrate a rapid time-course response to growth plate injury, consisting of four phases [355, 356]. These include an initial inflammatory response (from hours to up to day 3 after injury), a mesenchymal response (3 to 7 days), an osteogenic response (7 to 14 days), and a bone-remodelling phase (25 to 35 days following injury) **(Figure 1.5)**. These findings were consistent with previous injury studies in the growth plate, using rabbits [350, 357, 358], rats [348] and mice [346], which all showed that a bony bridge could be forming or completely formed 1 to 3 weeks after drill-hole surgery.

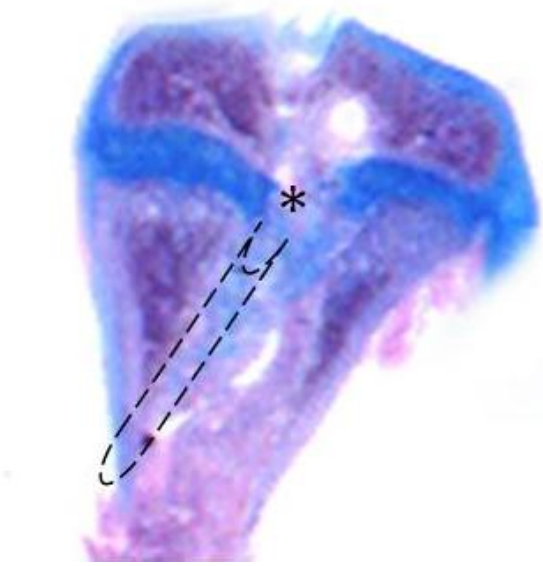

Figure 1.4 Drill-hole growth plate injury model.

Barbara stained section of a rat's injured proximal tibial growth plate. The growth plate was made accessible after introducing a cortical window in the metaphysis using a 2-mm wide dental bur. A centralized disruption of the growth plate was then made after the 2-mm wide dental bur was passed, via the cortical window, through the entire width of the growth plate, into the epiphyseal region. * Denotes the centralized growth plate defect. Dashed lines represent drill track. Image was adopted from Xian CJ et al (2004) *J Orthop Res*.

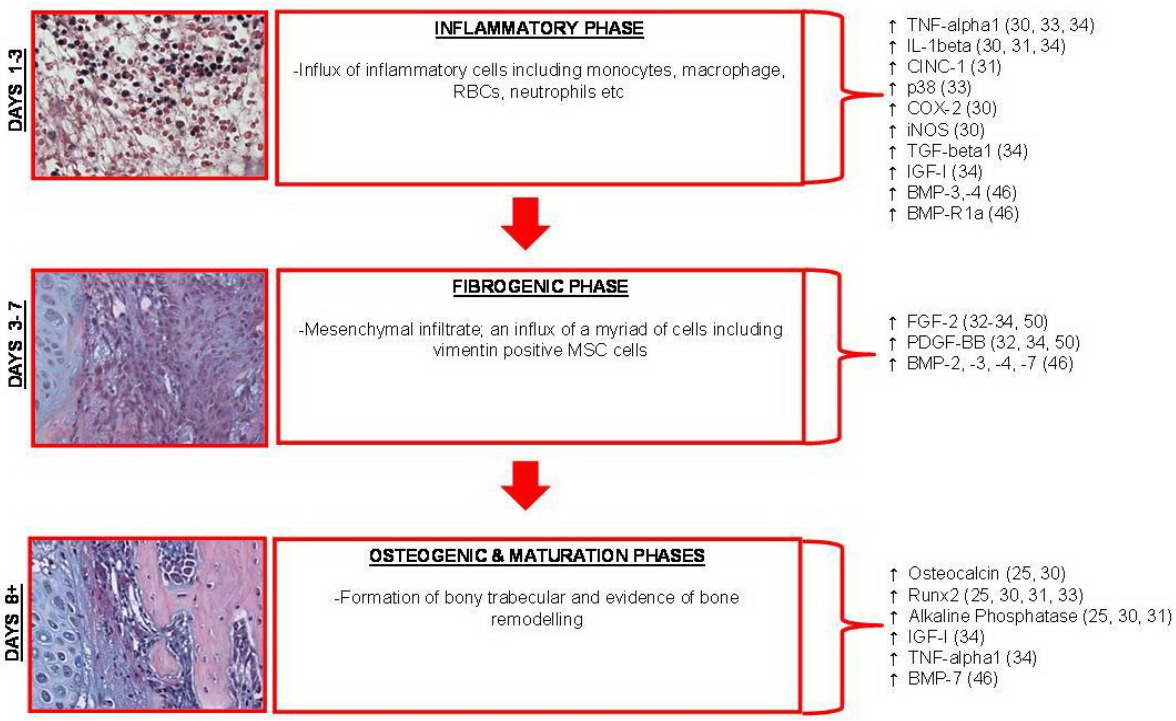

Figure 1.5 Growth plate injury repair responses and gene expression.

The rapid time-course response to growth plate injury consists of four phases, including inflammatory, fibrogenic, osteogenic and maturation phases on days 1-3, 3-7 and 8 onwards, respectively. Elevated expression of genes has been identified at each repair event, including cytokines, inflammatory mediators and growth factors. The figure was kindly provided by Dr. R Chung and adopted from Chung R et al (2011) *Front Biosci*.

Lee et al (2000) and Xian et al (2004) have demonstrated that bone bridge formation does not involve endochondral ossification, in the mouse and rat models, respectively, but is a result of direct bone formation by the process of intramembranous ossification [346, 347]. Xian et al (2004) has demonstrated an infiltration of vimentin-positive mesenchymal cells into the injury site, entering from the metaphysis and epiphysis. Many cells also expressed Runx2 or core-binding factor alpha 1 (cbfa1), which is a transcription factor for bone cell differentiation and a marker for osteoprogenitor cells and preosteoblasts [347]. Consequently, some cells from the mesenchymal infiltrate then differentiate into bone cells (osteoblasts), evident by the expression of alkaline phosphatase by preosteoblasts and osteocalcin by mature osteoblasts lining trabecular bone surface [347]. Lee et al (2000) and Zhou et al (2004) confirm a direct bone formation response during bone bridge formation through gene expression analysis [346, 349]. Lee et al (2000) showed that bone bridge formation at the injury site did not result in a change in expression of collagen II, Ihh, and VEGF, molecules characteristic of endochondral bone formation. Zhou et al (2004) also demonstrated a lack of up-regulation of cartilage specific genes, Sox-9 and collagen-2a, as examined using quantitative RT-PCR.

In subsequent studies using a similar model but with a smaller injury site, despite the fact that direct bone formation was the major mechanism of bony repair, Arasapam et al (2006) found an increased expression of cartilage related genes including collagen II, collagen X and Sox-9 as well as increased expression of bone-related genes (osteocalcin and cbfa1) [359]. This suggests an involvement of both endochondral and intramembranous ossification in the bony repair of the injured growth plate cartilage [355, 356, 359]. Similarly, Chung et

al (2006) found an increase in the gene expression of Sox-9 and collagen-2a following growth plate injury, and presence of positive staining for collagen II and collagen X in the injury site, supporting the presence or involvement of endochondral ossification mechanism in bone bridge formation [360].

1.5.3 Growth plate injury responses and gene expression

Understanding the genes involved in the repair events of the injured growth plate is important in elucidating the healing mechanisms. This information would provide assistance in studying their potential functions in mediating repair of the growth plate and to devise potential therapeutic means to enable cartilage regeneration in the fractured growth plate. Interestingly, the patterns of gene expression identified in the injured growth plate cartilage (discussed below) were found to have some similarities with those of previous bone fracture studies [355, 356].

Zhou et al (2004) examined the possible involvement of inflammatory cytokines and growth factors on the initial inflammatory response and subsequent bone repair phases. During the inflammatory response, IL-1β expression peaked at 8 to 16 hours post injury, and TNF-α and TGF-β1 levels peaked on day 1. Consistently, Chung et al (2006) found an increase in the levels of TNF-α (2-fold), IL-1β (2-fold) and TGF-β1 (3.5-fold) on day 1. Furthermore, IL-1β was up-regulated 30-fold and TNF-α 45-fold on day 1 in a similar study [359]. An up-regulation of FGF-2 and PDGF-B was found during late inflammatory phase [360] and during the mesenchymal response [349]; and during the osteogenic and bone remodelling

stages, levels of TNF-α, FGF-2 and TGF-β1 rose again during days 25 to 35, suggesting their potential involvement in bone bridge remodelling [349].

TNF-α is involved in the initial innate immune response to tissue injury, including growth plate injury [349, 361]. TNF-α is also required for regulating bone formation and remodelling in normal bone physiology and during bone fracture repair [362, 363]. The role of TNF-α in the activation of a mitogen activated protein kinase (MAPK), p38, and subsequent bone repair of the injured growth plate was examined in rats treated with a TNF-α antagonist [361]. Interestingly, p38 activation was blocked in rats treated with a TNF-α antagonist, suggesting a potential role of TNF-α in p38 activation. TNF-α inhibition also reduced mesenchymal infiltrate, cell proliferation and FGF-2 expression on day 8, suggesting that TNF-α signalling is important in mesenchymal infiltration and proliferation at the injury site. In the absence of TNF-α signalling there was also an increase in the expression of cbfa1, osteocalcin and increased bone formation at the injury site [361]. TNF-alpha has been shown to inhibit osteoblast differentiation *in vitro* and has been shown to activate p38, a MAPK shown to be important in regulating cell migration and stimulating bone cell differentiation *in vitro* [361]. This study suggests that TNF-α signalling has an inhibitory effect on bone cell differentiation and bone formation at the injured growth plate [361].

Recently, a study demonstrated the inhibition of platelet-derived growth factor receptor (PDGF-R) signalling during the fibrogenic phase of bone bride formation and found PDGF signalling has a role in MSC infiltration, cartilage and bone formation and bone remodelling

[364]. This was evidenced by decreased MSC infiltrate (day 4), bony trabeculae, cartilaginous repair tissues, osteoclasts and bone marrow (day 14) and supported by decreased mRNA expression of cartilage and bone-related genes [364].

Ngo et al (2006) examined the expression of BMPs and receptors (BMP-R) at the injured growth plate. Using Real Time RT-PCR, this study revealed slight increases and decreases in mRNA expression levels of various BMPs (BMP-2, 3, 4, 6 & 7) over the time-course of injury, as compared to uninjured rats. Similarly, slight changes in expression were observed for BMP-R1a and BMP-R2, but overall, for all genes, expression changes were not significant. However, immunostaining for BMPs and their receptors was positive amongst inflammatory cells, infiltrating mesenchymal cells and differentiated osteoblasts lining bony trabeculae and marrow cells [365], suggesting potential involvement of BMPs and receptors during the various healing responses after growth plate injury.

Arasapam et al (2006) examined the effects of inhibiting two inflammatory mediators, cyclo-oxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) on the injury-induced inflammatory response and subsequent phases in bone bridge formation. Their activities were blocked with celecoxib and aminoguanidine, respectively, 2 days prior to and 7 days after drill-hole injury. Real Time RT-PCR revealed an up-regulation of COX-2 on days 1 and 4 and iNOS on day 1, in untreated rats. COX-2 or iNOS blocking using specific inhibitors significantly reduced the inflammatory infiltrate on day 1, and increased mesenchymal tissue proportions but decreased cartilaginous tissue proportions on day 8, suggesting that COX-2 and iNOS may play a role in the differentiation of mesenchymal cells

into cartilage cells [359]. Bone remodelling also appeared delayed with smaller bone marrow proportions on day 14, suggesting that COX-2 and iNOS could play a role in promoting bone remodelling at the injury site [359].

Furthermore, to examine the potential role of neutrophil-mediated inflammatory response in the repair of the injured growth plate, a neutrophil-neutralizing antiserum was administered to rats following growth plate drill-hole injury [360]. Results suggested that after injury, neutrophils have a role in enhancing the chondrogenic differentiation of mesenchymal cells within the injury site.

The selection for analysis of the aforementioned genes and pathways involved in bone bridge formation was through assumption that similar responses would occur in bony repair after growth plate injury as would in bone fracture healing. The problem arises from a lack of detailed understanding of the cellular and molecular mechanisms involved in bone bridge formation in the injured growth plate. Systematic or more powerful analytic methods (such as microarrays) are required to focus on the global identification of genes potentially involved in various healing phases and bone bridge formation. Furthermore, the above gene expression studies are also limited since samples from these studies were collected from the whole injured growth plate, which means that in these studies RNA was being isolated not only from the injury site but also from the uninjured area of the growth plate. Therefore, this method of tissue collection for gene expression analysis is not specific enough for a true representation of potentially differentiated expressed genes in the injury site and thus may down-play their roles as the injured area was only a small proportion of the tissue being

analysed. Laser capture microdissection allows for the contamination-free isolation of the injury site only, and combined with microarray analysis, would be a powerful way to identify potentially differentiated expressed genes and to understand mechanisms and pathways involved in the bony repair of the injured growth plate.

1.5.4 Growth plate injury repair responses compared to bone fracture healing

Bone fracture healing consists of cellular events that closely resemble those observed in the repair of the injured growth plate [355, 356]. The processes and their approximate timing during fracture healing include inflammation (days 0-4), intramembranous ossification (days 4-16), chondrogenesis (days 6-18), endochondral ossification (days 8 to 21) and bone remodelling (days 14-21) [366-369]. Also, similar to the repair of the injured growth plate, the cellular mechanisms and molecular pathways that regulate bone fracture repair are still yet to be fully elucidated due to the complexity of the healing responses. The molecular mechanisms known to regulate skeletal tissue formation during embryonic development have shown to be recapitulated during the fracture healing process [370-373]. These molecular mechanisms involve several extracellular matrix components and growth factor gene families. Fracture repair slows with advancing age; nonetheless, as examined by DNA microarrays, nearly all genes presently associated with bone metabolism showed the same response to fracture healing regardless of the age of the rats [374].

1.5.4.1 Bone fracture healing, gene array analyses and major signalling pathways

In recent years, the understanding of fracture healing at the molecular level has greatly improved with the introduction of microarray technologies. Several studies have used gene

expression arrays [366, 369, 375], or protein arrays [368], to identify genes, functional proteins and subsequent pathways participating in bone regeneration. Hadjiargyrou et al (2002) was one of the first to use microarray techniques to examine the transcriptional activity occurring during bone regeneration [369]. Using a rat fracture model over 21 days, data demonstrated that thousands of genes were activated during bone repair, the majority being known, but still a large number identified as functionally unknown/novel genes. Many known genes could be grouped to identify signalling pathways crucial for the repair, with the Wnt/beta-catenin, the hedgehog, and the BMP signalling pathways being the significant players [369].

Li et al (2005) found the IGF family to be very important in the early stages of bone repair and found many genes to be also correlated well with those identified by Hadjiargyrou et al (2002). In the analysis of the translational regulation of bone repair, eighteen pathways were highlighted as participating during bone regeneration [368]. Of these, four pathways were up-regulated during three or more phases of bone repair, including the ERK/MAPK, NF-κB, PDGF, and T-cell receptor signalling pathways [368].

Despite these data, array studies currently have contributed only a small proportion to the gained knowledge of the genes and subsequent signalling pathways involved in bone fracture repair, as follow-up studies on the thousands of genes only have begun. From reviewing the other literature, key players have been found to include pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α [362, 363]. Growth and differentiation factors, including a BMP-associated network, made up of BMPs, BMP receptors, and BMP-related

factors [376]. Other important members of this group include TGF-β [377, 378], PDGF [379, 380], FGF [381, 382] and IGF [372, 383]. Angiogenic factors are also critical and include VEGF [384, 385]**,** metalloproteinases, angiopoietin and pleiotrophin [386]. More recently, the Wnt signalling pathway has also been implicated in being critical in bone fracture repair [387].

1.5.4.2 Bone fracture healing and critical roles of BMP signalling

Bone morphogenic proteins (BMPs), members of the TGF-ß superfamily, have become a key focus in the investigation and manipulation of bone fracture repair. During bone repair, various BMPs and BMP receptors (BMPRs) are localized and expressed at callus forming sites. The mRNA expression of BMP-4 is up-regulated during the early stages of fracture repair [388-390]. BMP-2 was shown to be expressed at the very early stages of fracture repair [388], however another study found that BMP-2 was unaffected by fracture [390]. Immunohistochemistry revealed a dramatic increase in staining for BMP-4 and –2 in primitive mesenchymal and chondrocytic cells during the process of endochondral ossification and in osteoblasts laying down woven bone [388]. This was similarly observed for areas of the callus undergoing intramembranous ossification [388]. Onishi et al (1998) showed that BMP-4, –2 and -7 was strongly stained in the thickened periosteum in the early stages of fracture repair. Staining for these BMPs was also exhibited during endochondral ossification as well in newly formed trabecular bone [391]. Therefore, differences in the involvement of BMPs during fracture repair are apparent when comparing mRNA expression data to immunohistochemical findings. Differences are also found in the analysis of BMPRs in fracture repair. Like BMPs, there is obvious involvement, but results from

immunostaining suggest a more significant role [390, 392]. The variation in BMP and BMPR expression during fracture healing may be due to differing analytical techniques and/or differences in the generation of fractures. Kloen et al (2003) examined the expression of BMP signalling components in human fracture tissue. All BMPs examined (BMP-2, -3, -4, -7) were immunopositive in osteoblasts within the callus samples, with colocalization with BMPR-1A, -B, and –11. Positive staining for BMP receptor-regulated Smads in the osteoblasts suggests that BMP signalling is activated in the human fracture callus [138].

BMP-related factors hepatocyte growth factor (HGF), sonic hedgehog (Shh), and noggin are shown to regulate the actions of BMPs and BMPRs during fracture healing [376]. HGF was activated and expressed at the fracture site and was shown to increase the expression of BMPRs in mesenchymal cells at the injury site [393]. Shh mRNA was synthesised by fracture callus cells and co-localized with BMP-4 in the early phase of fracture repair (day 2) but not at later stages (day 12), in closed fractures of 5-week-old mice [394]. Several papers show that exogenous application of BMP antagonist noggin down-regulates BMP activity and subsequent bone formation [395, 396]. In addition, when noggin expression was suppressed in mice with critical-sized calvarial defects, bone regeneration was enhanced at 2 and 4 weeks post-injury [397]. Therefore, these studies do suggest a critical involvement of BMPs during fracture repair, but demonstrate that BMPs do not act alone and are a part of a BMP associated network [376].

Several studies have shown that that the administration of recombinant BMPs can accelerate the healing of critical-sized bone defects in a variety of animal species, including humans

[398-400]. Inactivation of BMP-2 in a limb-specific manner, before the onset of embryonic skeletal development, had an extremely negative effect on postnatal bone function and fracture healing [401]. By 13 weeks of age, all mice experienced spontaneous bone fractures, which all failed to demonstrate normal fracture healing responses, and more interestingly, long after these fractures occurred (20 weeks), the bones failed to heal [401]. This study showed that the early reparative process involving new chondrogenesis does not begin and that mesenchymal progenitors at the fracture site remain undifferentiated in the absence of BMP-2. It was shown that the levels of other BMPs at the fracture site were comparable with or without BMP-2 present, and could not compensate for the lack of BMP-2. Therefore, this study is the first to show that BMP-2 is the crucial BMP for the initiation of bone healing [401].

Considering all of the above, the use of BMPs to treat bone fractures has undergone considerable research in clinical trials. The clinical effectiveness of BMP-2 and 7 have been more favourably investigated and have shown success, particularly in the treatment of tibial fractures [398, 402, 403].

1.5.4.3 Bone fracture healing and angiogenesis

The development of a vascular supply is essential for tissue repair, including bone fracture healing [404]. However, the mechanisms regulating angiogenesis during fracture repair are not well understood [405-407]. The administration of anti-angiogenic agents are shown to prevent fractures from healing properly, whereas treatments with pro-angiogenic agents are shown to promote bone formation. For example, administration of the angiogenic inhibitor

TNP-470 completely prevented femoral fracture healing in rats, with the lack of formation of both callus and periosteal woven bone [406]. In addition, mice femoral fractures treated with a neutralising VEGF receptor (Flt-IgG) exhibited decreased angiogenesis, bone formation, callus mineralization and had drastically reduced osteoblast activity [384]. Conversely, VEGF was shown to stimulate bone repair, in part, through its direct effects on osteoblast differentiation [384]. Furthermore, rabbits with critical sized radial defects, filled with VEGF165-GAM, showed partial or total bone regeneration, in comparison to control rabbits with no defect healing, typical of atrophic non-unions [408]. Eckardt et al (2005) demonstrated that rabbits with nonunion fracture can be treated with VEGF but failed to reunite after receiving carrier control treatment [385]. Thus, VEGF is important in the regulation of new blood vessel formation and bone turnover during bone regeneration [384].

Other molecules shown to play an essential role in angiogenesis during bone fracture repair include placental growth factor (PIGF), CNN1, hypoxia inducible factor-1 (HIF-1), matrix metalloproteinases (Mmps), angiopoietin (Ang 2), pigmented derived factor (PEDF), pleiotrophin, VEGF inhibitor (VEGFI) and Tie 1. PIGF, a VEGF homolog, was identified as being essential in mediating all aspects of bone fracture repair [409]. Mice lacking PIGF had a massive accumulation of cartilage in the callus and a lack of bony bridging, suggesting that VEGF does have a significant role in controlling the proliferation of osteoprogenitors and their differentiation into bone forming osteoblasts [409]. A paracrine loop, where VEGF mediates the up-regulation of CNN1 in osteoblasts, was identified after the inhibition of CNN1 prevented bone fracture healing in mice [405]. The expression of CNN1 was identified in newly formed osteoid, attracting endothelial cells and promoting angiogenesis

during the reparative phase of fracture healing [405]. HIF-1α, a transcription factor that regulates cellular responses to hypoxia, as occurs during bone fracture, was up-regulated during fracture repair and appears to have a critical role in angiogenesis at 10 days post fracture [410].

