The Effect of Recombinant Human Osteogenic Protein-1 on Growth Plate Repair in a Sheep Model

by

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Thesis Summary

The growth plate is a unique cartilaginous structure located towards the ends of children's long bones. It is responsible for the longitudinal growth of the bones through the proliferation, maturation and hypertrophy of chondrocytes, the specialised cells of the growth plate. The growth plate has a limited ability to undergo repair, and damage to it often results in limb angulation deformities and/or growth arrest due to the formation of a bone bridge spanning the growth plate region. The Langenskiöld procedure is a surgical treatment for growth plate injuries that involves the removal of the bone bridge and replacement with autologous fat. This prevents further bone formation and allows future growth of the bone.

Osteogenic protein-1 (OP-1) is a member of the transforming growth factor beta (TGF-β) superfamily and is well known for its ability to promote bone formation in diaphyseal defects. However, more recent research has demonstrated that OP-1 can also promote the proliferation of chondrocytes and the synthesis of a cartilage matrix both *in vitro* and *in vivo*. This growth factor has been successfully used to enhance healing of articular cartilage and thyroid defects *in vivo*.

The aim of this study was to further investigate the histological and molecular changes that occur to the remaining growth plate and defect site following the Langenskiöld procedure in the presence and absence of recombinant human OP-1 (rhOP-1). A sheep model was utilised in which a section of the growth plate was ablated and filled with autologous fat. Half of the animals had rhOP-1 (350 μg) injected at the interface of the remaining growth

plate and the defect. The animals were sacrificed in triplicate at days 7, 14 and 56, and the tissues processed for histological and molecular characterisation.

Following the Langenskiöld procedure, the total limb growth continued at an equivalent rate in both the rhOP-1 treated and untreated groups compared to the normal animals that did not undergo the Langenskiöld procedure. There were no differences in the formation of bone, medial spurs or fibrocartilage observed between the rhOP-1 treated and untreated groups. However, the rhOP-1 treated animals displayed limited fibrous capsule formation around the fat implant compared to the untreated animals. The animals that were treated with rhOP-1 did show a significant increase in the height of the growth plate adjacent to the defect compared to the height at the most distal aspect. The majority of the growth plate height increase was observed within the resting zone, and as there was no significant change in the number of cells present within the area, this was attributed to an increase in extracellular matrix synthesis by the resting chondrocytes.

Immunohistochemical analysis demonstrated that the growth plate adjacent to the defect displayed molecules consistent with the cartilage phenotype, including collagen types II and X, biglycan and glycosaminoglycan epitopes from chondroitin sulphate, chondroitin-4-sulphate and keratan sulphate. The presence of these molecules in both groups suggests that rhOP-1 does not have an adverse effect on molecules indicative of the chondrogenic phenotype. However, the expression of type I collagen, osteopontin and decorin was detected in the chondrocytes adjacent to the defect in the rhOP-1 treated animals at day 7 and the untreated animals at day 56. These molecules are usually indicative of an osteogenic phenotype and suggest a modulation of chondrocyte phenotype within the growth plate. These molecules were detected in both the rhOP-1 treated and untreated

groups suggesting that the phenotypic switch was not a direct result of the rhOP-1 treatment. Rather, treatment with rhOP-1 accelerated the response, with the molecules appearing at day 7 compared to day 56.

In conclusion, administration of rhOP-1 in conjunction with the Langenskiöld procedure initiated a complex response in the growth plate adjacent to the defect. There was a significant increase in growth plate height, suggesting this growth factor may be beneficial in regenerating the growth plate following injury. However, rhOP-1 also accelerated the osteogenic response that was observed in the untreated animals. Therefore, the use of rhOP-1 in the treatment of growth plate injuries may be of limited value. The osteogenic properties of this growth factor have the potential to cause accelerated bone formation due to the osteogenic phenotype the growth plate chondrocytes adopted.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution 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.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Belinda Jane Thomas

February 2004

This thesis is dedicated to the memory of

David Roy Thomas,

Frederick George Moll

and

Amelia Lousie Rix

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Abbreviations

APES	3-aminopropyltriethoxysilane
bp, kb	base pairs, kilo base pairs
BSA	bovine serum albumin
°C	degrees Celsius
dH_2O	deionised water
DIG	digoxigenin
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbant assay
g, mg, µg, ng	grams, milligrams, micrograms, nanograms
K-wires	Kirschner wires
L, ml, µl	litre, millilitre, microlitre
MGP	matrix γ-carboxyglutamic acid protein
mm, µm	millimetre, micrometre
M, mM	moles per litre, millimoles per litre
µCi	micro Curie
OD	optical density
opm	oscillations per minute
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pH	hydrogen ion concentration
% (w/v)	percent weight per volume
% (v/v)	percent volume per volume
rhOP-1	recombinant human osteogenic protein-1
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dedecyl sulphate
SSC	saline sodium citrate
Tris	Tris (hydroxymethyl) amino methane
U	units
UV	ultra violet
V	volts

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Chapter 1

Introduction and Literature Review

1.1 Summary of Project Introduction

The growth plate is a unique cartilaginous structure located at both ends of children's long bones until skeletal maturity is reached. This structure is responsible for the elongation of bone through the proliferation, maturation and hypertrophy of chondrocytes, the specialised cells within the growth plate.

Damage to the growth plate through sporting and playground injuries or traffic accidents are a significant causal factor in abnormalities of bone length and angulation. Injuries to the growth plate can result in the formation of bone across the growth plate that limits further growth of the limb. The current treatment for limb length discrepancies is mechanistic, correcting the angulation or length discrepancy after the deformity has occurred. The Langenskiöld procedure is a surgical treatment for removing and preventing the reformation of bone at the site of growth plate injury. However, this procedure often fails due to incomplete resection or reformation of the bone bridge. Recent advances delineating the regulation of growth plate function have led to the belief that biological repair mechanisms may be possible.

Osteogenic protein-1 (OP-1), a member of the transforming growth factor-beta (TGF-β) superfamily, has been shown to stimulate cartilage formation at sites of injury. Previous research within our laboratory has demonstrated that the addition of recombinant human OP-1 (rhOP-1) into growth plate defects can promote expansion of the remaining growth plate. However, this expansion was restricted due to the formation of bone within the injury site.

The focus of this study was to use rhOP-1 in conjunction with the Langenskiöld procedure to promote the regeneration of a functional growth plate following injury. Histological and molecular studies of the remaining growth plate and the injury site were conducted.

1.2 Structure of Developing Bones

The bones of the body play several fundamental roles, including structural support, protection of the body's internal organs (Recker, 1992; Price *et al.*, 1994), and to facilitate movement of parts of the body (Price *et al.*, 1994). At the molecular level, bone serves as a reservoir for calcium, phosphorus, sodium, magnesium and carbonate, factors critical to the maintenance of normal cell and tissue function (Recker, 1992, Price, 1994 #5). In addition, the bone also provides a site for haematopoiesis (blood cell production; (Price *et al.*, 1994)).