Mmp 2, 9, 13, and 14 were expressed at high levels during fracture healing, with maximal levels during the chondrogenic phase of fracture repair [386]. After culture comparison of fracture callus cells from wild type and TNF-α deficient mice, it was revealed that TNF-a treatment specifically induced the expression of Mmp9, Mmp14, VEGFI and Ang 2 [386]. Interestingly, TNF-α treatment greatly increased the anti-angiogenic factor VEGFI, and *in vivo*, in mice deficient for TNF-α, there was a strong inhibition of VEGFI. This shows that VEGFI is directly under the control of TNF-α and acts as a major negative regulator during fracture repair, particularly at day 10 where microarray analysis showed a 60-fold induction [386].

The above studies indicate that there has been a tremendous improvement in the understanding of the cellular and molecular mechanisms controlling bone fracture healing. Although the previous studies have suggested that the bony repair at the growth plate injury site may have similar repair mechanisms and use some similar cellular and molecular machinery as in bone fracture healing, mechanism for growth plate bony repair is hardly adequate.

1.6 Project rationale, aims and hypotheses

An undesirable outcome to growth plate fracture is the bony repair of the injured cartilage at the fractured area. Consequently, children often incur skeletal angular deformities and growth arrest. Current corrective surgical treatments for these outcomes are highly invasive, and therapeutic interventions are not possible as little is known about the mechanisms and pathways that lead to bone bridge formation. Using a rat model, some previous studies have shown sequential inflammatory, fibrogenic, osteogenic and bone maturation responses involved in the bony repair of the injured growth plate. However, structural changes in the growth plate, at both the injury site and at the non-injured area, have not been closely examined previously, and little is known about the molecular mechanisms underlying the bony repair. Therefore, this PhD study, using a rat tibial growth plate injury model, aimed to examine effects of growth plate injury on the structure and composition of the injured growth plate in a longitudinal study using micro-CT and histology. Microarray analysis of the injury site only collected using laser capture microdissection was used to identify potential cellular and molecular mechanisms involved in bone bridge formation. In addition, Real-Time RT-PCR on adjacent uninjured growth plate was used to examine potential cellular/molecular changes at the uninjured area and on whole growth plate scrapes to examine potential involvement of Wnt signalling in bone bridge formation. This information may one day lead to the development of a biological treatment to enable cartilage regeneration in the fracture site of the growth plate.

Using a rat growth plate injury model, the aims of this project were to:

(1) To examine effects of growth plate injury on the structure and composition of the injury site and uninjured area in a longitudinal study using micro-CT and to examine potential cellular/molecular changes at the uninjured area.

(2) To identify molecular pathways for the bony repair of the injured growth plate using microarray analysis of the injury site.

(3) To examine the potential involvement of the Wnt signalling pathway in bone bridge formation.

It is hypothesised that growth plate injury will cause changes in the structure, composition and gene expression at the injury site and uninjured area of the growth plate, and some key genes and signalling pathways known critical in bone fracture healing may be present during the bony repair of the injured growth plate.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Materials used in chapter 3 (published article)

Histochemical Reagents

Xylene Ethanol (Absolute)	Merck KGaA (Darmstadt, Germany)
DPX	distyrene-tricresyl-phosphate-xylene
Alcian Blue	Sigma-Aldrich (NSW, Australia)

Histochemical Solutions:

Kindly donated by the Department of Histopathology at the Children, Youth and Women's Health Service (South Australia, Australia). They were made using distilled water and were as follows:

Mayer's Haematoxylin	0.2% haematoxylin C.I. 75290, 5% ammonium aluminium sulphate, 0.02% sodium iodate, 5 mM citric acid, 5% chloral hydrate
Stock Eosin Y	1% eosin Y C.I. 45380, 0.5% potassium dichromate, 10% saturated aqueous picric acid, 10% absolute ethanol
Working eosin solution	50mls of stock eosin and 50 mls of water
Alcian Blue	1% alcian blue dissolved in 3% (v/v) glacial acetic acid in water
Lithium Carbonate	10 mls of saturated stock solution and 40 mls of distilled water

Additional:

Anaesthetic (per mL)	160 ul of ketamine, 100 µl of xylazine made up in sterile PBS

2.1.2 Aditional materials used in chapters 4 (published article) and chapters 5

Additional Materials:

Ethanol, (Absolute), Molecular Biology Grade	Merck KGaA (Darmstadt, Germany)
Ethanol (70% & 80%)	Ethanol (Absolute), Molecular Biology Grade, diluted in RNase Free water
10% (v/v) Neutral Buffered Formalin (NBF)	10% (v/v) Formalin, 0.22 M NaH_2PO_4, 0.45 M Na_2HPO_4 in d. H_20
Agarose, DNA grade	Progen Industries Ltd. (Darra, Qld, Australia)
Ethidium bromide	Ameresco (Ohio, USA)

2.2 Gene expression profiling (in addition to Chapter 4; published paper)

2.2.1 Drill-hole injury model

Thirty, 6-week-old male Sprague-Dawley rats (Laboratory Animal Services, University of Adelaide), weighing approximately 100 grams, underwent experimental drill-hole growth plate injury in the proximal tibia of both hind legs. Under the anesthesia isofluorane (Abbott Australasia Pty Ltd., NSW, Australia), the growth plate was made accessible after introducing a cortical window in the metaphysis using a 2-mm wide dental bur. A centralized disruption of the growth plate was then made after the 2-mm wide dental bur was passed, via the cortical window, through the entire width of the growth plate, into the epiphyseal region. The wound was irrigated with 0.9% saline (BDH Laboratory Supplies, Poole, England) before being closed with metal clips. This study was performed under the guidelines of the National Health and Medical Research Council of Australia and with approval from the Animal Ethics Committee of the Women's and Children's Hospital Adelaide.

2.2.2 Time-course specimen collection

Six rats were sacrificed by a carbon dioxide overdose 1, 4, 8 and 14 days post injury. These time-points were selected for gene expression analysis of the inflammatory response, 1 day post-injury; the mesenchymal response, 4 days post injury; bone formation, 8 days post injury and bone formation and maturation, 14 days post injury.

2.2.3 Sample preparation

The proximal tibia from both hind legs was harvested for laser capture microdissection (LCM). To prepare samples for LCM, all surrounding soft tissue around the top half of the

tibia was removed using scissors. Using a scalpel blade, the hard cortical bone surrounding the growth plate and metaphysis was gently peeled off. The top of the epiphysis was also gently sawed off to ensure that all hard bone was removed. The remaining tibia was placed into an OCT mould (Tissue-Tek® Cryomold® standard, ProSciTec, Qld, Australia), covered with OCT (Tissue-Tek®, ProSciTec, Qld, Australia) and wrapped in aluminum foil. The mould was immersed in a dish containing isopentane (Merck KGaA, Darmstadt, Germany) and the dish was lowered into liquid nitrogen to snap freeze the bone. Moulds were stored at –80°C until needed for sectioning.

2.2.4 Cryostat sectioning

Samples were transported to Adelaide Microscopy on dry ice and only removed when ready to cut. Samples were cut on a cryostat-microtome at –23°C at 6 µm using RNase-free techniques. Tissue sections were carefully pressed onto the membranes of metal LEICA slides. Slides were kept on aluminum foil, on dry ice, until they were ready to be used for LCM. Three sections were collected per slide, and 5 slides were used to collect tissue for each animal.

Metal slides were used over glass or plastic slides because they were reusable and when collecting samples, they can be attached to a slide holder which kept the slide warm enough to enable 3 sections to be collected per slide. Slides were reused by first soaking the slides in 70% ethanol, allowing the old glue (aquadere) holding the previous membrane, to be easily scraped off using a scalpel blade. Slides were rinsed in ethanol and dried in a dust free environment. Once dry, the edges of the slides were traced with glue and pushed down onto

a tightly laid sheet of membrane and left to dry overnight. Slides were individually collected from the sheet using a scalpel blade and stored in a dust free environment.

2.2.5 Laser capture microdissection

RNase-free techniques were employed when using the Leica AS LMD system at Adelaide Microscopy. First, a slide was placed upside down onto a slide holder and a sterile 0.5 ml tube was placed into the tube holder, with the tube's cap positioned to collect samples. Subsequently, the tube's cap was filled with 65 µl of Buffer RLT, the first step in the protocol of total RNA isolation from microdissected cryosection, from the Rneasy® Micro Handbook (Qiagen, Clifton Hill, Victoria). Both the slide and tube holder were slid into position on the microscope.

The Leica AS LMD system was connected to a database so that before and after shots could be taken during cutting. Settings were adjusted for the laser to optimally cut bone sections. On a blank area of the slide, the laser was then calibrated on 10x magnification. Using the tracing selection, line, and cut, the area within the injury site was traced using a mouse. After initiating the cut, the laser cut around the pre-determined path, excising the tissue from the rest of the sample, which would fall by gravity into a designated tube. If the sample did not fall after cutting once, the move and cut option was used to break any remaining joins.

After collection was completed for one animal, the sample volume in the tube was adjusted to 75 µl, after the addition of 5 µl of Buffer RLT and 5 µl of carrier RNA. The sample was then vortexed for 30 seconds. At this point in the Qiagen protocol, the tube can be stored at –80°C for up to several months. Therefore, for convenience, the tube was kept on dry ice

whilst samples were collected for additional animals. The tubes were then stored at –80°C until ready to proceed with the remaining steps in the extraction protocol.

2.2.6 RNA extraction, yield and purity

Tubes were removed from storage at -80°C and thawed at room temperature. A volume of 75 µl of 70% ethanol was added to the homogenised lysate and mixed well by pipetting. The hymogenised lysate with ethanol was applied to an Rneasy MiniElute Spin Column centrifuged for 15 seconds at 10, 00 x g, for absorption of RNA to the membrane. A volume of 350 µl of Buffer RW1 was added to the column and centrifuged for 15 seconds at 10, 00 x g to wash the column. The on-column DNase treatment option was used to remove any possible traces of DNA contaminates by pipetting a DNase 1 incubation mix (Qiagen Clifton Hill, Victoria) directly onto the silica-gel membrane inside the column and left to incubate at room temperature for 15 minutes.

The column was washed with Buffer RW1 and then with 500 µl of Buffer RPE, and were centrifuged for 15 seconds at 10, 00 x g after each wash. A volume of 80% ethanol was added to the column and centrifuged for 2 minutes at 10, 00 x g for 2 minutes to dry the silica-gel membrane. The cap of the spin column was opened and the column was centrifuged at full speed for 5 minutes to ensure that the silica-gel membrane was completely dried. Total RNA was eluted from the column with 11 µl of RNase-free water. The tube was spun for one minute at maximum speed in a microcentrifuge to collect the RNA in a 1.5 ml collection tube. The dead volume of the RNeasy MinElute Spin Column is 2 µl and therefore elution gave an eluate of 9 µl.

To assess RNA yield and purity, 1 µl of each sample was run on a RNA 6000 Nano LabChip Kit on an Aligent 2100 Bioanalyser Nanochip at Adelaide Microarray Facility. The electrophoretic data revealed the ribosomal bands, 18s and 28s but a ratio and RIN number could not be calculated for all samples due to very low concentrations of RNA. The concentration, gel images and electrophoretic data for each sample can be viewed in **Appendix 1.1**. Due to a lack of sample volume, the RNA analysis could not be repeated on a Picochip, which would have provided ratios and RNA integrity numbers for all samples. However, a further check for purity was performed following RNA amplification.

2.2.7 RNA amplification

RNA amplification was performed using a commercially available kit called Ovation™ RNA Amplification Systmen V2 (NuGEN Technologies, Inc). This kit generates 4-7 µg of antisense, single-stranded cDNA product from 5 to 100 ng of total RNA. All reagents and reaction tubes were kept on ice unless otherwise instructed.

2.2.7.1 First strand cDNA synthesis

The total RNA to be amplified was adjusted with water so that 8 ng of total RNA was present in 5 µl in a 0.2 ml PCR tube. Two microliters of first strand primer mix was added to the 5 µl of total RNA sample. The PCR tubes were flick mixed 6-8 times and spun in a 4-degree microcentrifuge at 74, 000 x g. Tubes were than placed in a pre-warmed thermal cycler to run a pre-set program for primer annealing. Samples were incubated in the thermal cycler for five minutes at 65°C and then the tubes were snap-cooled on ice. The PCR tubes were flick mixed 6-8 times and spun in a 4-degree microcentrifuge at 74, 000 x g. A master

mix was prepared after combining first strand buffer mix with first strand enzyme mix in a 0.5 ml capped tube. Volumes were adjusted to suit the number of reactions being made up as depicted in the table below.

First Strand Buffer Mix (single reaction)	**First Strand Enzyme Mix** (single reaction)
12 µl	1 µl

The master mix was mixed by pipetting and spun down briefly at 4°C in a microcentrifuge at 10,000 x g. Reaction tubes were spun briefly in a microcentrifue to which then 13 µl of the first strand master mix was added to each tube. The PCR tubes were flick mixed 6-8 times and spun in a 4-degree microcentrifuge at 74, 000 x g. Tubes were placed in a pre-warmed thermal cycler and a program was run to perform first strand synthesis. Samples were incubated at 48°C for 60 minutes, followed by 70°C for 15 minutes and cooled to 4°C. Once the temperature had reached 4°C, the tubes were removed and flicked 6-8 times, and spun briefly in a microcentrifuge.

2.2.7.2 Second strand cDNA synthesis

A master mix was made after the addition of second strand buffer mix with second strand enzyme mix in a 0.5 ml capped tube. Volumes were adjusted to suit the number of reactions being made up as depicted below.

Second Strand Buffer Mix (single reaction)	**Second Strand Enzyme Mix** (single reaction)
18 µl	2 µl

The master mix was mixed by pipetting and was then spun down briefly. Twenty microliters of the second strand master mix was added to each reaction tube. Tubes were mixed by flicking and spun down in a microcentrifuge. Reaction tubes were then incubated in a thermal cycler to carry out second strand synthesis. Tubes were first incubated at 37°C for 30 minutes, followed by 75°C for 15 minutes and then cooled to 4°C. Upon completion of second strand synthesis, tubes were mixed by flicking and then spun down.

2.2.7.3 SPIA™ Amplification

A master mix was made by sequentially adding SPIA™ Buffer Mix, SPIA™ Primer Mix, water and SPIA™ Enzyme mix into a 1.5 ml capped tube. Volumes were adjusted to suit the number of reactions being made up as depicted in the table below.

SPIA Buffer Mix	**SPIA Primer Mix**	**Water**	**SPIA Enzyme Mix**
72 µl	4 µl	4 µl	40 µl

***For a single reaction**

The master mix was mixed by pipetting and spun down briefly. A volume of 120 µl of the SPIA™ master mix was added to each of the second strand reaction tubes and mixed well by pipetting, followed by brief centrifugation. The 160 µl reaction volume was split into two 80 µl volumes, in new chilled 0.2 ml PCR tubes, and then spun briefly.

2.2.7.4 cDNA purification, yield and purity

The amplified SPIA cDNA product was purified using Zymo Research Clean and Concentrator™ 25, recommended by NuGEN as one of four methods to use to purify the amplified cDNA. 160 µl of the amplified cDNA was added to 320 µl of DNA binding buffer in a 1.5 ml tube. After a brief vortex and spin, the entire volume was loaded onto a Zymo-Spin 11 Column, attached to a collection tube. The column and collection tube was centrifuged for 10 seconds at 10, 000 x g in a microcentrifuge. The flow through was discarded and the column was placed back in the same collection tube.

The sample was washed with 200 µl of room temperature 80% ethanol. The column and collection tube was then centrifuged for 10 seconds at 10, 000 x g in a microcentrifuge. The flow-through was discarded and the collection tube reused. The sample was washed again with 200 µl of 80% ethanol. The column and collection tube was centrifuged for 30 seconds at 10, 000 x g. The flow though and collection tube was discarded. To remove any residual wash buffer from the tip of the column, the column tip was blotted onto filter paper. The column was then placed into a 1.5 ml microcentrifuge tube.

A volume of 30 µl of water was added to the centre of each column and left to stand for 1 minute at room temperature. The column and collection tube was centrifuged for 30 seconds at 10, 000 x g, whereby approximately 30 µl of purified cDNA was collected. The samples were mixed by a brief vortex and centrifugation and 1.5 µl was put into a 0.5 ml microcentrifuge tube for measuring cDNA yield and purity. The remaining purified cDNA was stored at –20°C for later use in fragmentation and labelling.

The purity and concentration of the cDNA was measured using a NanoDrop ND 1000 spectrometer, using 1 absorbance unit at 260 nm of single-stranded DNA = 33 µg/ml as the constant. Before measuring the samples, the NanoDrop was blanked with 2 µl of water and measured to ensure a flat baseline was achieved. Blanking was repeated if necessary. Both pedals were cleaned with a lab wipe, before loading 1.5 µl of the first sample, and cleaned between subsequent samples. Samples were considered good quality when ratios of greater than 1.8 were achieved. These samples were sent to the Australian Genome Research Facility LTD in Victoria for labelling, fragmentation and hybridisation to Affymetrix gene chips.

2.2.8 Microarray analysis

2.2.8.1 cDNA labelling and hybing to Affymetrix Rat Genearrays

Rat cDNA quality was ascertained using the Agilent Bioanalyser 2100 using the NanoChip protocol. A total of 1.5 µg of cDNA was labelled with Biotin using the Kreatech ULS labelling kit (GEA-001). After coupling the biotin, the reaction was cleaned using the KREApure columns in the kit. The labelled cDNA samples were then fragmented using

DNase I as recommended by the Affymetrix process. The fragmented cDNA was quality checked using the Agilent Bioanalyser 2100 using the NanoChip protocol. Samples that passed this checkpoint were then prepared for hybridisation to the *Rat 230 version 2.0* GeneChip by preparing a probe cocktail (cRNA @ 0.05 µg/µl) that includes 1x Hybridisation Buffer (100mM MES, 1M NaCl, 20mM EDTA, 0.01% Tween-20), 0.1mg/ml Herring Sperm DNA, 0.5mg/ml BSA, and 7% DMSO.

A total hybridisation volume of 220 µl is prepared for each sample and 200 µl loaded into a *Rat 230 version 2.0* GeneChip. The chip is hybridised at 45°C for 16 hours in an oven with a rotating wheel at 60 rpm. After hybridisation the chip is washed using the appropriate fluidics script in the Affymetrix Fluidics Station 450. Upon completion of the washing, the chips are then scanned using the Affymetrix GeneChip Scanner 3000. The scanner operating software, GeneChip Operating Software (v.1.4, Affymetrix), converts the signal on the chip into a DAT file, which will then be used for generating subsequent CEL and CHP files for analysis.

2.2.8.2 Bioinformatics

To ascertain if the array had worked sufficiently to continue with further analysis, a quality check (QC) preliminary analysis was undertaken whereby the samples were required to conform to several parameters and quality checks (**Appendix 1.2**). QC preliminary analysis revealed that day 1 was not suitable for further analysis due to poor sample quality **(Appendix 1.2.1)**. All the chips were normalised using Robust Multichip Averaging and then normalised, per gene, to the median.

The significantly differentially expressed genes obtained from fold-change analysis were represented. Since there were no replicates, statistical analysis of the data was not possible. The differentially expressed genes were derived from the following comparisons. Genes were selected from condition Day 8 that had normalized data values that were greater or less than those in condition Day 4 by a factor of 2-fold. Genes selected from condition Day 14 that had normalized data values that were greater or less than those in condition Day 4 by a factor of 2-fold. Genes selected from condition Day 14 that had normalized data values that were greater or less than those in condition Day 8 by a factor of 2-fold. Genes were further grouped into functional categories using microarray analysis programs GoStat [411], Onto-Express [412] and David [413, 414], and Pathway-Express (PE) was used to identify potential pathways involved in bone bridge formation [415]. The top differentially expressed genes (either up- or down-regulated) were tabulated for different time-point comparisons. Listing the top 25 genes ensured that only genes that were increased or decreased by a considerable amount were included in the discussion.

2.3 Wnt signalling

2.3.1 Drill-hole injury

Thirty, 6-week-old male Sprague-Dawley rats, weighing approximately 100 grams, underwent drill-hole growth plate injury in the proximal tibia of both hind legs. Injury was introduced after a 2-mm wide drill was passed centrally into the growth plate through a cortical window in the metaphysis (detailed in **2.2.1**) [347]. This study was performed under the guidelines of the National Health and Medical Research Council of Australia and with

approval from the Animal Ethics Committee of the Women's and Children's Hospital Adelaide.

2.3.2 Time-course specimen collection

Six rats were sacrificed by a carbon dioxide overdose at 0, 1, 4, 8, 14 and 25 days after injury. These time-points have been selected to allow each stage in bone bridge formation to be examined. The days selected for analyses correspond to the following stages in bone bridge formation: day 1 (inflammatory response), day 4 (mesenchymal response), days 8 and 14 (bone formation) and day 25 (bone maturation). Day 0 is representative of an absence of tissue within the growth plate injury site. Growth plate cartilage was collected from the right proximal tibiae after gently breaking open the epiphysis to expose growth plate tissue on the surface of the epiphysis and/or metaphysis. Using a small surgical scalpel blade, growth plate samples were scraped from the exposed surfaces, inclusive of material formed within the injury site. Samples were snap-frozen and stored at -80°C for RNA extraction. Left proximal tibiae were collected and fixed in 10% formalin for 24 hours and decalcified for 4 days in Immunocal solution (Decal Corporation). Tibiae were bisected longitudinally and processed for paraffin embedding. Sections were cut for paraffin tissue blocks and collected on SuperFrost Plus coated slides for immunohistochemical analysis.

2.3.3 RNA extraction

To extract total RNA, TRI reagent (Sigma, NSW, Australia) was added to frozen growth plate samples and isolated using a needle and syringe. Extracted RNA was then treated with DNase I (Ambion, USA) to degrade contaminating DNA. The concentration and quality of

RNA was determined using a spectrophotometer. Due to the small amount of total RNA that can be purified from whole growth plate scrapes, RNA from the six rats at each time-point were pulled into groups of three. Each pool consisted of RNA from two rats to make up a total amount of 3 µg. cDNA was synthesized for the multiple pools using random decamers (Geneworks, SA, Australia) and superscript-II Rnase H RT (Stratagene, La Jolla, CA).