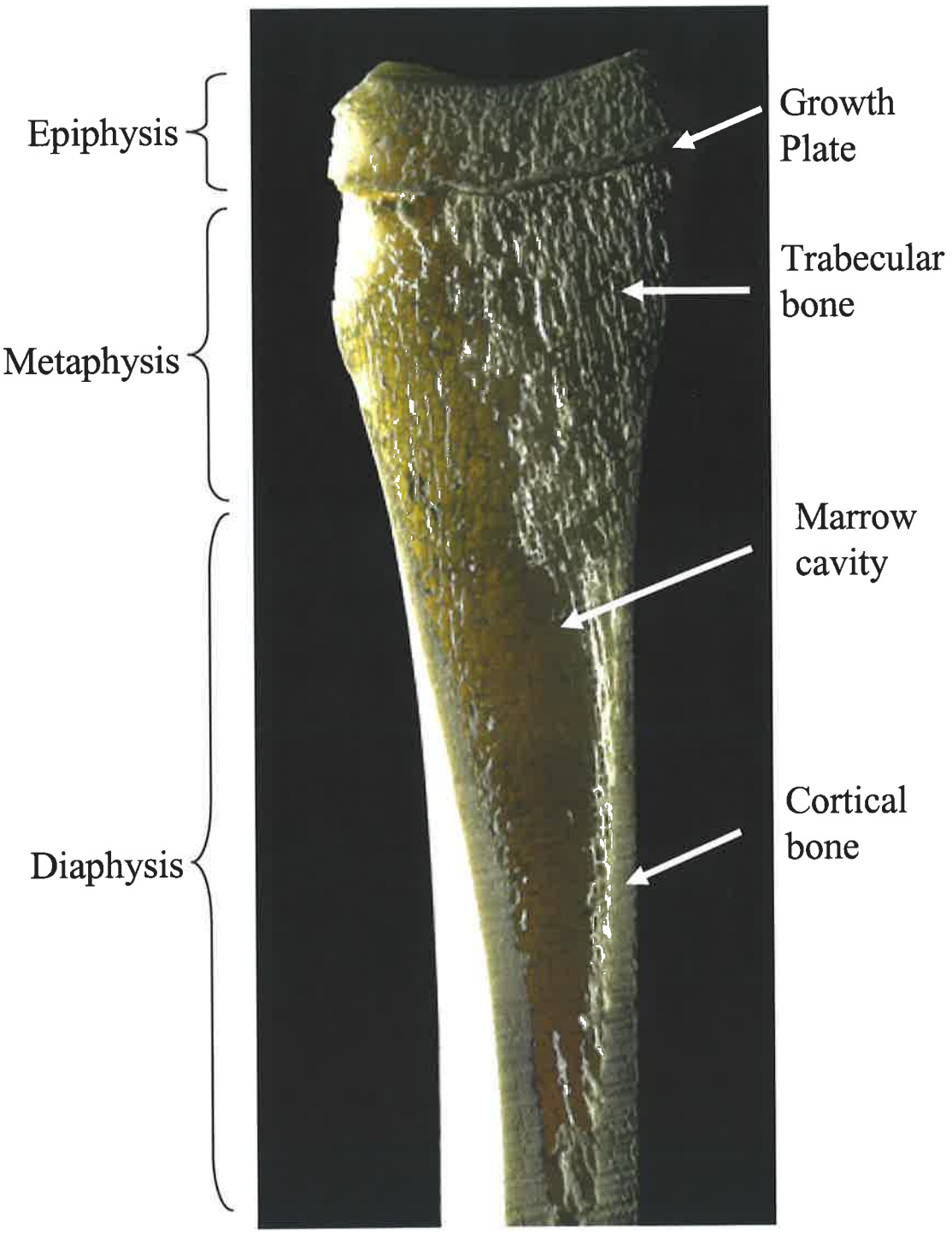
1.2.1 Anatomy of Bones

The main components of developing bones are the epiphysis, growth plate (also known as the physis), metaphysis and diaphysis (Fig. 1.1). Each region has specific functions that contribute towards the formation and growth of bones, as discussed below.

The articular cartilage of the epiphysis is located at the ends of the long bones to provide the joints with a low friction surface, and together with lubrication provided by the synovial fluid, it provides wear characteristics required for a continuous gliding motion (Mankin *et al.*, 1994). Articular cartilage functions to absorb mechanical shock and distributes the applied load onto the bony supporting structures below (Muir, 1995). The primary structure of articular cartilage is a large extracellular matrix with a sparse

Figure 1.1: A human proximal tibia showing the major anatomical regions of a long bone. The growth plate is located between the epiphysis and metaphysis. Examples of trabecular and cortical bone are also shown.

Photo kindly provided by Dr. Krystyna Zarrinkalam.


Epiphysis
Metaphysis
Diaphysis
Growth Plate
Trabecular bone
Marrow cavity
Cortical bone

population of chondrocytes, with the main components being type II collagen, aggrecan and water (Muir, 1995). Also within the epiphysis is the secondary centre of ossification, which is important in determining the shape and form of the joint surface (Iannotti *et al.*, 1994).

The growth plate is a cartilaginous structure located distal to the epiphysis that is responsible for the longitudinal growth of the long bones in children. The specialised cells of the growth plate, the chondrocytes, undergo a tightly controlled proliferation and maturation process that produces the calcified cartilage onto which bone is deposited. This will be discussed in detail in section 1.3.

Immediately below the growth plate the mineralised cartilaginous matrix is replaced by bone to form the primary spongiosa of the metaphysis (Iannotti, 1990; Recker, 1992; Price *et al.*, 1994). This bone is remodelled by the synchronised process of osteoclastic resorption and osteoblastic bone formation and continues into the diaphysis. The process of funnelisation results in the narrowing of the diameter of the metaphysis to meet the diaphysis of the bone (Brighton, 1978; Iannotti, 1990; Recker, 1992).

The diaphysis is the major portion of the bones and is where the marrow cavity forms (Iannotti, 1990). During development, the cells around the diaphysis differentiate into osteoblasts and begin to form the periosteum, a collar of bone surrounding the diaphysis. Blood vessels penetrate to gain access to the calcified cartilage and bring osteoclasts along with elements that will form marrow (Recker, 1992). The marrow cavity is an important source of haematopoietic-derived bone cells, including osteoclasts (Buckwalter *et al.*,

1995), and the blood vessels in the marrow form a critical part of the circulatory system in bone.

Surrounding the growth plate of the long bones are the perichondrial ring of LaCroix and the groove of Ranvier. The perichondrial ring functions to limit the lateral growth of the growth plate and provides strong mechanical support at the bone-cartilage junction to resist compressive forces across the growth plate (Shapiro *et al.*, 1977; Iannotti, 1990). The groove of Ranvier flows into and merges with the resting zone of the growth plate, and contributes to the latitudinal growth of the growth plate (Iannotti *et al.*, 1994).

1.2.2 Composition of Bone

Bone is composed of an organic phase (approximately 23% of the dry weight of bone), which gives bone its form and contributes to its ability to resist tension, and an inorganic phase (approximately 77% of the dry weight of bone), which primarily resists compression (Buckwalter *et al.*, 1995). Collagen, predominantly type I, comprises 90% of the organic matrix while the remaining 10% is a mixture of non-collagenous glycoproteins and bone-specific proteoglycans with functions that may influence matrix organisation, mineralisation, or the behaviour of bone cells (Recker, 1992; Buckwalter *et al.*, 1995). The inorganic phase of bone has two functions. Firstly, it acts as an ion reservoir, maintaining the extracellular fluid concentrations within the necessary ranges critical for biochemical reactions and physiological functions, including nerve conduction and muscle contraction. Secondly, the calcium-phosphate crystals provide bone with its strength and rigidity (Buckwalter *et al.*, 1995).

1.2.3 Types of Bone

At the microscopic level, two forms of bone are present: woven and lamellar. Woven bone is the immature or primitive form and is found in the embryo, the newborn, the fracture callus and the metaphysis of the growing bones. Lamellar bone is the mature form and begins forming about one month after birth. By one year of age, woven bone has been resorbed and replaced by lamellar bone (Singh, 1978; Recker, 1992).

Structurally, the woven and lamellar types are combined to form trabecular (spongy or cancellous) and cortical (dense or compact) bone. Trabecular bone has a greater surface area than cortical bone, and is found in areas including the metaphysis and epiphysis. The spicules of trabecular bone are arranged to form a three dimensional branching lattice which is aligned along areas of physical stress. Cortical bone has four times the density of trabecular bone and forms an outer collar around trabecular bone and the marrow cavity. Cortical bone is subject to greater bending and torsional forces as well as compressive forces (Recker, 1992; Price *et al.*, 1994; Buckwalter *et al.*, 1995).

1.2.4 Mechanisms of Bone Synthesis

Bone is produced via two distinct pathways, intramembranous ossification and endochondral ossification. Intramembranous ossification occurs at the periosteal surfaces of all bones, as well as in parts of the skull, pelvis and clavicles. This process begins with the condensation of nests of primitive mesenchymal cells and their differentiation into osteoblasts. These osteoblasts secrete osteoid, the protein matrix of bone, and bury themselves within the matrix, which then calcifies. The collagen fibrils of the matrix are randomly arranged, and the resulting bone formed is woven bone. The woven bone engulfs

blood vessels and organises itself into broad, flattened plates. Concurrently, the woven bone is replaced by highly organised lamellar bone (Iannotti, 1990; Recker, 1992).

Endochondral ossification describes bone that arises from a cartilaginous template and occurs within the growth plates and fracture callus. The primary ossification centre of a long bone develops in the centre of what will become the diaphysis of the bone. The cartilage within this area begins to calcify and the chondrocytes die, and at the same time cells within the collar around the diaphysis differentiate into osteoblasts and form the bone of the periosteum. Blood vessels penetrate the periosteum, bringing osteoclasts and osteoblasts to remove and replace the calcified cartilage with lamellar bone. At the time of birth, a secondary ossification centre appears separating the growth plate from the articular cartilage. The growth plate continues to form the calcified cartilage template for bone formation until skeletal maturity when the growth plate closes and no longer produces calcified cartilage (Iannotti, 1990; Recker, 1992).