2.3.4 Real Time quantitative RT-PCR

A SYBER Green Real Time PCR assay was used to analyse the expression of genes involved in Wnt signalling using rat gene-specific primers, excluding LRP-5 that was designed from mouse mRNA **(Table 2.1)**. Cyclophilin-A (Cyc A) was used as the internal reference gene [349]. Primers (GeneWorks, Thebarton, Australia) were designed using Primer Express® Software v2.0 (Applied Biosystems). Gel electrophoresis in 3% agarose was used to visualize and identify the RT-PCR product of appropriate size using Puc19 (**Appendix 1.3**), a 100-base pair ladder (GeneWorks, Thebarton, Australia).

Real Time RT-PCR was performed using Applied Biosystems 7300 RT-PCR system (Warrington, UK), using the Absolute Quantification program. Amplification of DNA product was measured by fluorescence of SYBR® Green fluorescent dye (Invitrogen, Vic, Australia). Data is presented as relative expression levels at each time-point to enable comparisons between the different phases of growth plate injury and to make the qPCR data more comparable to results obtained from microarray analysis. Consequently, components of the Wnt signalling pathway that displayed a significant difference in mRNA expression

Table 2.1. Primer Sequences used for Real Time RT-PCR. Prepared at a 40 nmol scale and were desalted.

Gene	Forward (5' → 3')	Reverse (5' → 3')	Product (bp)	Accession # (NCBI)
Cyc A	CGTTGGATGGCAAGCATGTG	TGCTGGTCTTGCCATTCCTG	95	M19533
LRP-5	TCAGGAGCGCATGGTGATA	CGCTATATTGAGTCAGGCCAAA	62	6678715
LRP-6	CAGTCAGAAGAGCGCCATCAAC	ACGTTCCGAAGGCTGTGGATA	87	34858854
SFRP-1	CCCGAGATGCTCAAATGTGAC	AGATGTTCGATGATGGCCTCC	152	XM_001072532
SFRP-4	CATGTGTTATGAGCGGCGTTC	TGCGGCTGGCTATTTGCTT	155	NM_053544
Wnt-10b	AGAATGCGGATCCACAACAAC	TCCAACAGGTCTTGAATTGGC	112	NM_001108111
Wnt-5a	AGGACCACATGCAGTACATTGG	TGCATCACCCTGCCAAAGA	126	34871497
Ck2	AGATGACTACCAGCTTGTTC	AATGTCTGCAAGTGTGATGAT	172	P19139
Fzd1	AGTCTCTACTTCTTCAGCAT	AACACACTCCACTGAGTACGT	210	Nc_005103

between different phases of growth plate injury have been represented as a fold-change in **Table 5.1**.

Real Time RT-PCR results were expressed as mean ± SEM. Statistics were calculated by a one-way analysis of variance (ANOVA) with a Tukey's post hoc test, using GraphPad Instat (version 5.02.). Significance was assumed when $P<0.05^{*}$, $P<0.01^{**}$ and $P<0.0001^{***}$.

2.3.5 Microarray analysis of Wnt signalling components

Pathway analysis of normalised data obtained from microarray analysis of growth plate injury (section 2.2), revealed a strong participation of the Wnt signalling pathway at all time-points analysed. Participation of genes of the Wnt signalling pathway were identified using the microarray analysis programs, Go-Stat, Onto-Express and DAVID. Wnt genes that were significantly identified in two or more programs were deemed highly involved and were grouped and summarised in relation to their function in bone and/or cartilage.

CHAPTER 3

Structural and molecular analyses of bone bridge formation within the growth plate injury site and cartilage degeneration at the adjacent uninjured area

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BONE 49 (2011) 904-912

STATMENT OF AUTHORSHIP

STRUCTURAL AND MOLECULAR ANALYSES OF BONE BRIDGE FORMATION WITHIN THE GROWTH PLATE INJURY SITE AND CARTILAGE DEGENERATION AT THE ADJACENT UNINJURED AREA

BONE 49 (2011) 904-912

MACSAI, C.E. (Candidate)

Conducted animal trials, tissue collection, micro-CT, histology, interpreted data, wrote manuscript.

I hereby certify that the statement of contribution is accurate.

Signed.....*Date*.....

HOPWOOD, B.

Performed Real-Time PCR, helped in data interpretation.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed.....*Date*.....

CHUNG, R.

Performed Nick Translation staining, helped in data interpretation.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed.....*Date*.....

FOSTER, B.K.

Supervised development of work.

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Signed.....*Date*.....

XIAN, C.J.

Supervised development of work, helped in data interpretation, critical manuscript evaluation, and acted as corresponding author.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed.....*Date*.....

Macsai, C.E., Hopwood, B., Chung, R., Foster, B.K. & Xian, C.J. (2011) Structural and molecular analyses of bone bridge formation within the growth plate injury site and cartilage degeneration at the adjacent uninjured area.
Bone, v. 49 (4), pp. 904-912

CHAPTER 4

Microarray expression analysis of genes and pathways involved in growth plate cartilage injury responses and bony repair

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BONE 50 (2012) 1081-1091

STATMENT OF AUTHORSHIP

MICROARRAY EXPRESSION ANALYSIS OF GENES AND PATHWAYS INVOLVED IN GROWTH PLATE CARTILAGE INJURY RESPONSES AND BONY REPAIR

BONE 50 (2012) 1081-1091

MACSAI, C.E. (Candidate)

Conducted animal trials and tissue collection, performed experiments, interpreted data, and wrote manuscript.

I hereby certify that the statement of contribution is accurate.

Signed.....*Date*.....

GEORGIOU, K.R.

Conducted some confirmation RT-PCR experiments.

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FOSTER, B.K.

Supervised development of work.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

Signed.....*Date*.....

ZANNETTINO, A.C.W.

Supervised development of work and provided critical manuscript evaluation.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

Signed.....*Date*.....

XIAN, C.J.

Supervised development of work, assisted with animal trials and tissue collection, helped in data interpretation, critical manuscript evaluation, and acted as corresponding author.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

Signed.....*Date*.....

Macsai, C.E., Georgiou, K.R., Foster , B.K., Zannettino, A.C.W., Xian, C.J. (2012) Microarray expression analysis of genes and pathways involved in growth plate cartilage injury responses and bony repair.
Bone, v. 50 (5), pp. 1081-1091

CHAPTER 5

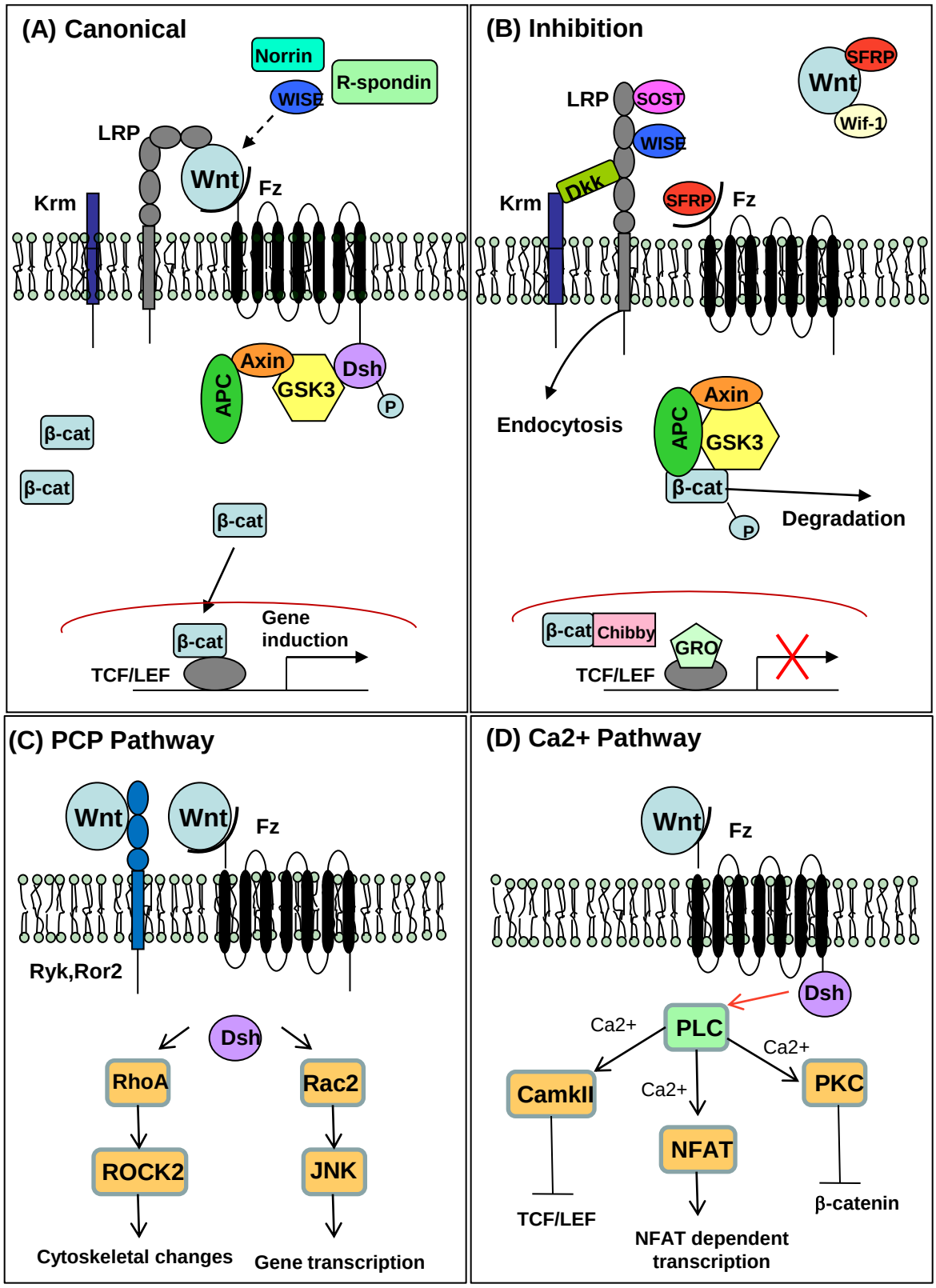
Expression of Wnt genes at the injured growth plate cartilage in young rats

5.1 Introduction

One pathway that could be potentially important in regulating the bony repair of injured growth plate cartilage is the Wnt signalling pathway. Wnt signalling not only has an important role in regulating developmental processes, including embryogenesis and organogenesis, but also has an essential role throughout life, particularly in regulating bone formation and bone mass. In addition, Wnt signalling plays an important role in bone fracture repair. Wnt signalling regulates bone formation and repair by controlling mesenchymal stem cell proliferation, osteoblast differentiation, proliferation and function, chondrocyte differentiation and proliferation, as well as osteoclast formation and function [133].

Wnt signalling is comprised of two pathways, the canonical pathway and the non-canonical pathway **(Figure 5.1)**. Activation of the canonical pathway is initiated when a Wnt molecule binds to Frizzled (Fzd) receptors and low density lipoprotein receptor-relatead protein-5/6 (LRP-5/6) co-receptors **(Figure 5.1A)**. Wnt binding activates the intracellular protein Dishevelled (Dsh) which mediates the inhibition of a cytoplasmic complex composed of glycogen synthase kinase-3 (GSK-3), Axin, β-catenin and adenomatous polyposis coli (APC). Dsh causes the collapse of this multi-protein complex by inhibiting the phosphorylation of β-catenin by GSK-3 [416]. As a result, β-catenin is not targeted for degradation, but is released and stabilised, allowing its nuclear transport and gene expression induction via binding to T-cell factor (TCF)/lymphoid enhancer-binding factor 1 (Lef1) transcription factors. In the absence of Wnt signals, the multi-protein complex mediates the proteasomal degradation of β-catenin, thus inhibiting transcription of Wnt target genes such

Figure 5.1 The canonical Wnt/β-catenin pathway and non-canonical Wnt pathways. (A) Activation of the canonical pathway is initiated when Wnt binds to Frizzled (Fz) receptors and low density lipoprotein LRPs-5/6 (LRP-5/6) co-receptors. This interaction is transmitted through Dishevelled (Dsh), and inhibits a cytoplasmic complex composed of GSK-3, Axin and APC, leading to a block in β-catenin phosphorylation by GSK-3. β-catenin accumulates in the cytoplasm and then enters the nucleus, where it stimulates TCF/LEF dependent transcription. Additional activators of the canonical pathway include Norrin, R-spondin and WISE. (B) The canonical pathway can be inactivated through a variety of inhibitors, mediating the proteasomal degradation of β-catenin via its phosphorylation, inhibiting gene transcription. Inhibitors include, Dkks, SOST, WISE, SFRPs, Wif-1, Chibby and Groucho (Gro). (C) Signalling via the planner cell polarity pathway (PCP) leads to the activation of small GTPases, RhoA and Rac2, which act through their target proteins, ROCK and JNK to regulate cytoskeletal changes. (D) The calcium-releasing pathway (Ca^{2+} pathway) negatively regulates the Wnt/β-catenin pathway, whereby the release of intracellular calcium activates protein kinase C (PKC), calmodulin dependent protein kinase 2 (CamK2) and nuclear factor of activated T cells (NFAT), which repress β-catenin signalling via different mechanisms. Figure adapted and modified from Macsai et al (2008), J. Cell. Physiol (**215**: 578-587).


(A) Canonical
Norrin
R-spondin
WISE
LRP
Wnt
Fz
Krm
Axin
APC
GSK3
Dsh
P
β-cat
β-cat
β-cat
Gene
induction
β-cat
TCF/LEF
(B) Inhibition
SFRP
Wnt
Wif-1
LRP
SOST
WISE
Dkk
Krm
SFRP
Fz
Endocytosis
Axin
APC
GSK3
β-cat
P
Degradation
β-cat
Chibby
GRO
TCF/LEF
(C) PCP Pathway
Wnt
Wnt
Fz
Ryk,Ror2
Dsh
RhoA
Rac2
ROCK2
JNK
Cytoskeletal changes
Gene transcription
(D) Ca2+ Pathway
Wnt
Fz
Dsh
Ca2+
PLC
Ca2+
Ca2+
CamkII
NFAT
PKC
TCF/LEF
NFAT dependent
transcription
β-catenin

as c-myc and cyclin D1 **(Figure 5.1B)** [417]. The non-canonical pathway is poorly understood but functions in a β-catenin-independent manner, where Wnt binds only to Fzd receptors. However, Wnt binding activates at least two non-canonical pathways, including the JNK/Planar Cell Polarity pathway (PCP) **(Figure 5.1C)** [418] and the calcium-releasing pathway (Wnt/Ca^{2+}) **(Figure 5.1D)** [419].

It has been demonstrated that Wnt signalling, as shown in a RNA expression study, is activated during bone fracture repair in a rat closed fracture model [369]. This study identified the up-regulation of several Wnt genes, as well as several target genes, including Wnt-5a, Fzd, casein kinase II, β-catenin and phosphatase 2A; and c-myc, fibronectin, retinoic acid receptor gamma and connexin 43, respectively. A follow-up of this study revealed the up-regulation of additional Wnt signalling pathway genes, including Wnt-4, Wnt-5b, Dishevelled members 1-3(Dvl1-3), casein kinase 1, alpha 1 (CK1A1), Tcf1, LRP-5 and Wnt target genes such as Engrailed-1, peroxisome proliferator-activated receptor delta (PPARD) and CD44 [420].

The transcription factor Lef1 was down-regulated during the early phases of the repair process and during maximal bone formation. Lef1 is a known repressor of cbfa1, therefore the down-regulation of Lef1 was deemed necessary for bone repair to occur [420]. In addition, β-catenin and Dishevelled (Dsh) proteins were localized in proliferating and differentiating chondrocytes and osteoblasts within the fracture callus. Interestingly, studies with knockdown of Dsh isoforms (1-3) (*in vitro*) indicated that Dsh was essential for regulating chondrocyte proliferation and differentiation via the canonical pathway [420].

Wnt-1- induced secreted protein 1 (Wisp1) may play a role during bone fracture repair, as demonstrated in a mouse fixed femur fracture model [223]. Wisp1 was expressed in mesenchymal cells surrounding the site of injury not long after fracture, suggesting a possible role in the recruitment of mesenchymal cells to the fracture site. Wisp1 was also expressed in osteoblasts lining the periosteum and the woven bone within the callus, suggesting a role for Wisp1 in bone matrix formation during bone regeneration [223].

A recent study demonstrated that β-catenin signalling plays a crucial role in fracture healing [421]. In the early phases of fracture healing, β-catenin tightly regulates the differentiation of mesenchymal cells into osteoblasts and chondrocytes lineages. Once the cells have adopted an osteoblast phenotype, β-catenin signalling promotes osteoblastic differentiation and enhances osteogenesis **(Figure 5.2)**. Several Wnt ligands and receptors were also up-regulated during fracture repair including Wnt-4, 5b, 5a, 10b, 11 and 13, and the receptors Fzd-1, 2, 4 and 5, and LRP-6. While Wnt-4 and Wnt-10b are known canonical Wnt ligands and therefore it is likely that they are activating β-catenin signalling during fracture repair [421], the activation of non-canonical Wnt ligands Wnt-11 and Wn-5a suggests that the non-canonical pathway also participates in the repair process. However, treatment with Dkk-1, an antagonist of the β-catenin pathway, suppressed fracture repair, suggesting that the canonical pathway is the dominant mechanism regulating β-catenin during bone healing and that Wnt/ β-catenin signalling pathway plays a crucial role in fracture healing [421]. Therefore, these studies suggest that the Wnt signalling pathway could play an important role in regulating bone fracture repair.

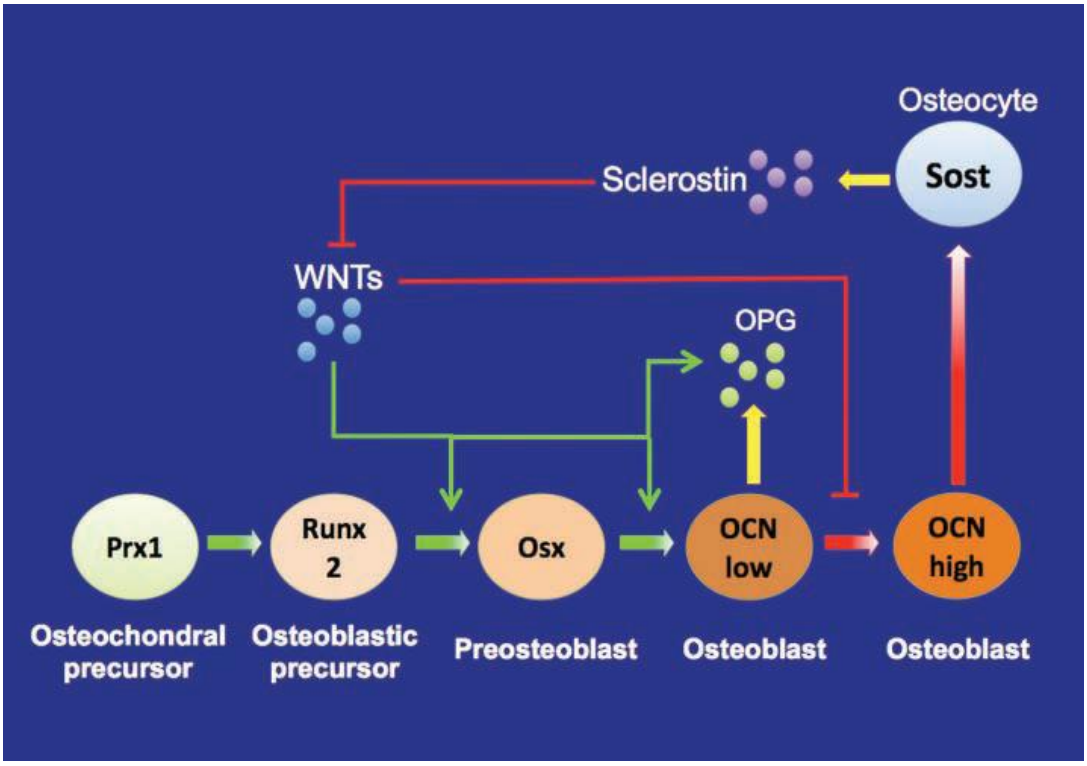


Figure 5.2 Wnt/β-catenin signalling and osteoblast differentiation. Wnt/β-catenin signalling is required during the early stages of commitment to the osteoblast lineage and early osteoblast differentiation. Wnt signalling is necessary for the differentiation of committed osteoblastic/mesenchymal precursors into pre-osteoblasts. Signalling via β-catenin has a negative effect on osteoclast differentiation and regulates bone remodelling by inducing OPG expression. Wnt signalling inhibits the differentiation of osteoblasts into mature bone forming osteoblasts which express high levels of osteocalcin. Osteocytes (terminally differentiated osteoblasts) express sclerostin, inhibiting Wnt signalling via a negative feedback-loop. Figure adapted from Galli C *et al* (2010), J Dent Res (**89**(4):331-343, 2010).

Known inhibitors of the Wnt signalling pathway include the Dickkopf (Dkk) family, the secreted frizzled related protein (SFRP) family, Wnt inhibitory factor-1 (WIF-1), Wnt-1-induced signalling protein-1 (Wisp1), Kremen1 and sclerostin [133]. These inhibitors bind to Wnt directly and/or the Fz receptors to inhibit the action of Wnt in both the canonical and non-canonical pathways.

Whether Wnt signalling is involved in regulating growth plate bony repair remains unknown. Using quantitative real time RT-PCR and results from microarray data analysis, this study examined the expression profiles of Wnt signalling components in a time-course at the injured proximal tibial growth plate in rats to highlight the potential involvement of Wnt signalling in bone bridge formation.

5 .2 Results

5.2.1 Time-course mRNA expression of Wnt signalling components

The mRNA expression of some Wnt signalling components was initially examined by quantitative real time RT-PCR at 0, 1, 4, 8, 14 and 25 days post-injury from dissected whole growth plate tissues. These days were selected to correspond to the following stages in bone bridge formation: day 0 (no tissue in injury site), day 1 (inflammatory response), day 4 (mesenchymal response), days 8 and 14 (bone formation) and day 25 (bone maturation). Growth plate cartilage was collected from the right proximal tibiae after gently breaking open the epiphysis to expose growth plate tissue on the surface of the epiphysis and/or metaphysis. Using a small surgical scalpel blade, growth plate samples were scraped from the exposed surfaces, inclusive of material formed within the injury site. This method has

been routinely used when investigating molecular mechanisms involved in bone bridge formation [349, 359-361, 365] and thus was firstly used to investigate the potential involvement of the Wnt signalling pathway in the repair of the injured growth plate. Data is presented as relative expression levels at each time-point to enable comparisons between the different phases of growth plate injury and to make the qPCR data more comparable to results obtained from microarray analysis. Consequently, components of the Wnt signalling pathway that displayed a significant difference in mRNA expression between different phases of growth plate injury have been represented as a fold-change in **Table 5.1**.

There was a significant increase in the mRNA expression of Wnt-10b at day 25 (bone maturation phase) when compared to days 8 and 14 (bone formation) ($P<0.05$) **(Figure 5.3A)**, by 3.11 and 2.57-fold, respectively **(Table 5.1)**. The mRNA expression of Wnt-5a was significantly increased at day 25 when compared to day 0 (3.5-fold), day 1 (6.16-fold), day 4 (6.54-fold), and days 8 (5.44-fold) and 14 (5.45-fold), ($P<0.0001$) **(Figure 5.3B)**. Similarly, there was a significant increase in LRP-5 **(Figure 5.4A)** and LRP-6 **(Figure 5.4B)** 25 days post-injury when compared to 0, 1, 4, 8 and 14 days post-injury ($P<0.0001$) **(Table 5.1)**. Interestingly, a steady increase in the mRNA expression of SFRP-1 was observed between days 1 to 25, but no significant differences were found ($P>0.05$) **(Figure 5.5A)**. There was no change observed in the mRNA expression of SFRP-4 across the time-course of bone bridge formation **(Figure 5.5B)**.

Table 5.1. Real Time RT-PCR analysis of Wnt signalling genes in a time-course of growth plate injury.

Group	Wnt Genes	Gene Symbol	Fold Change
D0 & D25	Low Density Lipoprotein Receptor-Related Protein-5	LRP-5	3.760
	Low Density Lipoprotein Receptor-Related Protein-6	LRP-6	3.161
	Wingless-type MMTV integration site family, member 5a	Wnt-5a	3.148
D1 & D25	Low Density Lipoprotein Receptor-Related Protein-5	LRP-5	8.759
	Low Density Lipoprotein Receptor-Related Protein-6	LRP-6	5.028
	Wingless-type MMTV integration site family, member 5a	Wnt-5a	6.155
D4 & D25	Low Density Lipoprotein Receptor-Related Protein-5	LRP-5	9.061
	Low Density Lipoprotein Receptor-Related Protein-6	LRP-6	4.861
	Wingless-type MMTV integration site family, member 5a	Wnt-5a	6.537
D8 & D25	Low Density Lipoprotein Receptor-Related Protein-5	LRP-5	6.690
	Low Density Lipoprotein Receptor-Related Protein-6	LRP-6	4.260
	Wingless-type MMTV integration site family, member 10b	Wnt-10b	3.111
	Wingless-type MMTV integration site family, member 5a	Wnt-5a	5.441
D14 & D25	Low Density Lipoprotein Receptor-Related Protein-5	LRP-5	10.265
	Low Density Lipoprotein Receptor-Related Protein-6	LRP-6	4.064
	Wingless-type MMTV integration site family, member 10b	Wnt-10b	2.565
	Wingless-type MMTV integration site family, member 5a	Wnt-5a	5.446

Several components of the Wnt signalling pathway were analysed across the time-course of bone bridge formation using Real Time RT-PCR. Genes that displayed a significant difference in mRNA expression between different phases of growth plate injury have been represented ($P<0.05$, one-way ANOVA, Tukeys post-hoc test). There was a significant increase in the mRNA expression of LRP-5, LRP-6 and Wnt-5a at day 25 when compared to 0, 1, 4, 8 and 14 days post-injury. There was also a significant increase in the mRNA expression of Wnt-10b at day 25 when compared to days 8 and 14.

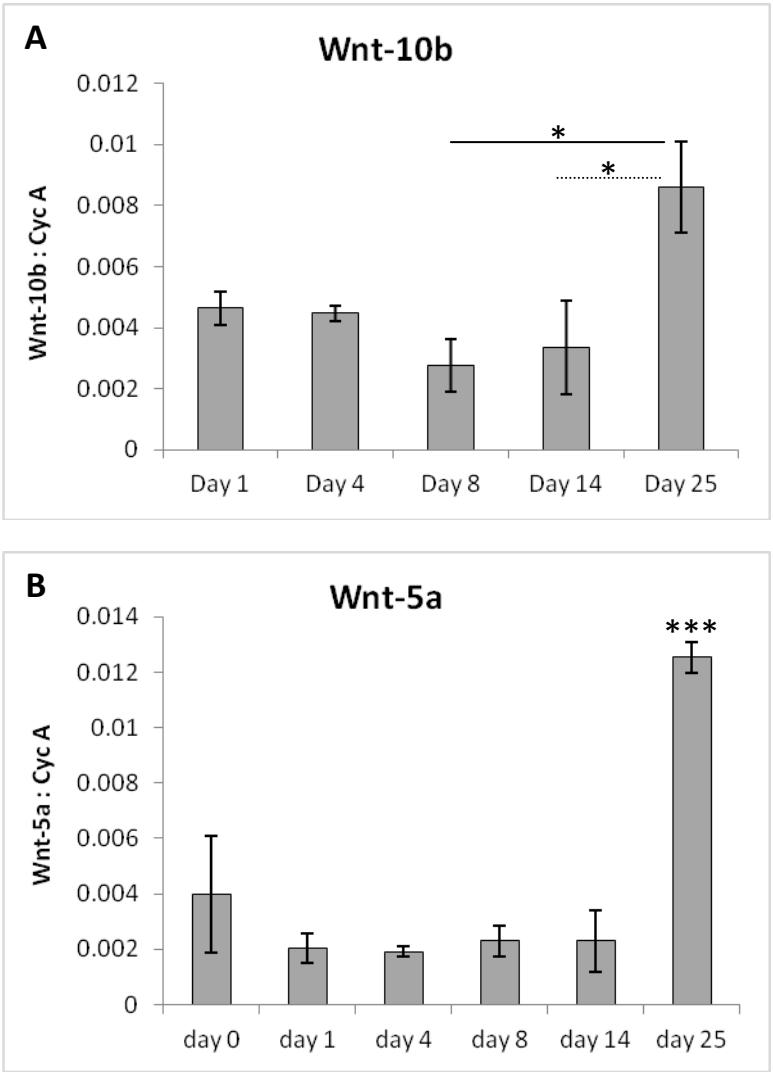


Figure 5.3 Time-course mRNA expression of Wnt-10b and Wnt-5a after growth plate injury. (A) There was a significant increase in the mRNA expression of Wnt-10b at day 25 when compared to days 8 and 14 ($P<0.05^{*}$). (B) The mRNA expression of Wnt-5a was significantly higher at day 25, compared to days 0, 1, 4, 8 and 14 ($P<0.0001^{***}$). Data is presented as average ± SEM of 3 pooled samples, with 2 animals per pool. Statistics were calculated by a one-way analysis of variance (ANOVA) with a Tukey's post hoc test.

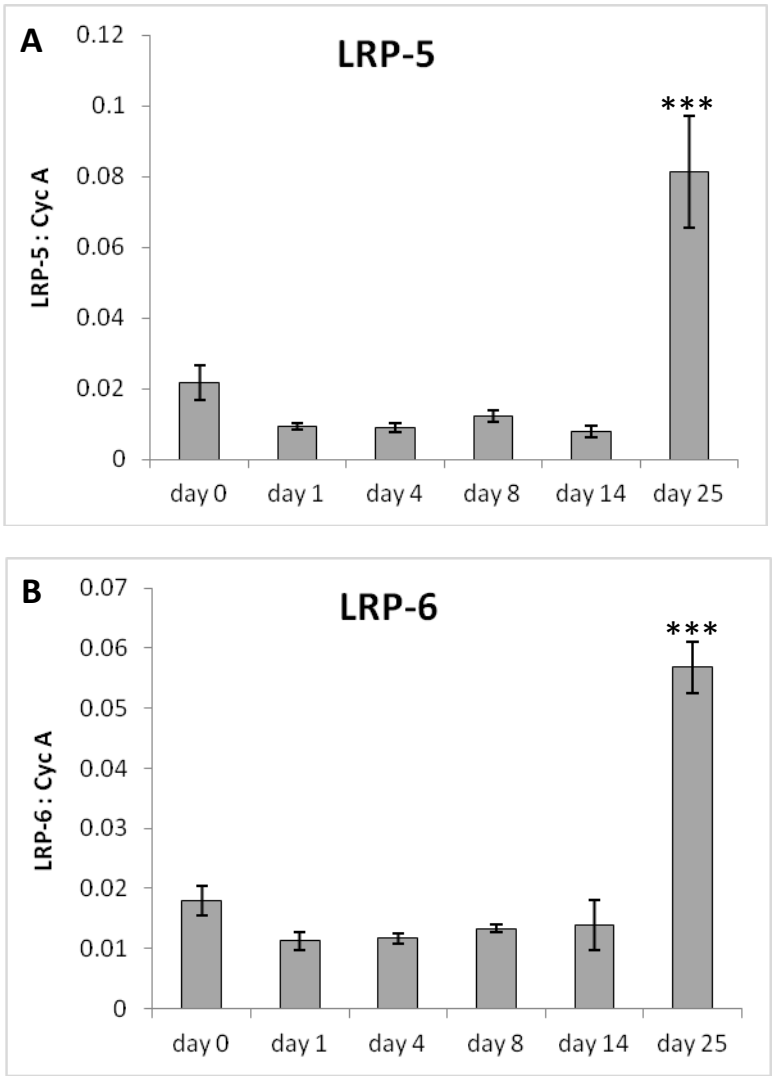


Figure 5.4 Time-course mRNA expression of LRP-5 and LRP-6 after growth plate injury. (A) The mRNA expression of LRP-5 was significantly increased on day 25, compared to 0, 1, 4, 8 and 14 days post-injury ($P<0.0001^{***}$). (B) The mRNA expression of LRP-6 was significantly higher at day 25, compared to days 0, 1, 4, 8 and 14 ($P<0.0001^{***}$). Data is presented as average ± SEM of 3 pooled samples, with 2 animals per pool. Statistics were performed by a one-way analysis of variance (ANOVA) with a Tukey's post hoc test.

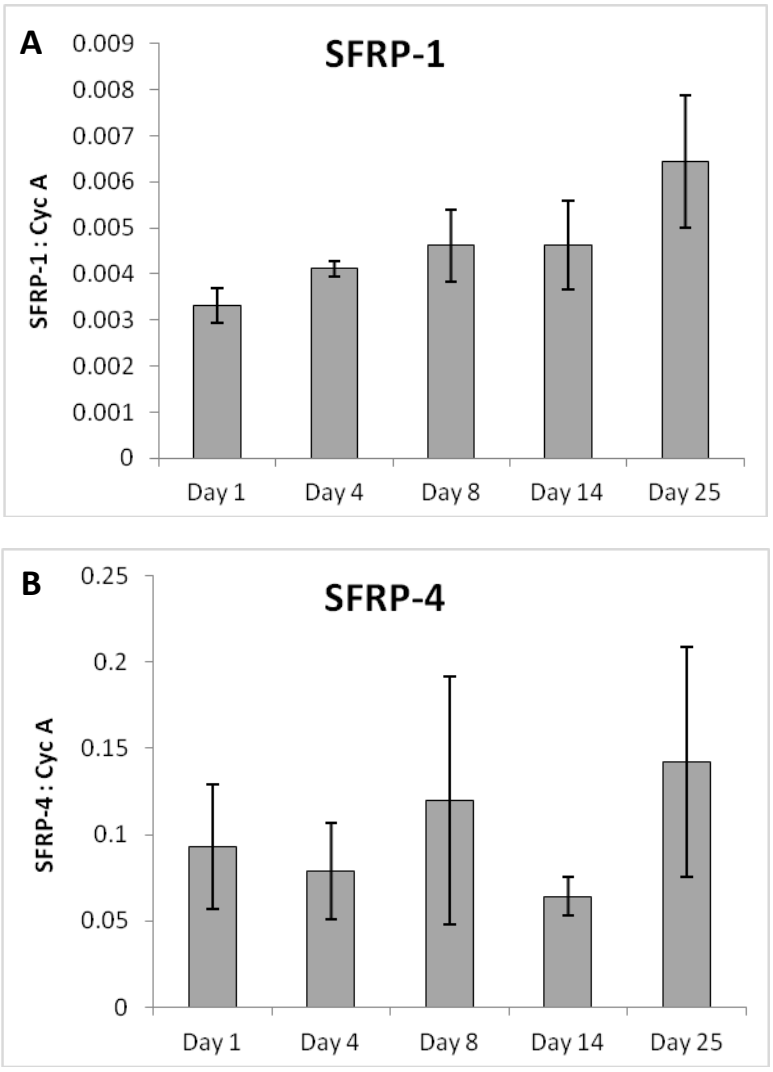


Figure 5.5 Time-course mRNA expression of SFRP-1 and SFRP-4 after growth plate injury. (A) A steady increase in the mRNA expression of SFRP-1 was observed between days 1 to 25, but was not statistically significant (P>0.05). (B) There was no change in the mRNA expression of SFRP-4 across the time-course of bone bridge formation. Data is presented as average ± SEM of 3 pooled samples, with 2 animals per pool. Statistics was a one-way analysis of variance (ANOVA) with a Tukey's post hoc test.

5.2.2 Microarray analysis of Wnt genes differentially expressed in a time-course of growth plate injury

Microarray analysis of the growth plate injury site tissue isolated using laser capture microdissection identified many genes involved in the Wnt signalling pathway. Differentially expressed genes identified in the microarray study were grouped into functional categories using microarray analysis program's Onto-Express and David. The submitted gene lists had normalized data values greater or less than their comparison by a factor of 2-fold. Onto-Express identified Wnt signalling as having an important role in all stages of bone bridge formation. A complete list of Wnt genes identified in one or both (overlapping) programs are tabulated in **Table 5.2** (day 4 versus day 8) ($P<0.01$), **Table 5.3** (day 4 and day 14) ($P<0.1$ & $P<0.01$) and **Table 5.4** (day 8 and day 14) ($P<0.05$).

Wnt genes overlapping in analysis programs are summarised in more detail in **Table 5.5** and described in detail in regards to their known functions in bone and cartilage in **Table 5.6**. Overlapping Wnt components identified at day 4 versus day 8 **(Table 5.5)** included secreted frizzled-related protein 4, secreted frizzled-related protein 1, catenin beta1, and casein kinase 2 alpha 1 polypeptide. At day 4 versus day 14 **(Table 5.5)**, secreted frizzled-related protein 1, casein kinase 2 alpha 1 polypeptide, transcription factor 7 t-cell specific, frizzled homolog 1, lymphoid enhancer binding factor 1and secreted frizzled-related protein 4 were identified. Lastly, Wnt genes identified at day 8 versus day 14 **(Table 5.5)** included, frizzled homolog 1, frizzled homolog 2, Wnt1 inducible signalling pathway protein 1, carboxypeptidase Z, lymphoid enhancer binding factor 1, transcription factor 7 t-cell specific, and casein kinase 2 alpha 1 polypeptide.

Table 5.2. Microarray analysis of Wnt signalling components for Day 4 vs Day 8 comparison.

Day 4 vs. Day 8	
sclerostin domain containing 1 cadherin, EGF LAG seven-pass G-type receptor 2 DIX domain containing 1 microphthalmia-associated transcription factor	Onto-Express **P< 0.00925**
secreted frizzled-related protein-4 **secreted frizzled-related protein-1** **catenin (cadherin associated protein), beta 1** **casein kinase 2, alpha 1 polypeptide**	**Overlapping**
similar to nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 1 low density lipoprotein receptor-related protein-6 (predicted) similar to nemo like kinase wingless-type mmtv integration site family, member 2b; secreted frizzled-related protein-2 presenilin 1 seven in absentia 1a calcium/calmodulin-dependent protein kinase ii beta subunit creb binding protein 88kda, protein phosphatise 3, catalytic subunit, alpha isoform casein kinase 1, alpha 1 axin2 calcium/calmodulin-dependent protein kinase 2 gamma protein phosphatise 3, regulatory subunit b, alpha isoform (calcineurin b, type i)	DAVID **P<0.0041**

Wnt genes differentially expressed at Day 4 versus Day 8, as analysed using microarray analysis programs, Onto-Express and DAVID ($P<0.05$). These are genes that are selected from Day 8 that have normalized data values that are greater or less than those from Day 4 by a factor of 2-fold. Genes that were identified in both programs were identified as overlapping and are highlighted in the table in bold.

Wnt signalling component genes identified at Day 4 versus Day 14 as analysed using microarray analysis programs Onto-Express and DAVID ($P<0.05$). Several genes were found to overlap between the two programs (highlighted in bold). These are genes that are selected from Day 14 that have Normalized Data values that are greater or less than those from Day 4 by a factor of 2-fold.

Table 5.3. Microarray analysis of Wnt signalling components for Day 4 vs Day 14 comparison.

Day 4 vs. Day 14	
Carboxypeptidase Z DIX domain containing 1 microphthalmia-associated transcription factor	Onto-Express **P<0.0924**
secreted frizzled-related protein-1 **casein kinase 2, alpha 1 polypeptide** **transcription factor 7, t-cell specific** **frizzled homolog 1** **lymphoid enhancer binding factor 1** **secreted frizzled-related protein-4**	**Overlapping**
calcium/calmodulin-dependent protein kinase ii gamma protein kinase c, gamma calcium/calmodulin-dependent protein kinase ii, delta wingless-type mmtv integration site 5a phospholipase c, beta 1 axin2 phospholipase c, beta 1 ras-related c3 botulinum substrate 2 protein kinase c, gamma protein phosphatase 2 (formerly 2a), regulatory subunit b (pr 52), alpha isoform cyclin d1 protein kinase c, beta 1 protein phosphatase 3, catalytic subunit, alpha isoform calcium/calmodulin-dependent protein kinase ii gamma wnt inhibitory factor 1 creb binding protein; lymphoid enhancer binding factor 1 cyclin d3 ras-related c3 botulinum toxin substrate 1 similar to nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 1 calcium/calmodulin-dependent protein kinase ii, delta secreted frizzled-related protein 2 wingless-type mmtv integration site family, member 2b casein kinase 1, alpha 1 dishevelled associated activator of morphogenesis 2 (predicted)	DAVID **P<0.0033**

Table 5.4. Microarray analysis of Wnt signalling components for Day 8 vs Day 14 comparison.

Day 8 vs. Day 14	
cadherin, EGF LAG seven pass G-type receptor 2 catenin, beta-interacting protein 1 DIX domain containing 1 sclerostin domain containing 1	Onto-Express **P<0.01578**
frizzled homolog 2 **frizzled homolog 1** **wnt1 inducible signalling pathway protein 1** **carboxypeptidase Z** **lymphoid enhancer binding factor 1** **transcription factor 7, T-cell specific** **casein kinase 2, alpha 1 polypeptide**	**Overlapping**
junction plakoglobin casein kinase 1, gamma 1 ng22 protein; wingless-type mmtv integration site 5a axin2 complement component 4a dix domain containing 1 (predicted) transducin-like enhancer of split 1, homolog of drosophila e(spl) (predicted) heat shock 70kd protein 1a; neuraminidase 1 ng23 protein wingless-type mmtv integration site family, member 2b chloride intracellular channel 1 casein kinase 1, alpha 1	DAVID **P<0.013**

Many Wnt signalling component genes were identified using microarray analysis programs, Onto-Express and DAVID ($P<0.05$) at Day 8 versus Day 14. Several genes were found to overlap between the two analysis programs (highlighted in bold). These are genes that are selected from Day 14 that have Normalized Data values that are greater or less than those from Day 8 by a factor of 2-fold.

Table 5.5. Microarray analysis of Wnt signalling component genes differentially expressed (overlapped in DAVID and Onto-Express microarray analysis programs) in a time-course of growth plate injury.

Group	Wnt Genes	Symbol	Gen bank	Fold Change
D4 & D8	Secreted frizzled-related protein 4	SFRP4	NM_053544	3.357
	Secreted frizzled-related protein 1	SFRP1	XM_224987	3.513
	Catenin (cadherin associated protein), beta 1	Ctnnb1	NM_053357	-2.178
	Casein kinase 2, alpha 1 polypeptide	Csnk2a1	NM_053824	-4.049
D4 & D14	Secreted frizzled-related protein 1	SFRP1	XM_224987	3.960
	Casein kinase 2, alpha 1 polypeptide	Csnk2a1	NM_053824	3.957
	Transcription factor 7, t-cell specific	Tcf7	XM_343891	-3.106
	Frizzled homolog 1	Fzd1	NM_021266	-2.494
	Lymphoid enhancer binding factor 1	Lef1	NM_130429	2.155
	Secreted frizzled-related protein 4	SFRP4	NM_053544	5.036
D8 & D14	Frizzled homolog 2	Fzd2	NM_172035	-2.770
	Frizzled homolog 1	Fzd1	NM_021266	-3.058
	Wnt1 inducible signalling pathway protein 1	Wisp1	NM_031716	2.516
	Carboxypeptidase Z	Cpz	NM_031766	4.635
	Lymphoid enhancer binding factor 1	Lef1	NM_130429	3.245
	Transcription factor 7, T-cell specific	Tcf7	XM_343891	-2.247
	Casein kinase 2, alpha 1 polypeptide	Csnk2a1	NM_053824	2.355

Several genes involved in the Wnt signalling pathway were identified at all time-points analysed that overlapped in DAVID and Onto-Express microarray analysis programs ($P \leq 0.01$). Comparisons between groups identified 4 genes at day 4 versus day 8, 6 genes at day 4 versus day 14 and 7 genes at day 8 versus day 14, respectively. The fold change was decreased (-) or increased ≥ 2-fold.

Table 5.6. Functions/Description of differentially expressed Wnt signalling component genes.

Gene	Description/Function	Reference
SFRP-1	Binds to Wnt and Fz receptors to inhibit both Wnt signalling pathways. Expressed and released by osteoblasts to inhibit osteoclast formation. Enhanced and prolonged trabecular bone accrual in knockout mice.	[449] [450]
SFRP-4	Inhibitor of both Wnt signalling pathways. Highly expressed in osteoblasts in late differentiation stages and thus could control maturation.	[451]
β-catenin	Induces early osteoblast differentiation but not at later stages. β-catenin determines the differentiation of MSCs into either cartilage or bone. β-catenin at high levels has a strong negative effect on osteoclast differentiation, controlling OPG expression.	[186, 187]
Csnk2a1	Encodes the catalytic subunit α of protein kinase CK2. CK2 is an inhibitor of apoptosis in rat articular chondrocytes. CK2 can regulate ALP activity and stimulate osteoblast differentiation via phosphorylation of the transcription factor Ikaros.	[188, 452]
Fzd1	Wnt co-receptor that initiates Wnt signal transduction. Cis-regulatory polymorphisms in the promoter region may have an effect on bone phenotype in humans.	[453]
Fzd2	During chick embryogenesis, Fzd2 is expressed in the proximal limb mesenchyme. Expressed on MSCs, derived from bone marrow, adipose tissues and umbilical cord blood.	[189, 454]
Wisp1	Over-expressed in synovial joints and cartilage in models of human and mouse osteoarthritis, inducing cartilage loss. Expressed in osteoblasts and osteoblastic progenitor cells during embryonic mouse limb development. Suppresses chondrocyte differentiation whilst promoting MSC proliferation and osteoblast differentiation. Potential role in fracture repair.	[195, 196, 455, 456]
Lef1	Lef1 is a known repressor of Runx2-mediated induction of osteocalcin transcription, having a functional binding site adjacent to Runx2 in the osteocalcin promoter. Lef1 expression decreases bone cell differentiation and opposes osteoblast maturation. Lef1 was down-regulated during the early phase of fracture repair and during maximal bone formation.	[457] [458] [446]
Tcf7	Expressed in osteoblasts during embryonic and postnatal development. Increased bone resorption in Tcf7 deficient mice, where OPG was identified as a target gene for Tcf proteins. Mouse embryos deficient in both Tcf7 and Lef1 have arrested limb bud development. Tcf7 inhibition enhanced *in vitro* MSCs osteogenesis.	[187, 459] [190]
CPZ	CPZ is co-expressed with Wnt-4 in growth plate chondrocytes. Over expression promotes terminal differentiation of chondrocytes. Thyroid hormone can regulate CPZ expression levels. Regulates skeletal development in the embryonic chicken.	[460] [461]

Genes that were up-regulated and down-regulated in a time-course of growth plate injury are described in relation to their bone/cartilage functions.

5.2.2.1 Pathway analysis

The significance of Wnt signalling was also examined using the Onto-Tools application, Pathway-Express. As described in section 4.2.5, Pathway-Express (PE) was used to identify potential pathways involved in bone bridge formation. PE employed a novel impact analysis method which ranks pathways based on an impact factor [415]. Specifically, the impact factor is based on the magnitude of the expression change of each gene, their position within the pathway, and their interaction and regulation of other genes within the pathway [415]. This type of analysis was considered advantageous because it considered the effect of each differentially expressed gene on other genes within a given pathway. The highest ranked pathways were presented with a gamma p-value ≤ 0.1. At day 4 versus day 8, Wnt signalling was ranked number 12 among all potential pathways involved, with an assigned impact factor of 5.28 **(Chapter 4, Table 6)**. At day 8 versus day 14, the Wnt signalling pathway was ranked 8 with an impact factor of 5.69 **(Chapter 4, Table 8)**. The Wnt signalling pathway was identified at day 4 versus day 14 but had a gamma p-value greater than 0.1 and therefore was not listed **(Chapter 4, Table 7)**.

5.3 Discussion

Wnt signalling has an essential role in regulating bone formation and remodelling during embryonic development and throughout postnatal and adult life. Specifically, Wnt signalling regulates bone formation by controlling embryonic cartilage development and postnatal chondrogenesis, osteoblastogenesis, osteoclastogenesis, endochondral bone formation, and bone remodelling [422, 423]. Furthermore, Wnt signalling is activated during bone fracture repair and plays a crucial role in regulating bone regeneration [420, 421, 424, 425].

Therefore, Wnt signalling was hypothesised to be potentially important in regulating the bony repair of the injured growth plate cartilage.

In the current study, a time-course of tibial growth plate injury in young rats was used to investigate the potential role of Wnt signalling in the bony repair of the injured growth plate cartilage. An initial examination of Wnt components using Real-Time RT-PCR revealed significant changes in mRNA levels of Wnt-5a, Wnt-10b, and LRP-5 and 6 co-receptors in the injured growth plate tissue (from whole growth plate). Furthermore, microarray analysis of the injury site only, specifically isolated using laser capture microdissection (LCM) identified the differential expression of many genes involved in both the canonical and non-canonical Wnt signalling pathways. Overall, results strongly suggest that Wnt signalling plays an important role in the repair of the growth plate following drill-hole injury.

A limiting factor in this and in previous growth plate fracture studies is the method of the isolation of injured tissue. Samples used for analysis in Real Time RT-PCR were collected by scraping the whole growth plate (including the injury site and the surrounding uninjured areas). Expression data from RNA samples prepared from the scraped tissue may not specific enough for a true representation of the changes in gene analysis of the injury site and may down play their role as this tissue is only a small proportion of the tissue being analysed. Furthermore, changes observed in expression of Wnt genes could be due to a physical removal of tissue overestimating the importance of some genes. However, due to the improvements, availability and affordability in laser capture microdissection (LCM) and microarray technologies, these more modern methods were adopted at later stages in this

study. LCM allowed the isolation of the injury site only and combined with microarray analysis, was able to identify Wnt signalling as a major player in growth plate bony repair and bone bridge formation, identifying several Wnt genes as potential players. This approach was advantageous as Wnt signalling was identified using systemic gene expression profiling analysis in contrast to preselecting potentially important genes as performed before with Real Time RT-PCR analysis. Despite this, one major concern in the microarray study was the lack of biological replicates. Due to the small size of injury site and thus the limited ability to isolate large amounts of tissue with LCM from each injured growth plate, this meant that samples at each time-point had to be pooled in preparation for amplification and microarray analysis. As a consequence of not having biological replicates, statistical data could not be presented for the differentially expressed genes identified. As a result, differentially expressed genes were ranked based only on fold-change. If biological replicates had been included, variation in gene expression between animals could have been identified and genes presenting with poor reproducibility could have been excluded.

Although Pathway-Express analysis identified Wnt signalling across the time-course of bone bridge formation, it is important to note the potential importance of more highly ranked pathways. At day 4 versus day 8, 14 pathways were tabulated **(Chapter 4, Table 6)** and were dominated by pathways related to the immune system including antigen processing and presentation, leukocyte transendothelial migration, graft-versus-host disease etc. Similarly, at day 4 versus day 14 **(Chapter 4, Table 7)** and day 8 versus day 14 **(Chapter 4, Table 8)** pathways related to the immune system were prominent amongst those listed higher than Wnt signalling.

Wnt genes

Wnt-10b was selected for analysis using Real Time RT-PCR due its critical role in bone formation. Wnt-10b, expressed in mature osteoblasts and mesenchymal precursor cells, has an important role in bone formation. In Wnt-10b-/- mice, there was a decreased trabecular bone volume and serum osteocalcin level [426]. In addition, Wnt-10b is important in promoting the differentiation of mesenchymal precursor cells into osteoblasts and away from adipocytes and stimulating bone formation [426, 427]. In the current study, RT-PCR on tissue from growth plate scrapes revealed a significant increase in the mRNA expression of Wnt-10b at day 25 (bone maturation phase) when compared to days 8 and 14 (bone formation). It was originally proposed that the expression of Wnt-10b would increase as bone formation began on day 8, but this was not observed using this sample preparation and RT-PCR analysis method. It is possible that lower levels of Wnt-10b mRNA expression may be present during earlier stages of bone bridge formation due to a physical removal of growth plate tissue. Levels may be increased at day 25 due to the presence of mineralizing bone and mature bone cells expressing Wnt-10b in the growth plate injury site. Nonetheless, Wnt-10b was not found to be differentially expressed following microarray analysis at all time-points analysed.

Additionally, Wnt-10b promotes osteoblastogenesis via induction of the osteoblastogenic transcription factors Runx2, Dlx5, and osterix and suppression of the adipogenic transcription factors C/EBPα and peroxisome proliferator-activated receptor gamma (PPARγ). Microarray analyses revealed no changes in expression of these genes at day 4 versus day 8. However, there was a 2-fold increase in both Runx2 and PPARγ expression at

day 14 when compared to days 8 and 4, which is neither supportive of osteoblastogenesis nor adipogenesis. Thus, these and the above results are not strong enough to suggest that signalling through Wnt-10b plays an important role in bone bridge formation.

Wnt-5a signals through the non-canonical Wnt/calcium-releasing pathway. It was important to investigate the role of Wnt-5a in bone bridge formation as Wnt-5a has an important role in growth plate formation and function [419, 428], MSC maintenance [429-431] and osteoblast differentiation [432]. Wnt-5a is expressed at high levels in chondrocytes of the proliferative and prehypertrophic zones and is essential in controlling the pace of transition of proliferative chondrocytes into prehypertrophic chondrocytes, independently of the Ihh/PTHrP negative feedback loop [131, 132].

Real Time RT-PCR on whole growth plate samples revealed a significant decrease in Wnt-5a expression on day 0, day 1 (inflammatory phase), day 4 (fibrogenic phase), and days 8 and 14 (bone formation phase), when compared to 25 days post-injury. The decreased expression of Wnt-5a was found immediately following surgical injury (day 0), which strongly suggested that the decline in expression across the time-course was due to the physical removal of part of the growth plate tissue by the drill during surgery. Interestingly, similar to Wnt-10b, the expression of Wnt-5a was significantly increased at day 25. Wnt-5a, a non-canonical Wnt, is important in guiding MSCs down the osteogenic lineage [220, 221]. Wnt-5a stimulates osteogenic differentiation by signalling through Ror2 [432], which activates the small GTPase RhoA, and ultimately Runx2 expression [433-435]. This has been shown to be both necessary and sufficient in inducing osteogenic differentiation using

osicallary fluid flow *in vitro* [436] and mechanical stimulation *in vitro* [432]. Furthermore, RhoA activation inhibits both adipogenic and chondrogenic differentiation, whilst stimulating the commitment of MSCs to the osteogenic lineage [434, 435, 437], via tension generated by RhoA in the actin cytoskeleton [221]. Consequently, a significant increase in Wnt-5a expression would be expected at day 4 and day 8 during the mesenchymal and early bone formation responses, respectively. Therefore, Wnt-5a either lacks a significant role in the bony repair of the injured growth plate or the method of sample preparation for Real Time RT-PCR was not specific enough to allow such changes to be observed. An increase towards normal levels by day 25 may be due to presence of bone marrow within the injury site, where Wnt-5a and Frizzled5, a receptor ligand pair, are shown to be important for limb bud and bone marrow stem cell development [438].

Interestingly, microarray analyses, with the injury site specimens only, revealed a 2-fold increase in Wnt-5a expression at days 4 and 8 when compared to day 14. These findings may be explained in reference to recent studies identifying Wnt-5a as important in the maintenance of human MSCs *in vitro* and in the enhancement of osteoblast differentiation *ex vivo* [220, 221]. Higher levels found during the mesenchymal response (day 4) is consistent with the presence of high numbers of infiltrating MSCs in the injury site. Furthermore, at day 8, in addition to infiltrating MSCs, early bone formation is occurring requiring osteoblast differentiation, which are both consistent with the functions of Wnt-5a. Importantly, results also demonstrate that Wnt-5a is expressed in the injury site during the time-course of bone bridge formation, thus supporting a role for Wnt-5a in osteogenesis and bone fracture repair, in addition to its known function in the regulation of the growth plate.

Receptors

LRP-5 and LRP-6 co-receptors are essential in the activation of the Wnt/β-catenin signalling pathway and have a crucial role in bone mass determination and bone metabolism [439-444]. Therefore, it was deemed necessary to investigate the role of these receptors in bone bridge formation using Real Time RT-PCR.

PCR data with samples from whole growth plate scrapes revealed a significant decrease in LRP-5 and LRP-6 mRNA expression from days 0 through to 14 in comparison to day 25. Again, the reduction in mRNA expression could be attributed to a physical removal of growth plate tissue. However, LRP-5 is expressed by osteoblasts of the endosteal and trabecular bone surface and regulates osteoblastic proliferation, survival and activity [445]. Thus, it was surprising that levels were not found to be higher at day 14, where bone formation is rapidly occurring with the presence of many trabeculae. However, much higher levels were found at day 25, supporting signalling through LRP-5 and LRP-6 co- receptors in the regulation of bone formation. Microarray data revealed a 2.71-fold increase in the expression of LRP-6 at day 8, when compared to day 4. It was surprising that an up-regulation was not found between other time-points compared.

The complexity of the Wnt signalling pathway has greatly increased with the controversial finding that the crucial regulation of bone by LRP-5 is not a direct action, but is regulated via the gut [446, 447]. Tryptophan hydroxylase 1 (Tph1) in the gut synthesizes serotonin/5-hydroxytryptamine (5-HT) which is released into the circulation to be taken up by bone cells expressing cell surface receptors 5-$HT_{1B,\ 2A}$ and $_{2B}$ and possessing the plasma membrane

transporter (5-TT) [447]. LRP-5 expression inhibits the synthesis of 5-HT in the duodenum, decreasing 5-HT blood levels. At high circulating levels, 5-HT inhibits the function of LRP5, inhibiting CREB expression and subsequently osteoblast proliferation [447].

Due to these findings, the potential involvement of 5-HT signalling in bone bridge formation was investigated through analysis of microarray data. Subsequently, a decrease in the expression of 5-HT receptor 2A and 2B was found at day 8 when compared to day 4 and at day 14 when compared to day 4. This is supportive of the literature where higher levels of osteoblast proliferation would be required at day 8 during early bone formation. However, an increase in 5-HT was found at day 14 in comparison to both day 4 and day 8. An increase in the uptake of 5-HT at day 14 is logical where lower levels of osteoblast proliferation would be occurring in comparison to day 4 and day 8.

Interestingly, it was found that wild type and LRP-5 knockout mice fed a diet containing 75% less tryptophan than normal had decreased circulating levels of 5-HT and subsequently, LRP-5 knockout mice had normalized skeletal pathology [447]. Therefore, future studies in investigating methods to inhibit bone bridge formation could be the addition of Trp1 in the feed of injured rats, or the administration of serotonin injections into the circulation. Both methods could potentially inhibit bone bridge formation.

The differential expression of Frizzled 2 (Fzd2) was identified in two or more microarray analysis programs and supports a role for Wnt-5a in bone bridge formation. There are many Fzd receptors indentified in humans and mice, however only Fzd1 and Fzd2 have been

identified in the rat. In the rat, Fzd2 has been shown to interact with Wnt-5a through the Wnt/Calcium^{2+} pathway to elicit calcium release [448]. In support of a down-regulation of Wnt-5a, Fzd2 was found to be decreased by 2.77-fold at day 14 in comparison to day 8. Signalling through the calcium pathway negatively regulates the Wnt/β-catenin pathway, whereby the release of intracellular calcium activates protein kinase C (PKC), calmodulin dependent protein kinase 2 (CamK2) and nuclear factor of activated T cells (NFAT), which repress β-catenin signalling via different mechanisms [449-451]. Therefore, signalling via this pathway may be down-regulated at day 14 to increase osteoblast differentiation as well as regulate bone remodelling via canonical Wnt signalling.

A significant increase in Fzd1 expression was found at day 14 in comparison to day 4 (2.49), whereas a significant decrease was observed at day 14 when compared to day 8 (-3.06). In mice, Fzd1 has an antagonistic role in Wnt/β-catenin signalling, suppressing the expression of alkaline phosphatase in MSCs, the translocation of β-catenin to the nucleus and Wnt-3a induced TCF signalling [452]. Therefore, an increase in Fzd1 at day 14 would be consistent with the role of β-catenin, where it does not induce later stages of differentiation and consistent with expression levels of Wnt-5a/Fzd2 [214]. In reference to the literature, an increase in Fzd2 expression (in conjunction with Wnt-5a) and Fzd1 at day 8 in comparison to day 14 may be negatively regulating the levels of β-catenin dependent transcription to allow osteoblast maturation and thus early bone formation during this early osteogenic response of bone bride formation. Consequently, lower expression levels at day 4 suggest an expansion of MSCs within the injury site and their commitment to the osteoblast lineage. An

increase in canonical Wnt signalling in MSCs increases their rate of proliferation via autocrine and paracrine mechanisms [215, 453, 454].

Transcription factors

The non-canonical calcium signalling pathway can also inhibit Tcf/Lef transcription factors, including Tcf7 and Lef1. Following translocation of β-catenin to the nucleus, β-catenin binds to Tcf7/Lef1 to displace transcription inhibitors bound to Tcf7/Lef1, enabling the transcription of target genes [455]. Therefore, levels of Tcf/Lef are directly influenced by levels of nuclear β-catenin and thus Wnt binding to LRP-5 and LRP-6 co-receptors. Microarray analysis revealed a higher level of Lef1 expression during the mesenchymal response at day 4 in comparison to day 14 (bone formation and maturation responses). During early bone formation, Lef1 collaborates with Runx2 to regulate the expression of fibroblast growth factor 18 (FGF-18) [456], a recently identified direct target gene of the Wnt signalling pathway [456]. FGF-18 has a role in expanding the early osteoblast population, expressed at sites that coincide with sites of osteoblast development, where β-catenin levels are increased [456]. Thus, higher levels of Lef1 expression at day 4 would be required to stimulate and expand the MSC population in preparation of repairing the injury site with a bony bridge. Furthermore, Wnt/β-catenin signalling via Tcf/Lef1 transcription factors is necessary for the commitment of osteoblastic/mesenchymal precursors to the osteoblast lineage and their differentiation into early osteoblasts (expressing low levels of osteocalcin) [457-459]. Consequently, higher levels of β-catenin were found at day 4, when compared to day 8, thus supporting the commitment of osteoblastic precursors to the osteoblast lineage and early events of osteoblast differentiation occurring during the

mesenchymal response. Inhibition of Wnt/β-catenin signalling is required for full maturation of osteoblasts into osteocalcin expressing cells [457] and therefore lower levels of β-catenin at day 8 may support the formation of mature bone-forming osteoblasts required in the early events of bone bridge formation.

The expression of Lef1/Tcf7 are shown to be down-regulated as cells stop proliferating and differentiate into osteoblasts [460], which supports lower levels at day 14 where a high level of bone formation is occurring, requiring high numbers of mature osteoblasts. In addition, Lef1 is a known repressor of Runx2-mediated induction of osteocalcin transcription and therefore osteoblast maturation, having a functional binding site adjacent to Runx2 in the osteocalcin promoter [456, 460, 461]. Thus, high levels of Lef1 at day 14 would have an inhibitory effect on bone formation. Lef1 was found to be down-regulated during the early phase of fracture repair and during maximal bone formation [420]. Therefore, the down-regulation of Lef1 expression at day 14 when high levels of bone formation is occurring is supportive of the requirement of high levels of osteoblast differentiation. In contrast, higher levels of Lef1 expression were found at day 14 in comparison to day 8. Bone formation is in early stages by day 8 and thus lower levels would be consistent with the need to have high levels of osteoblast differentiation and maturation in comparison to day 14, where bone formation and remodelling are already well underway.

At day 14, there was a significant increase in the expression of Lef1 (3.25-fold), but a significant decrease in Tcf7 expression (-2.25), in comparison to day 8. Embryonically, Lef-1 is expressed in predominantly skeletal structures, whereas, Tcf7 is expressed in pre-

cartilaginous cells [420]. Therefore, Tcf7 may be down-regulated due to a lack of chondrocyte proliferation, whereas Lef1 may be stimulating the proliferation of osteoblast precursors in the injury site. A deficiency in Tcf7 has shown to increase bone resorption in mice and enhance osteoblast differentiation in mesenchymal stem cells *in vitro* [218, 462]. Therefore, the decrease in Tcf7 at day 14 may be due to a demand for bone resorption where bone has begun remodelling by day 14. Expression of Tcf7 was down-regulated at day 14 when compared to day 4. It is possible that Tcf7 is being expressed in cartilage debris in the injury site at day 4 and higher levels at day 14 are required to enhance bone formation.

Antagonists/Agonists

Real-Time RT-PCR and microarray analysis both identified a possible role for Wnt antagonists, SFRP-1 and SFRP-4, in the bony repair of the injured growth plate. SFRP-1 is shown to be an inhibitor of bone formation, signalling through both canonical and non-canonical pathways. Bodine et al (2004) found the deletion of SFRP-1 prolonged and enhanced trabecular bone accrual in adult mice (13-52 weeks), by potentiating osteoblast proliferation and differentiation and reducing osteoblast and osteocyte apoptosis [463].

Real-Time RT-PCR revealed a steady increase in mRNA expression of SFRP-1 over the time-course of growth plate injury, with highest levels of expression observed at 25 days. As an inhibitor of bone formation, increasing SFRP-1 expression may be co-commitant with a decline in the rate of bone formation occurring as bone reaches the remodelling phase, commencing at approximately day 25. Microarray analysis also found that SFRP-1 was up-regulated where levels were higher at day 8 in comparison to day 4 (3.51-fold increase) and

at day 14 in comparison to day 4 (3.96-fold increase). This was consistent between analysis programs. Biological significance for the higher levels of SFRP-1 at later days of bony repair is supportive of the time-course observed in bone bridge formation.

SFRP-4 is also known to inhibit bone formation, where SFRP-4 was shown to be a negative regulator of peak bone mineral density in mice, through inhibiting Wnt signalling [464]. Similar to findings with SFRP-1, microarray analysis revealed that SFRP-4 expression was 3.36 fold higher at day 8 compared to day 4 and 5.04-fold higher at day 14 compared to day 4, which overlapped in analysis programs. PCR found the mRNA expression of SFRP-4 to be very inconsistent between animals at all time-points analysed.

Wnt-1-induced secreted protein 1 (Wisp1) is a member of the CCN family of growth factors and is identified as a secreted Wnt antagonist. A significant up-regulation (2.52-fold) in Wisp1expression was identified at day 14 (compared to day 8). This was not surprising as Wisp1 is expressed in mesenchymal stem cells, bone marrow stromal cells (BMSC) and osteoblasts during embryonic mouse limb development, promoting osteoblast differentiation, whilst suppressing chondrocyte differentiation [223, 465]. This was also found in human BMSC *in vitro* [224] and recapitulated in a fracture repair mouse model, suggesting a role for Wisp1 in bone fracture repair [223]. Participation of Wisp1 in the bony repair of the injured growth plate is very relevant where bone formation is induced and cartilage formation suppressed. Furthermore, Wisp1 expression is shown to increase in cells as they reach a more mature osteoblast phenotype. At day 14, there is a high level of bone formation, whereas at day 8, bone formation is in its early stages. Thus a higher number of

bone cells would be present at day 14, potentially accounting for the increased levels of Wisp1 expression.

Another gene that was identified as having significant importance in bone bridge formation was Csnk2a1, where Csnk2a1 was differentially expressed at all time-points analysed in microarray analysis. Csnk2a1 encodes the catalytic subunit α of CK2, a serine/theronine kinase that is composed of two catalytic subunits (α1, α2) and two regulatory subunits (β) [466, 467]. A role for the catalytic subunit alone in osteogenesis and/or chondrogenesis has not been recognized as yet, however CK2 has been shown to regulate ALP activity and stimulate osteoblast differentiation via phophorylation of the transcription factor Ikaros [216]. Furthermore, CK2 was shown to be an inhibitor of apoptosis in rat articular chondrocytes [468]. Csnk2a1expression was significantly decreased at day 8 in comparison to day 4 (4.05-fold), increased at day 14 in comparison to day 4 (3.96-fold) and increased at day 14 when compared to day 8 (2.36-fold). The reason for the differential expression of this subunit alone is not understood.

In summary, the Wnt signalling pathway, a critical regulator of skeletal development, was evident across all time-points analysed and included Wnt signalling inhibitors (SFRP-1, SFRP-4 and Wisp1), frizzled receptors (Fzd1 and Fzd2), transcription factors (Lef1 and Tcf7), β-catenin, Cpz and Csnk2a1. On the whole, at day 4, there was an increased expression of Wnt-5a and β-catenin and decreased levels of Fzd1expresion which could suggest that during the mesenchymal response, Wnt-5a signals through the PCP pathway to enhance the commitment of MSCs to the osteogenic lineage through cytoskeletal changes

that may increase β-catenin-dependent transcription. At day 8, both canonical and non-canonical pathways were stimulated. Signalling was evident via both LRP-5 and LRP-6 co-receptors and through the non-canonical Ca^{2+} pathway, as demonstrated by a significant increase in the Wnt-5a/Fzd2 ligand pair, as well as increased Fzd1 expression. Consequently, both pathways appear to be working together to regulate a balance between osteoblast proliferation and osteoblast maturation to allow early bone formation to occur. Overall, at day 14, there was a suppression of the Ca^{2+} pathway, with decreased expression of Wnt-5a and Fzd2, potentially increasing β-catenin-dependent transcription, bone cell differentiation and regulating bone remodelling. Expression of Wnt signalling inhibitors (SFRP-1, SFRP-4 and Wisp1) was increased during bone bridge formation in early and late bone formation, which are likely be involved in regulating the balance between osteoblast development/bone formation and bone resorption. Overall, it cannot be stipulated whether there is an overall inhibition or stimulation of Wnt signalling. It is the balance and complex interactions between the canonical and non-canonical pathways which occur to regulate β-catenin-dependent transcription. This balance plays a significant role in the bony repair of the injured growth plate cartilage.

CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS, and FUTURE DIRECTIONS

6.1 General Discussion and Conclusions

The growth plate cartilage, located at the ends of each long bone, is responsible for the longitudinal growth of the skeleton, which occurs via a process called endochondral ossification. The growth plate is the weakest structure in the developing long bone, and is therefore a common place for injury. The most common injury to the growth plate occurs as a result of fracture. When the fracture involves the entire width of the growth plate, bony repair or bone bridge formation often occurs, replacing the fractured area with mature trabecular bone, an area previously made up of only cartilage [335, 336]. Evidently, this bony bridge disturbs the process of endochondral ossification and often results in skeletal angular deformities and growth arrest. Using a rat model, previous studies have demonstrated a time-course of bone bridge formation consisting of sequential inflammatory, fibrogenic, osteogenic and bone maturation responses. However, structural changes in the growth plate, at both the injury site and at the non-injured area, have not been closely examined and little is known about the cellular and molecular mechanisms underlying the bony repair. Therefore, using a rat tibial growth plate injury model, this PhD study aimed to examine effects of growth plate injury on the structure and composition of the injured growth plate in a longitudinal study using micro-CT and histology. Microarray analysis of the injury site only collected using laser capture microdissection was used to identify potential cellular and molecular mechanisms involved in bone bridge formation. In addition, Real Time RT-PCR on adjacent uninjured growth plate was used to examine potential cellular/molecular changes at the uninjured area and on whole growth plate scrapes to examine potential involvement of Wnt signalling in bone bridge formation.

A longitudinal study using micro-CT was used to examine the structure and composition of the injury site and uninjured growth plate area following trauma in the drill-hole injury model. Previous work has established that by day 7, a small bone bridge has formed, consisting of small trabeculae, which are increased in number and size between days 10 and 14 [347]. At 25 and 35 days post-injury, the bone bridge has matured and undergone remodelling, and has well-formed marrow [347]. By 14 days post-injury, the injury site is composed of a variety of tissue types including cartilage, fibrous mesenchymal cells, bone marrow and bone [347, 359-361]. The percentages of these tissue types that make up the injury site have been calculated from histological analysis of paraffin embedded sections, at singular time-points [359-361]. However, the progressive accumulation of bone that replaces the growth plate and/or injury site following drill-hole injury remains unknown, and there have been no studies that have investigated potential changes at the adjacent non-injured area of the growth plate. Therefore, using micro-CT, this study aimed to examine effects of growth plate injury on the structure and composition of the injury site and adjacent uninjured area. Our micro-CT study revealed that while the bone volume within the injury site at day 14 is small, there was a considerably larger bone volume at the injury site by 60 days post-injury. Therefore our in vivo micro-CT data has demonstrated a time-course of bone bridge formation, revealing bone bridge formation as only minimal at day 14 when compared to day 60.

Micro-CT analysis also revealed that bone bridge formation does not result in premature thinning of the growth plate. However, the formation of bony pinnacles in the adjacent uninjured area of growth plate, in 60% of injured animals, as observed at 60 days post-

injury, was an unexpected finding. Growth plate injury has previously been examined up to 35 days post-injury, and did not reveal pinnacle formation. RT-PCR analysis on adjacent uninjured growth plate tissue found an increase in bone matrix protein osteocalcin on day 60, supporting bone formation in the adjacent uninjured area. In addition, gene expression analysis with specimens of the adjacent non-injured cartilage at 7 and 14 days post-injury also suggested a decrease in chondrogenesis and an increase in chondrocyte apoptosis, where a significant down-regulation of Sox-9 and IGF-1 mRNA expression and a significant increase in FasL expression was observed, potentially contributing to growth plate degeneration. Therefore, this study demonstrated that growth plate injury can cause some degeneration of the non-injured area as a result of altered gene expression and cellular processes. However, whether the altered signalling and degeneration in the adjacent uninjured growth plate could contribute to the bone growth defects following a trauma injury remains to be studied.

In this study, there is a potential concern for having only one time point of normal growth plate control for RT-PCR analysis of bone and cartilage-related genes in the adjacent uninjured growth plate. Ideally, a normal growth plate control should be provided at each phase of growth plate injury. This would account for any potential changes in gene expression occurring in the normal growth plate with age. The normal growth plate control used in this study was collected from whole growth plate scrapes to coincide with 14 days post-injury. This time-point was selected to represent normal growth plate tissue at a mid-range between 7 and 60 days post-injury. Future experiments would benefit from adding aged-matched controls at days 7 and 60 to assess whether the expression of the various bone

and cartilage-related genes have been potentially over- or under- estimated in the adjacent uninjured growth plate. However, a previous study has demonstrated that the age of the rats between 6-11 weeks did not seem to influence the expression of IGF-I, TNF-α, and TGF-β1 [349].

To enhance the understanding of the mechanisms involved in growth plate bone bridge formation, this study used modern techniques, including laser capture microdissection and microarray analysis, to identify potential genes and molecular pathways involved in the bony repair of the injured growth plate. In this study, Wnt signalling was found to be the major bone-related pathway involved in the bony repair of the injured growth plate, identified across all time-point comparisons. Many other genes involved in skeletal development and osteoblast differentiation were also found to be differentially expressed, with a large proportion of these genes belonging to the BMP signalling pathway. The majority of genes identified within these pathways were not unexpected, having literature supporting a role for these genes in bone formation and bone fracture repair. BMP-2, BMP-7, chordin, chordin-like 2 and Id-1 were found to be important in early stages of bone bridge formation (day 4 and day 8), regulating MSC infiltration and osteoblast differentiation. Components of the Wnt signalling pathway, particularly β-catenin, Csnk2a1, SFRP-1, SFRP-4, Fzd1 and Fzd2 were also regarded as important during early phases. During later stages (day 14), active bone remodelling was prominent and was largely regulated by genes of the BMP signalling pathway (BMP-1 and BMP-6), Traf6, Fgfr1, osteopontin, Mmp9 and Wnt signalling, where several genes were up and down-regulated. Expression of Wnt signalling inhibitors (SFRP-1, SFRP-4 and Wisp1) were increased during both early and late osteogenic responses,

potentially regulating the balance between osteoblast development/bone formation and bone resorption. Osteocalcin expression was prominent at day 8, supportive of osteoblast development and bone formation. Findings were also suggestive of an overall increase in the canonical Wnt signalling pathway at days 4 and14, supported by increased expression of β-catenin and drecreased expression of Wnt inhibitors, and decreased expression of Fzd1 and Fzd2 and increased Lef1 expression, respectively. Lastly, literature has shown that the BMP and Wnt signalling pathways can cooperate to regulate bone formation [469-474], as also suggested in this study.

The major limitation of this study was sample size. At each comparison, the microarray data represent pooled results of several animals of the same time point, lacking the power of seeing potential animal variation. Although laser capture microdissection is a new and very specific technique for tissue isolation, it has the limited ability to isolate large amounts of tissue due to the small size of the injury site, thin sections and the difficulty in sectioning bone. Dissected samples at each time-point had to be pooled in preparation for amplification which required a minimum amount of starting RNA to generate enough cDNA for microarray analysis. As a consequence of not having biological replicates, statistical data could not be presented for the differentially expressed genes identified. As such, differentially expressed genes were ranked based only on fold-change. If biological replicates had been included, variation in gene expression between animals could have been identified and genes presenting with poor reproducibility could have been excluded. Furthermore, using Affymetrix arrays, fold change analysis can give some false positives which could have been identified if biological replicates had been included.

In addition, the microarray technique, despite being powerful in gene expression studies, has limitations as it cannot reveal alternative splicing and information downstream. Alternative splicing can lead to different mRNA molecules transcribed from one single gene. Alternative splicing of pre-mRNA can lead to dozens of variants. Therefore, analyses of molecular relationships and interactions downstream of the genomic information would be ideal. Proteonomics can evaluate changes in expression, structure, modifications as well as the interactions between various proteins in a tissue or a cell population. Future functional in vivo studies manipulating the function of genes of significant interest would also be desirable.

One pathway that could be potentially important in regulating the bony repair of injured growth plate cartilage is the Wnt signalling pathway, which has been shown by many studies to be important in regulating mesenchymal stem cell proliferation and bone cell differentiation [133, 422, 423]. Therefore, the final aim of this project was to examine the potential involvement of the Wnt signalling pathway in bone bridge formation. Using quantitative Real Time RT-PCR analysis, this study examined mRNA expression profiles of the Wnt signalling components in the injured proximal tibial growth plate (samples including both the injury site and non-injured area) 0, 1, 4, 8, 14 and 25 days after injury. PCR data with samples from whole growth plate scrapes revealed a significant decrease in Wnt-5a, LRP-5 and LRP-6 mRNA expression from days 0 through to 14 in comparison to day 25. The decreased expression of Wnt-5a, LRP-5 and LRP-6 was found immediately following surgical injury (day 0), which strongly suggested that the decline in expression across the time-course was due to the physical removal of part of the growth plate tissue by

the drill during surgery. A steady increase in SFRP-1 was observed after injury till day 25 and there was a significant increase in the mRNA expression of Wnt-10b at day 25 when compared to days 8 and 14. Although these results demonstrate changes in expression in Wnt signalling, Real Time PCR was unable to convincingly identify the involvement of Wnt signalling components in bone bridge formation. This was largely due to the method of the isolation of injured tissue. Samples used for analysis in Real Time RT-PCR were collected by scraping the whole growth plate (including the injury site and the surrounding uninjured areas). Thus, expression data from RNA samples prepared from the scraped tissue may not specific or sensitive enough for a true representation of the changes in gene analysis of the injury site and may down play their role as this tissue is only a small proportion of the tissue being analysed. Furthermore, changes observed in expression of Wnt genes could be due to a physical removal of tissue thus overestimating the importance of some genes, as evidenced by a significant decrease in expression at day 0. However, as stated above, microarray analysis of injured tissue obtained from laser capture microdissection identified Wnt signalling as one of the most critically important pathways involved in bone bridge formation at all time points analysed.

Finally, another limitation which should be taken into consideration was the animal model used throughout this study. The rat tibial growth plate injury model used to induce bone bridge formation does not mimic any of the Salter Harris Type injuries, although it is most similar type to the Salter-Harris type IV physeal fracture. Furthermore, the size of the injury inflicted in the rat growth plate using a drill exaggerates the extent to which the growth plate would be disrupted in the human clinical situation. In the animal model, it is advantageous

for the injury site to be larger in size to allow a sufficient amount of tissue to be collected for gene expression analysis. In addition, following growth plate injury in a child, the injured limb would initially be immobilised then stabalised until stable enough to mobilise. In contrast, following drill-hole injury, rats were free to move around and bear weight on their injured limbs. Therefore, the differences discussed above would potentially impact on the process of bone formation within the injured growth plate cartilage.

In conclusion, using a rat tibial growth plate injury repair model, this study has demonstrated that after an injury at the growth plate, bone formation within the injury site at day 14 is minimal, whereas the majority of bone is formed and remodeled by 60 days post-injury. Micro-CT also revealed the formation of bony pinnacles in the adjacent uninjured area at 60 days post-injury. Cellular and gene expression analyses on adjacent uninjured growth plate tissue found alterations in genes associated with chondrocyte apoptosis, proliferation and an increase in bone matrix osteocalcin on day 60, suggesting chondrocyte degeneration and supporting bone formation in the adjacent uninjured area. Microarray analysis of injured tissue obtained from laser capture microdissection identified two known major pathways involved in bone formation, including the BMP and Wnt signalling pathways (canonical and non-canonical). Literature has shown these pathways can cooperate to regulate bone formation, which is also suggested in this study.

6.2 Future Directions

Structural and molecular analysis revealed a potential involvement of the adjacent uninjured growth plate tissue in the clinical complications associated with growth plate injury, with

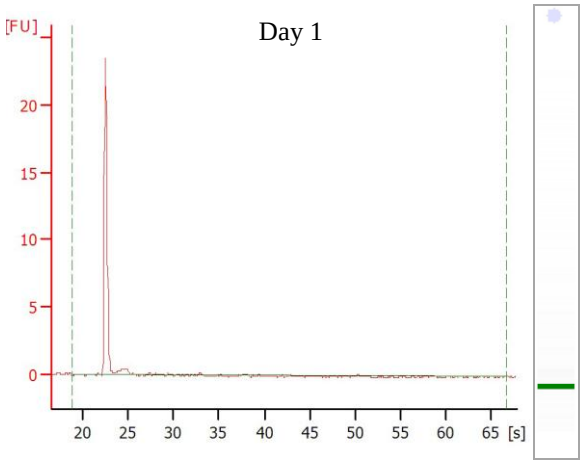
alterations found in cell proliferation, apoptosis, chondorgenesis and the presence of bone tethers. Further studies are required to explore the role of the adjacent uninjured growth plate in contributing to bone growth arrest, angulations of the bone, or potentially, to the formation of bone within the injury site. Growth plate chondrocytes may potentially secrete factors that influence the differentiation of mesenchymal stem cells within the injury site. A time-course analysis of changes in gene expression in the adjacent growth plate tissue using microarray analysis would greatly increase the understanding of its molecular/cellular changes and degeneration and potential associations with bone bridge formation. Furthermore, micro-CT proved an effective method to track the accumulation of bone within the injury site and sets the foundation for future studies aimed to reduce this bone formation in response to therapies.

This study identified two key pathways involved in bone bridge formation at the growth plate injury site, namely the Wnt and BMP signalling pathways, showing the differential expression of several genes within each pathway. In future studies, the functions of these differentially expressed genes could be examined in vitro for mesenchymal cell migration, proliferation and bone cell differentiation, using cell growth, migration and bone cell differentiation assays with bone marrow stromal cells and recombinant proteins or specific inhibitors. Furthermore, the *in vivo* roles of the two signal pathways in regulating these cell activities in the bony repair of growth plate injury site should be explored, particularly during the mesenchymal and osteogenic responses.

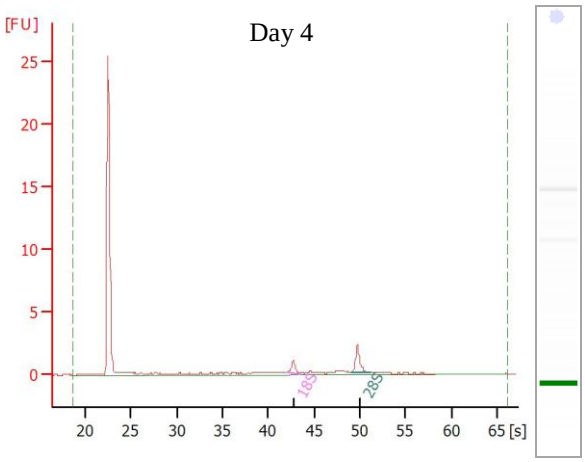
APPENDICES

Appendix 1Additional Results Supporting Chapters 3, 4 and 5

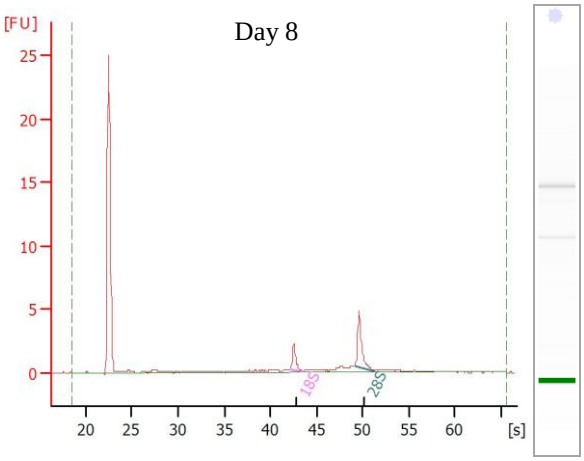
1.1 RNA yield and purity



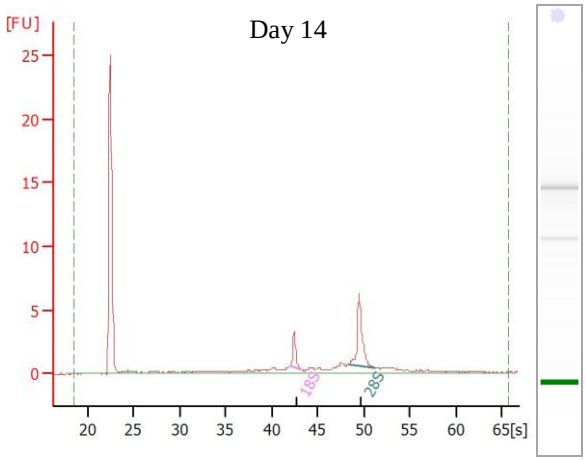
RNA Concentration: 2 ng/µl
rRNA Ratio [28s / 18s]: 0.0
RNA Integrity Number (RIN): N/A (B.02.03)



RNA Concentration: 6 ng/ul
rRNA Ratio [28s / 18s]: 0.0
RNA Integrity Number (RIN): N/A (B.02.03)



RNA Concentration: 4 ng/µl
rRNA Ratio [28s / 18s]: 1.9
RNA Integrity Number (RIN): N/A (B.02.03)



RNA Concentration: 16 ng/ul
rRNA Ratio [28s / 18s]: 1.7
RNA Integrity Number (RIN): 7.0 (B.02.03)

To assess RNA yield and purity, 1ul of each sample was run on a RNA 6000 Nano LabChip Kit on an Aligent 2100 Bioanalyser Nanochip at Adelaide Microarray Facility. The electrophoretic data revealed the ribosomal bands, 18s and 28s but a ratio and RIN number could not be calculated for all samples due to very low concentrations of RNA.

1.2 Affymetrix recommended quality checks

PRE-PROCESSING AND DIAGONISTIC PLOTS OF AFFYMETRIX CEL FILES

Summary

Expression data from CEL files were read into R software, and quality of the chips was assessed using various diagnostic plots, which were created using the following modules that are a part of the Bioconductor package.

- simpleAffy,
- AffyExpress,
- Limma, and
- Affy

Affymetrix recommended QC metrics were also considered.

Conclusion

Our findings suggest that the Day1 sample is of poor quality and should probably be removed from downstream analysis of the chips.

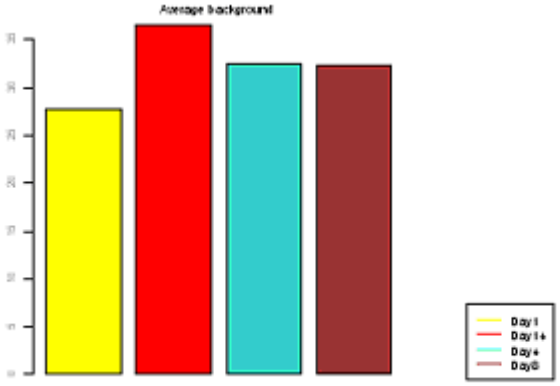
Affymetrix recommended QC

Affymetrix recommends a series of QC metrics that should be used to assess array data. In the publication GeneChip Expression Arrays: Data Analysis Fundamentals, available from http://www.affymetrix.com, Affymetrix gives guidelines on how to interpret these quantities and what cut-off values to use when determining whether an array is of acceptable quality or not. Some of the metrics are as follows

Average background

According to Affymetrix, the values should normally fall between 20 and 100 and chips being compared should ideally have comparable background values.

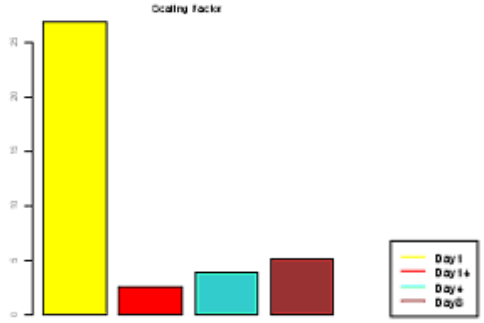
af1c1611-1-Day1-150708.CEL 27.73805
af1c1611-2-Day4-150708.CEL 32.39225
af1c1611-3-Day8-150708.CEL 32.25333
af1c1611-4-Day14-150708.CEL 36.58783



Scaling factor

Scaling factor depends on sample quality and target intensity and therefore Affyymetrix do not recommend any absolute threshold for determining if an array is of poor quality or not. Rather, they suggest that the factors should be similar among samples and not vary more than about 2 to 3-fold from each other.

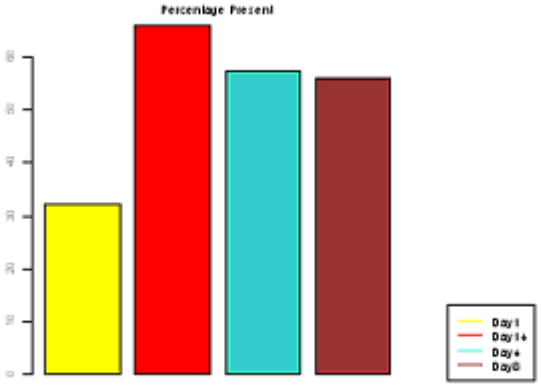
af1c1611-1-Day1-150708.CEL 5.3765979
af1c1611-2-Day4-150708.CEL 0.7799123
af1c1611-3-Day8-150708.CEL 1.0336816
af1c1611-4-Day14-150708.CEL 0.5109370



Percentage of present genes

This is a percentage of the number of probe sets called "Present" relative to the total number of probe sets on the array. This should be similar across all chips, except that in rare situations transcription is globally shut down or turned on under some conditions. Extremely low percentage values are a possible indication of poor sample quality but Percent-Present values depends on multiple factors such as cell type, probe array type, and overall quality of RNA.

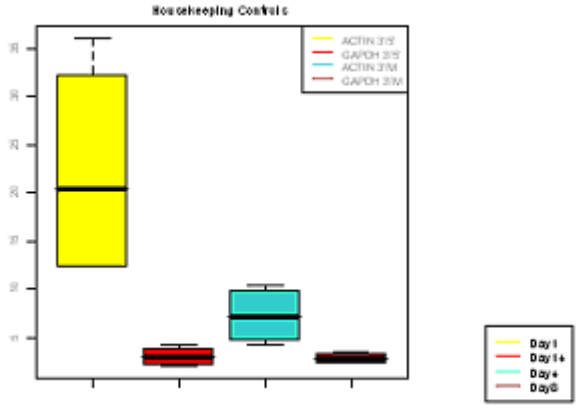
af1c1611-1-Day1-150708.CEL.present	32.09106
af1c1611-2-Day4-150708.CEL.present	57.45844
af1c1611-3-Day8-150708.CEL.present	56.04039
af1c1611-4-Day14-150708.CEL.present	66.06322



3' to 5' ratios for ß-actin and GAPDH.

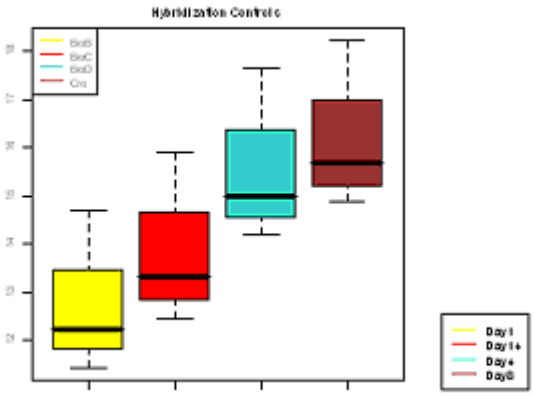
β-actin and GAPDH are used to assess RNA sample and assay quality. Signal values of the 3' probe sets for GAPDH and actin are compared to the Signal values of the corresponding 5' probe sets.The GAPDH ratio should be around 1 and the actin ratio should be less than 3. Note that if two-cycle amplification or NuGen amplification is used, this ratio could be much higher.

GAPDH ratio	
af1c1611-1-Day1-150708.CEL	3.529670
af1c1611-2-Day4-150708.CEL	2.479566
af1c1611-3-Day8-150708.CEL	3.317922
af1c1611-4-Day14-150708.CEL	2.479641
ß-actin Ratio	
af1c1611-1-Day1-150708.CEL	4.336923
af1c1611-2-Day4-150708.CEL	2.602629
af1c1611-3-Day8-150708.CEL	3.573380
af1c1611-4-Day14-150708.CEL	2.092398



Spike-in hybridization controls:

Prior to loading samples onto the arrays a number of control oligonucleotides are added and four of these are spiked into the sample mixture at different concentrations, thus allowing for later monitoring of the hybridisation performance. BioB, BioC, BioD, CreX should be called present.



af1c1611-1-Day1-150708.CEL.present	"P"
af1c1611-2-Day4-150708.CEL.present	"P"
af1c1611-3-Day8-150708.CEL.present	"P"
af1c1611-4-Day14-150708.CEL.present	"P"

A good quality chip spike-in-probes value increase roughly linearly with the logarithm of the concentration.

	AFFX-r2--Ec-bioB-3_at	**AFFX-r2-Ec-bioC-3_at**
af1c1611-1-Day1-150708.CEL	12.384276	13.56474
af1c1611-2-Day4-150708.CEL	9.879456	10.87883
af1c1611-3-Day8-150708.CEL	9.912636	11.11265
af1c1611-4-Day14-150708.CE	9.096590	10.12409

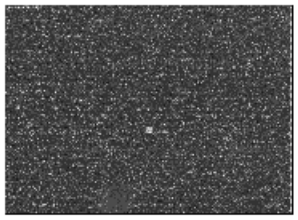
	AFFX-r2-Ec-bioD-3_at	**AFFX-r2-P1-cre-3_at**
af1c1611-1-Day1-150708.CEL	15.32423	15.90752
af1c1611-2-Day4-150708.CEL	12.55421	13.25073

af1c1611-3-Day8-150708.CEL	12.80438	13.45818
af1c1611-4-Day14-150708.CEL	11.86828	12.54202

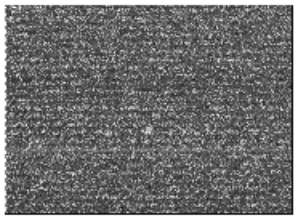
CEL Images

These plots are very useful in detecting spatial artifacts on the array surface or even in the scanner, which are very useful for controlling the quality of the array images. Each spot in the plot represents intensity values (on the log scale) measured for each probe in the physical position that the probe occupies on the array surface. In theory, these images generally should have dark and light bands. Very narrow scratches, however, are probably OK on most chips, as usually only one or two probes from a probeset will be affected. Blobs and large areas of discoloration on the chips signify poor quality.

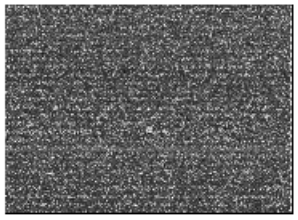
af1c1611-1-Day1-150708.CEL



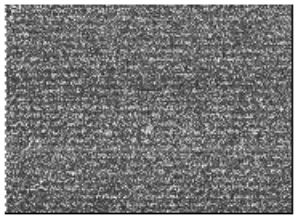
af1c1611-2-Day4-150708.CEL



af1c1611-3-Day8-150708.CEL

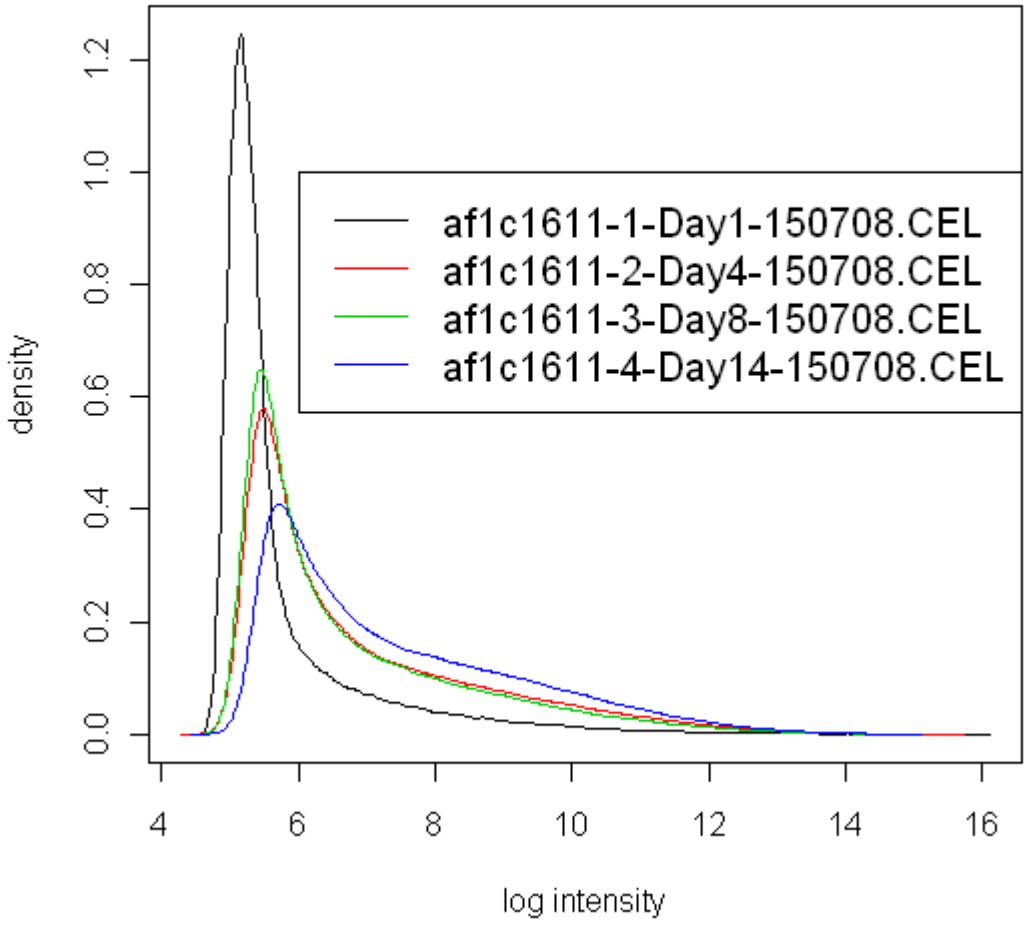


af1c1611-4-Day14-150708.CEL



Density Plot

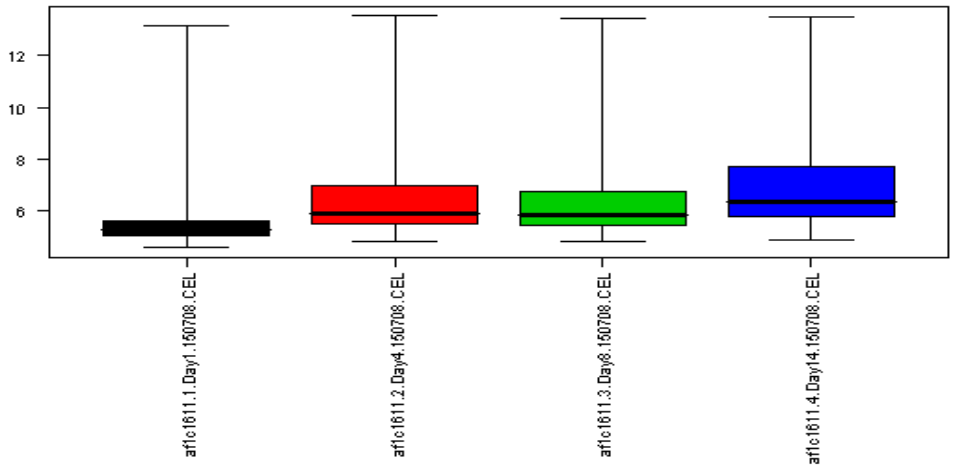
This plots the density of the PM probe intensities of each and every chip. This plot is useful to visualise differences in the distributions of the arrays.



Boxplot

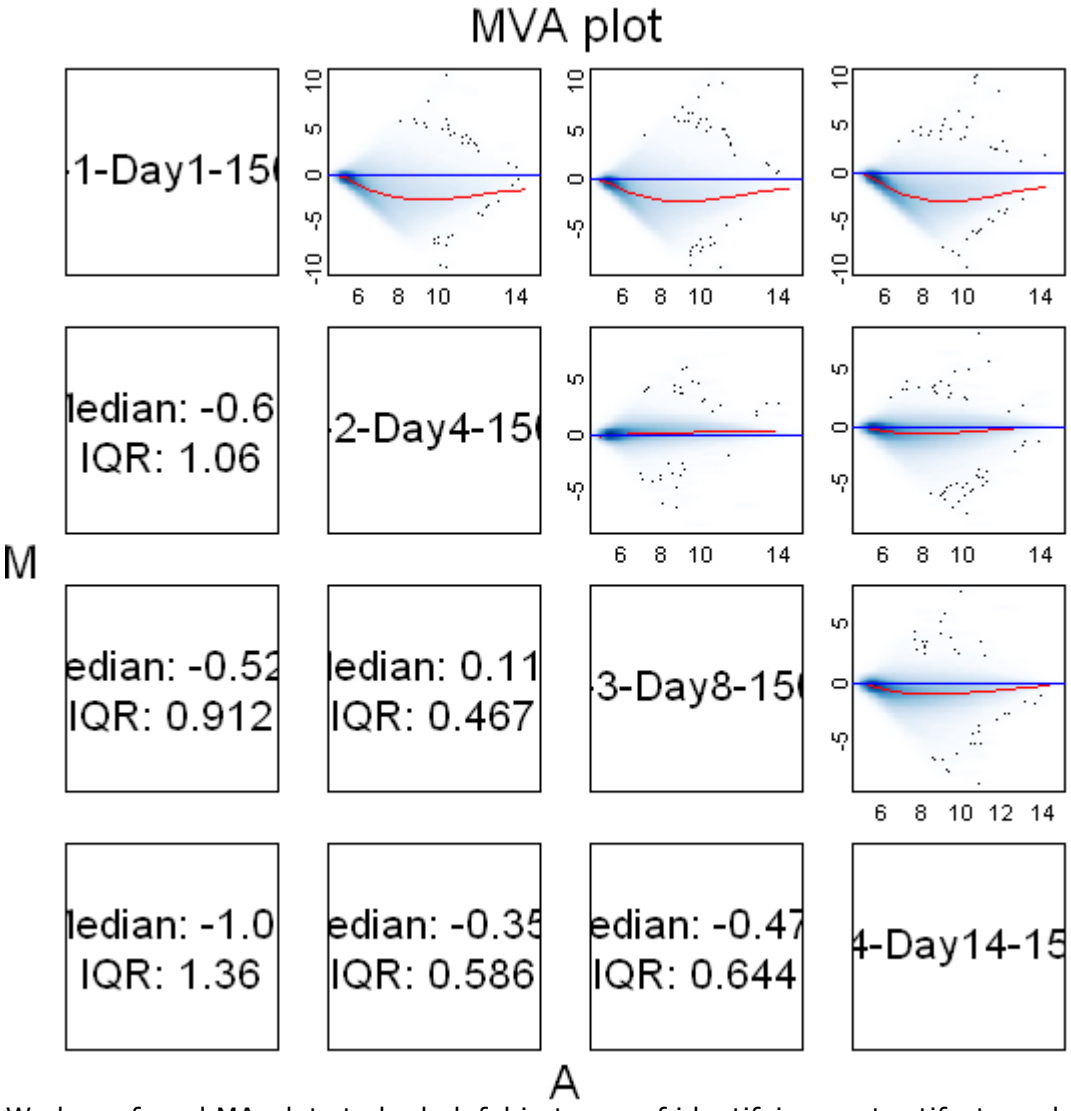
Boxplot makes it ideal as a means of comparing many samples at once, in a way that would be impossible for the histogram. Boxplots of the individual samples can be lined up side by side on a common scale, and the various attributes of the samples compared at a glance.

A Boxplot graph of the raw pm-intensities (log scale) is given for each array experiment. This plot may help us to evaluate the differences in the distributions of intensities across arrays. Above all we can distinguish if the arrays intensities are centered around the same values and estimate whether or not they have the similar dispersion. Day1 sample seems to be different from the other samples



MvA Plots

MA-plots are designed to compare two intensity measurements of raw microarray data. MA-plots represent the intensity difference (M) on the vertical axis and the average intensity (A) on the horizontal axis, usually on the logarithmic scale.

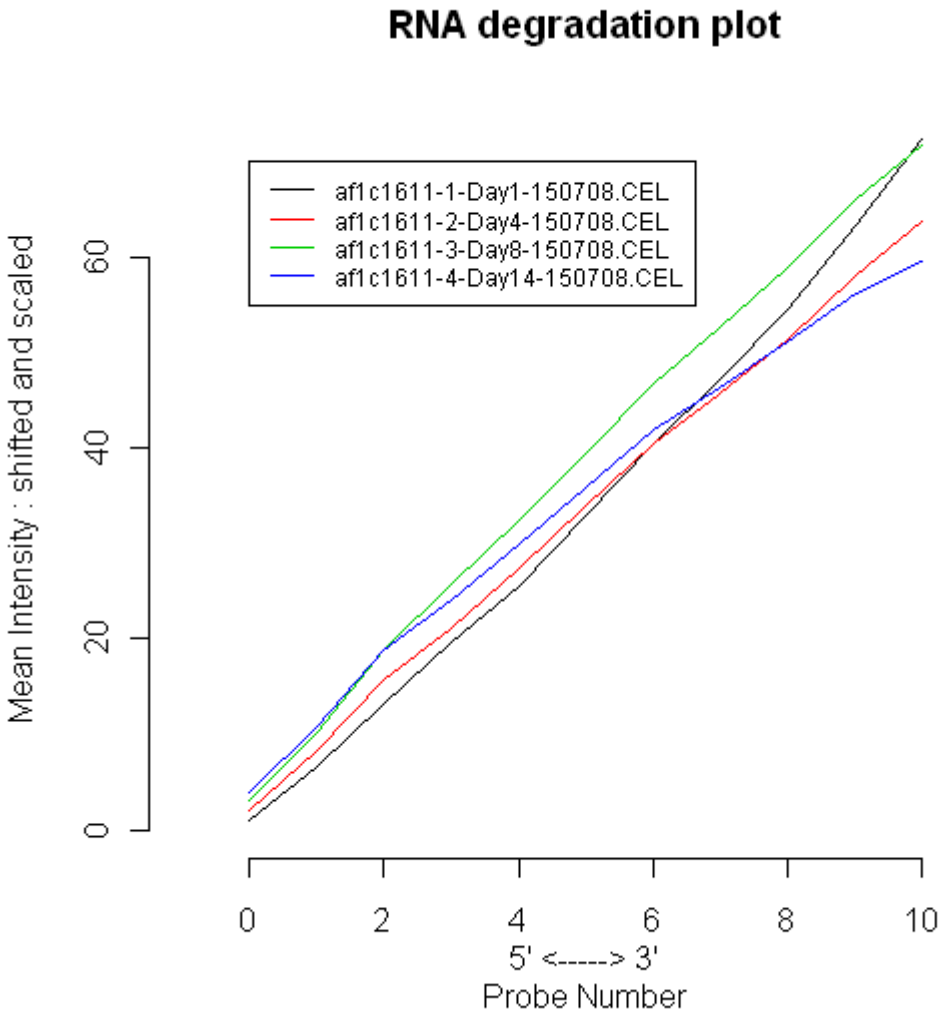


We have found MA-plots to be helpful in terms of identifying spot artifacts and detecting intensity-dependent patterns in the log ratios *M.* The red lines give a robust profile of the mean fold change as a function of average intensity. Ideally, we would expect the points in the plot to be concentrated around the horizontal axis M=0 for all values of A. Deviation from such ideal fit indicate big dependencies between the arrays that may need to be dismissed before any relevant biological information is drawn from the data. The loess curves do not deviate significantly from the M=0 axis for Day4, Day8, and Day14 chips indicating good replicate reproducibility.

RNA Degradation plot

This plot is supposed to give some idea of how much degradation of mRNA occurred, and how well the hybridisation step went. It assesses differences in the quality of the samples used in each array experiment. We should expect to find less chunks of RNA coming from 5' end than from the 3' end as degradation usually starts at the 5' end of the RNA molecule.

RNA degradation plots show expression as a function of 5'-3' position of probes. Probes are arranged by their proximity to the 5' end of the gene, for each chip. Probes matching close to the 5' end of the gene have lower intensity measures that the probes matching closer to the 3', and that is reflected in the graph.

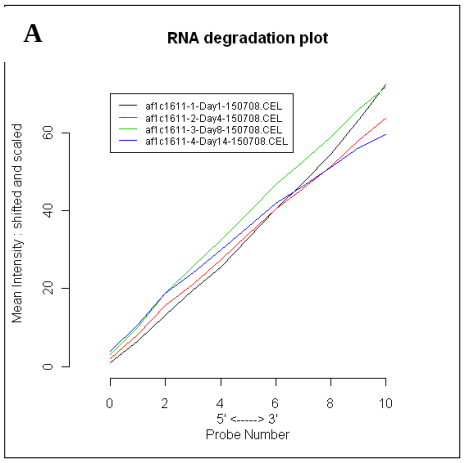


Conclusion

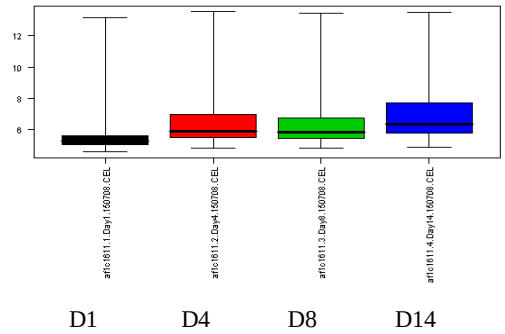
From the above QC metrics, we find that the Day1 sample is of poor quality.

1.2.1 Exclusion of Day 1 sample

A



B Boxplot- raw pm-intensities (log scale)

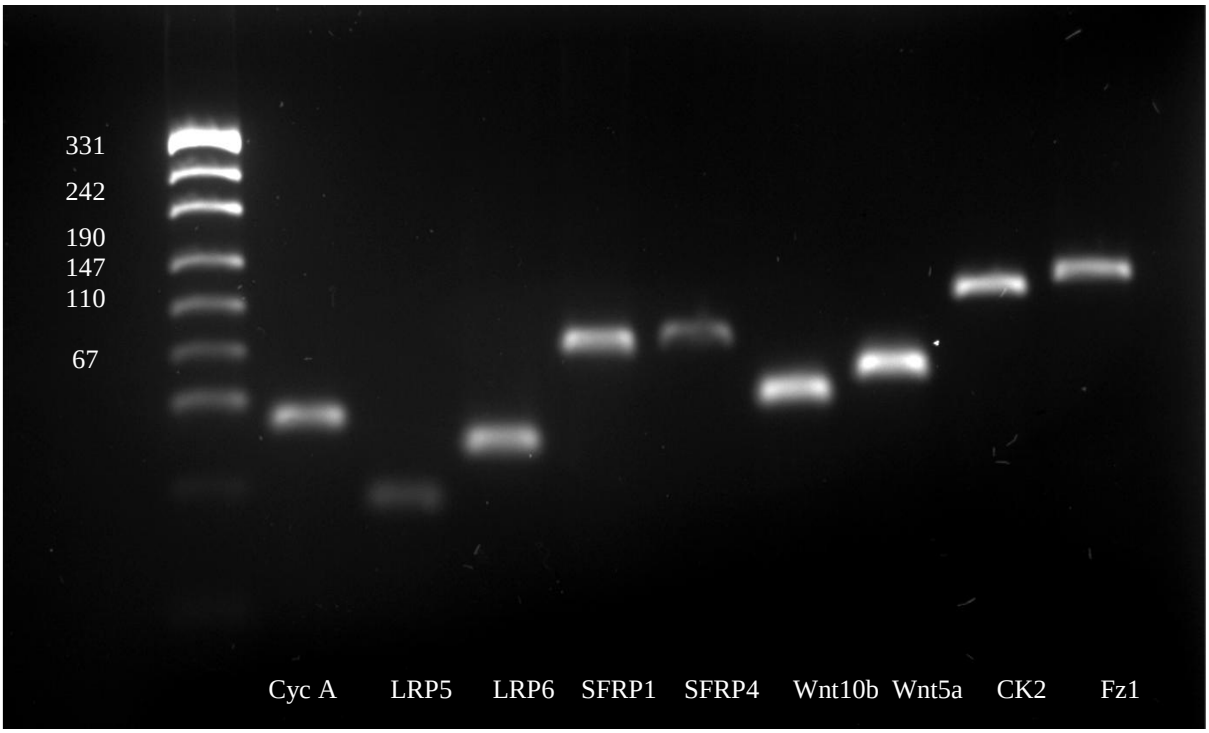


C QC metrics

Specimen	Background	Scaling Factor	Present Call (%)	GAPDH Ratio	B-actin Ratio
Day 1	27.74	5.38	32.09	3.53	4.34
Day 4	32.39	0.780	57.46	2.48	2.60
Day 8	32.25	1.03	56.04	3.32	3.57
Day 14	36.59	0.511	66.06	2.48	2.09

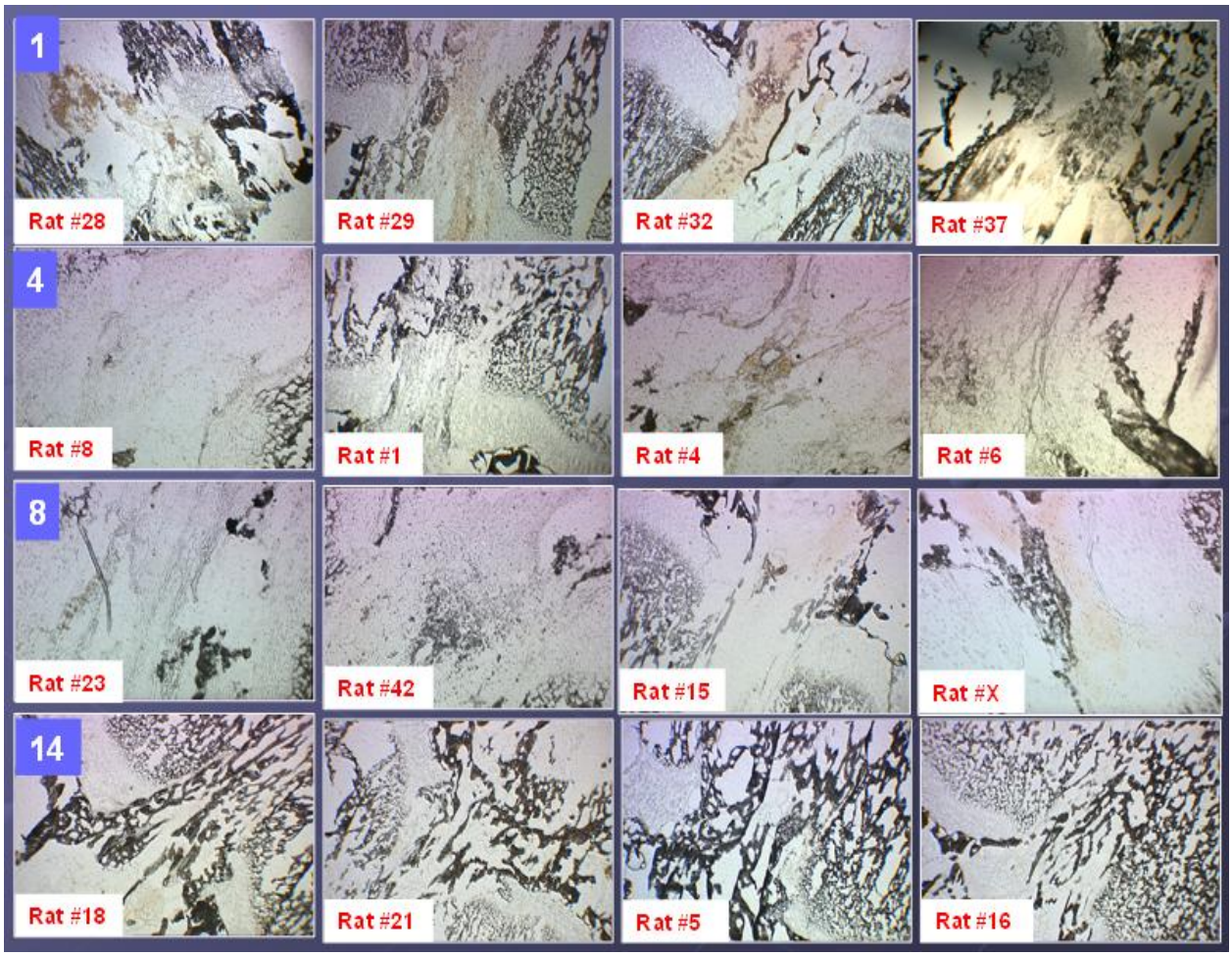
Expression data from CEL files were read into R software and quality of the hybridised chips were assessed using various diagnostic plots. Day 1 was excluded from further analysis due to poor sample quality. The RNA degradation plot (A) suggested that the RNA from Day 1 was degraded. The boxplot (B) and other QC metrics (C) also suggested that the Day 1 sample was of poor quality where values for samples Day 4, 8 and 14 were similarly dispersed, unlike the Day 1 sample. The Day 1 sample had the lowest RNA yield (Appendix 1.1), likely accounting for its poor quality. Refer to Appendix 1.2 for the complete set of QC metrics. Diagnostic plots were created from modules of the Bioconductor package, including simpleAffy, AffyExpress, Limma and Affy.

1.3 Gel electrophoresis



Gel Electrophoresis in 3 % agarose was used to visualize and identify the RT-PCR product of appropriate size using the molecular marker pUC19. Purified pUC19 DNA is digested to completion with HPA 11 restriction enzyme and is a molecular maker in the lower register, ranging from 26 base pairs, to 501 base pairs.

1.1 Laser capture microdissection images



1.5 Functions of known bone-related pathways and genes in relation to bone and cartilage formation and bone fracture repair.

Gene	Description/Function	Reference
Chrd	BMP antagonist (extracellular), binding directly to BMP-2,-4 and -7. Expression in MC3T3-E1 cells reduced bone sialoprotein and osteocalcin expression as well as ALP activity.	[475]
	Suppresses osteogenic differentiation. Knockdown in hMSC results in increased ALP expression and calcium mineral deposition.	[231]
	Activity enhanced or inhibited by twisted gastrulation (Tsg) depending on Tsg concentration.	[476, 477]
	Highly expressed in the resting zone of the growth plate.	[135]
Chrdl2	Involved in the down-regulation of BMP signalling.	
	Binds activin A and has a role in osteoblast differentiation in human tissues.	[478]
Id1	BMP2 induces transcription of Id1, to inhibit myogenesis to promote bone formation	[225, 479]
	Transuded by BMPR1A and mediated by binding of Smad1 and Smad4 to the BMP-2 responsive element in the human Id1 gene	[225, 480]
	Role in proliferation and differentiation of chondrocytes. Increased expression in MSCs forming cartilage of a mixed phenotype.	[481]
	Up-regulated in response to BMP-2, -6 and -9 expression.	[230]
BMP-6	BMP agonist expressed in growth plate, primarily in the hypertrophic zone with a role in chondrocyte maturation. Also expressed in osteoblasts (metaphysis) and osteoclasts (metaphysis, growth plate).	[134-136, 482] [134, 483]
	Functional cooperation with BMP-2, where BMP6 null mice had no effect on the newborn or adult skeleton, but when compounded with BMP $2^{+/-}$ mice, demonstrated growth retardation, decreased trabecular bone volume and suppressed bone formation.	[227-229]
	Stimulates osteogenic differentiation of MSCs.	
OPN	Bone matrix protein expressed by mature osteoblasts and osteocytes.	[20, 66]
	Found at active sites of bone metabolism. Mediates the attachment of osteoclasts to the bone surface leading to the formation of bone resorbing osteoclasts.	[65, 284, 314]
	Secreted by hypertrophic chondrocytes, forming part of the growth plate matrix.	[42]
	Negatively regulates hydroxyapatite formation.	[58]
	Increased expression in response to mechanical stress, during fracture healing and distraction osteogenesis.	[484-486]
OCN	Expressed by mature osteoblasts and recruits osteoclasts and/or osteoclast precursors for bone resorption, however, an absence does not impair bone resorption.	[19, 58, 59]
	The interactions of ATF4 and Runx2 and their binding to their sites on the osteocalcin promoter regulate osteocalcin mRNA levels and thus bone formation.	[255]
	Secreted by hypertrophic chondrocytes, forming part of the growth plate matrix.	[42]
	Binds calcium and hydoxyapatite and regulates the rate of mineralisation.	
	Up-regulated during bone fracture healing.	[58, 487]

Traf6	Initiates Rank signalling by binding within the cytoplasmic domain of Rank and initiating the expression of critical genes that regulate osteoclast differentiation and activation.	[488]
	In the cytoplasm, acts as a stimulator of osteoclast formation and activation, however in the nucleus, negatively regulates osteoclastogenesis.	[489]
FGFR1	Expressed in mesenchyme, hypertrophic chondrocytes, osteoblasts and osteoclasts.	[116, 490, 491]
	Negative regulator of skeletal growth, where enhanced activity causes Pfeiffer syndrome or osteoglophonic dysplasia, depending on the mutation.	[492, 493]
	Negatively regulates chondrogenesis. Expressed late in chondrocyte differentiation, initiating vascular invasion and ossification in the hypertrophic zone.	[116, 491]
	Essential for the differentiation and activation of osteoclasts in mice, where deletion results in decreased osteoclast number, activity and expression of TRAP and Mmp9 (osteoclast activity).	[494]
BMP-1	A protease that cleaves procollagen fibrils as well as the chordin/BMP complex, inactivating chordin and releasing BMP. Immunolocalised in the growth plate, primarily in late hypertrophic chondrocytes. Also expressed in osteoblasts (metaphysis) and osteoclasts (metaphysis, growth plate).	[136, 495]
BMP-2	BMP-2 null mice are embryonic lethal. BMP-2 $^{+/-}$ mice have a normal phenotype but when compounded with BMP-6 null mice, demonstrated growth retardation, decreased trabecular bone volume and suppressed bone formation.	[134, 483]
	Crucial for the initiation of bone healing. Absence causes spontaneous bone fractures which fail to heal. MSCs fail to differentiate and other BMPs are unable to compensate.	[401]
	BMP agonist immunolocalised at very early stages of fracture repair. Intense staining in primitive MSCs, chondrocytes, in osteoblasts laying down woven bone and osteoclasts, during enodchondral and imtramembranous ossification.	[388, 391]
	Immunolocalised in osteoblasts in human fracture tissue and used clinically to treat bone fractures.	[138, 496]
	Significant increase in expression following distraction osteogenesis (mechanical stretching).	[497]
	Time-course analysis of bone bridge formation revealed greatest mRNA expression at day 7 in the growth plate injury site.	[365]
	Expressed in growth plate at highest levels in hypertrophic zone, to induce endochonral bone formation.	[134, 135]
	Interacts closely with the Wnt signalling pathway. Inhibits multipotent cells from myogenic differentiation, stimulating osteoblast differentiation, via β-catenin Tcf-dependent transcription. BMP regulates ALP expression in MSCs indirectly via Wnt signalling.	[471, 472]
	BMP-2 regulates β-catenin levels by increasing the mRNA expression of Wnts and LRP and Fz receptors in MSCs and osteoblasts and by preventing the degradation of β-catenin.	[470, 472, 474]
Hgn	Nuclear protein of unknown function however is specifically expressed in undifferentiated hematopoietic cells.	[498] [499]
	Direct transcriptional target of HOXB4, promoting expansion of myeloid	

	progenitor cells.	
BMP-7	Expressed in growth plate, highest in proliferative and hypertrophic zones. Also expressed in osteoblasts and osteoclasts in the metaphysis.	[135, 136]
	Regulates bone formation by inducing MSCs to differentiate into osteoblasts, stimulating osteocalcin expression in mature osteoblasts and mineralised bone matrix formation.	[227, 500, 501]
	Immunolocalised in osteoblasts in human fracture tissue and clinically used to induce new bone formation in fracture repair.	[138, 496]
	Immunolocalised during early stages of fracture repair, during endochondral ossification. Expressed by osteoclasts in newly formed bone.	[391]
	Expressed predominantly in differentiated osteoblasts in the growth plate injury site and may play a role in bone remodelling/formation/resorption during bony repair.	[365]
Mmp9	Expressed by several cell types at the base of the growth plate and controls hypertrophic cartilage vascularisation and ossification. Endochondral ossification is delayed in the absence of Mmp9 in mice.	[76] [502]
	High expression in osteoclasts during mouse development.	
	Expression is important in regulating osteoclast activity.	

APPENDIX 2 – Personal Publications & Conference Abstracts

ROLES OF WNT SIGNALLING IN BONE GROWTH, REMODELLING, SKELETAL DISORDERS AND FRACTURE REPAIR

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STATMENT OF AUTHORSHIP

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2.2 Personal publications (2005-2012)

Journal Articles:

1. **Macsai CE**, KR Georgiou, BK Foster, AW Zannettinno, CJ Xian. (2012). Microarray expression analysis of genes and pathways involved in growth plate cartilage injury responses and bony repair. *Bone. May; 50(5): 1081-91.*

2. **Macsai CE**, Hopwood B, Chung R, Xian CJ (2011). Structural and molecular analyses of bone bridge formation within the growth plate injury site and cartilage degeneration at the adjacent uninjured area. *Bone.* Oct; 49(4):904-12.

3. **Macsai CE**, Foster BK, Xian CJ. (2008). Roles of Wnt signalling in bone growth, remodelling, skeletal disorders and fracture repair. *J Cell Physiol*. Jun; 215(3):578-87. Review.

4. Xian CJ, JC Cool, M Scherer, **CE Macsai**, M Covino, CM Fan, and BK Foster (2007). Mechanisms for methotrexate chemotherapy-induced bone growth arrest and osteopenia and protective effects of antidote folinic acid supplementary treatment. *Bone.* Nov; 41(5):842-50.

Book Chapters:

1. Cory J Xian, Rosa Chung, **Carmen E Macsai**, and Bruce K Foster (2008). Injury responses and repair mechanisms at the growth plate cartilage. Chapter in a review book; "Research Advances in Histochemistry and Cytochemistry. Published by Global Research Network, 2008.

2.3 Conference presentations

1. Wong DS, Chung R, **Macsai CE**, and Xian CJ. Inhibition of Wnt-β-catenin signalling promotes cartilage repair at injured growth plate in young rats. *ANZBMS*, Perth (2012).

2. R Chung, RC McCarty, FH Zhou, **CE Macsai**, S Gronthos, AC Zannettino, D Wong, BK Foster, Cory J Xian (2011). Pathophysiology for the Faulty Bony Repair of the Injured Growth Plate Cartilage and Potentials and Challenges for Cartilage Regeneration. *World Congress on Biotechnology 2011*, Hyderabad, India.

3. **Macsai CE**, B Hopwood, AC Zannettino, MA Scherer, BK Foster, & CJ Xian (2009). Micro-CT and micro-array analyses of growth plate cartilage injury responses and bony repair. *International Bone Mineral Society* 2009, Sydney, Australia.

4. Cory J Xian, FH Zhou, R Chung, **Macsai C**, M Scherer, BK Foster (2008). The injury-induced inflammatory response modulates downstream bony repair events in the injured growth plate cartilage. *International Conference on Osteoporosis and Bone Research* 2008, Beijing, China (Oral presentation).

5. **Macsai CE**, MA Scherer, BK Foster, A Zannettino and CJ Xian (2007). Expression of wnt signaling molecules at the injured growth plate cartilage in young rats. *European Calcified Tissue Society Scientific Conference 2007*. Copenhagen, Denmark.

6. Xian CJ, JC Cool, **CE Macsai**, M Covino, M Scherer, CM Fan, and BK Foster (2006). Mechanisms for methotrexate chemotherapy-induced bone growth defects. *European Calcified Tissue Society Scientific Conference 2006*. Prague, Czech Republic.

7. Xian CJ, JC Cool, **CE Macsai**, M Scherer, CM Fan, M Covino, and BK Foster (2006). Cellular mechanisms for methotrexate chemotherapy-induced bone growth arrest and osteoporosis. *Combined 3rd International Osteoporosis Foundation and ANZ Bone Mineral Society Scientific Conference*. Port Douglas, Qld.

8. Xian CJ, JC Cool, **CE Macsai**, M Covino, M Scherer, CM Fan, and BK Foster (2006). Mechanisms for methotrexate chemotherapy-induced bone growth defects. *2006 Clare Bone Scientific Meeting*, Clare, SA.

9. MA Scherer, **CE Macsai**, JC Cool, RC McCarty, BK Foster, S Gronthos and CJ Xian (2006). Short term methotrexate chemotherapy affects the size of the osteoprogenitor cell pool in the bone marrow of young rats. *2006 Clare Bone Scientific Meeting*, Clare, SA.

10. **Macsai CE**, M Scherer, BK Foster, A Zannettino, and CJ Xian (2006). mRNA expression of Wnt signalling molecules in the injured growth plate cartilage in rats. *2006 Clare Bone Scientific Meeting*, Clare, SA.

11. **Macsai CE**, M Scherer, BK Foster, A Zannettino and CJ Xian (2006). Expression of Wnt signalling molecules in the injured growth plate cartilage in rats. *2006 ASMR-SA Scientific Meeting*, Adelaide, SA (Oral presentation).

12. Ngo, T, Scherer, M, Zhou, FH, **Macsai, CE**, Foster, BK and Xian, CJ (2005). Expression of bone morphogenetic proteins and receptors at the injured growth plate cartilage in young rats. 29th *ANZ Matrix Biology Conference 2005*, Victor Harbor, South Australia.

13. Xian, CJ, Cool, J, Covino, M, **Macsai, CE** and Foster, BK (2005). Bone growth arrest and osteoporosis in young rats after acute chemotherapy with anti-metabolite 5-fluorouracil. 29th *ANZ Matrix Biology Conference 2005*, Victor Harbor, South Australia. (Oral presentation).

14. **Macsai CE**, M Scherer, J Cool, C Fan, R McCarty, S Gronthos, B Foster, CJ Xian (2005). Short term methotrexate chemotherapy affects proliferation of bone marrow osteoprogenitor cells. 29th *ANZ Matrix Biology Conference 2005*, Victor Harbor, South Australia. (Oral presentation).

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