Crucial to bone formation are the cells that synthesise and maintain the bone matrix. The various cells involved with bone formation and remodelling are derived from either a mesenchymal cell lineage (osteoblasts, osteocytes, bone-lining cells and chondrocytes) or a haematopoietic stem cell lineage (osteoclasts). These specialised cell types are different in morphometry, function and location, and are all required for the diverse functions of bone formation, resorption and repair. The undifferentiated mesenchymal cells reside within the bone canals, endosteum, periosteum and marrow, and have an irregular form, with a single nucleus, minimum cytoplasm and relatively few organelles (Buckwalter *et al.*, 1995). The haematopoietic stem cells are found within the marrow or circulating blood (Buckwalter *et*

al., 1995). It is the interaction of these different cell types that is responsible for the formation and maintenance of bones (Fig. 1.2).

1.2.4.1 Bone-lining cells

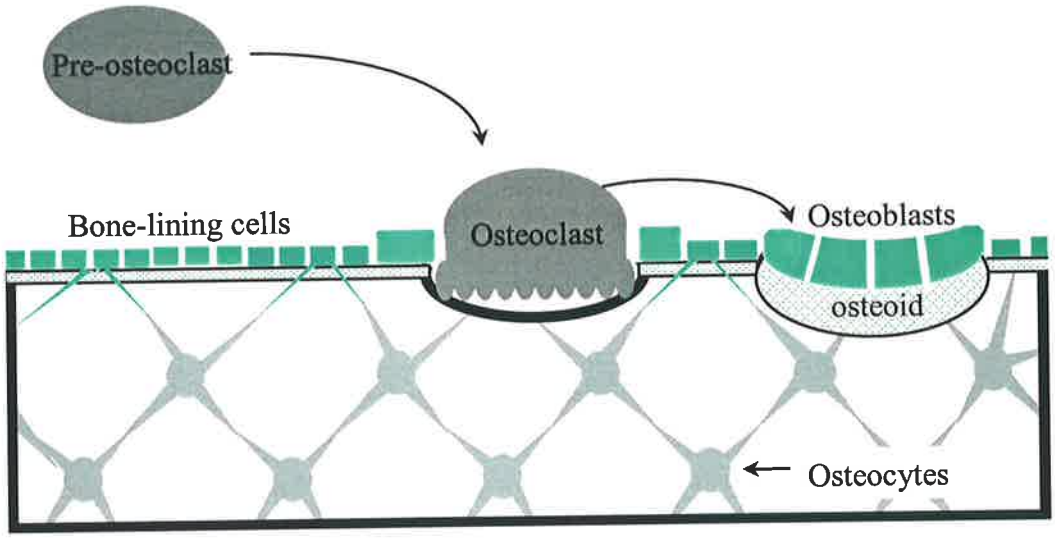
Bone-lining cells are also referred to as resting osteoblasts or surface osteocytes, and are found lying directly against the bone surfaces that are not undergoing bone formation or resorption (Marks Jr and Hermey, 1996). These cells have an elongated or flattened form, and have cytoplasmic extensions that penetrate the bone matrix and come into contact with the cytoplasmic extensions of osteocytes. The bone-lining cells are sensitive to parathyroid hormone (PTH), and exposure to PTH results in the cells contracting and secreting collagenases that are capable of removing the thin layer of osteoid that covers the mineralised matrix (McSheehy and Chambers, 1986). This is the first step in allowing osteoclasts to attach to the bone surface and begin resorption. Therefore, bone-lining cells play a role in attracting osteoclasts to specific sites and stimulating them to begin bone resorption (Buckwalter *et al.*, 1995).

1.2.4.2 Osteoblasts

Osteoblasts are the cells responsible for the formation and organisation of the extracellular matrix of bone and its subsequent mineralisation (Price *et al.*, 1994; Buckwalter *et al.*, 1995). Osteoblasts line the surface of bone and synthesise and secrete the organic matrix of bone. When in their active form, they are rounded and oval in shape and are separated from the mineralised matrix by an osteoid seam (Buckwalter *et al.*, 1995). At the conclusion of bone formation, the osteoblasts take one of three routes. Firstly, they can remain on the bone surface, decrease their synthetic activity and take on the flatter appearance of bone-lining cells. Secondly, they can surround themselves with matrix and become osteocytes,

Figure 1.2: Schematic representation of the bone cells responsible for the formation and maintenance of bone. The bone-lining cells are found lying directly against the bone surface and degrade the osteoid of the bone, allowing the osteoclasts to attach and begin resorption. The cytoplasmic extensions of these cells extend into the bone matrix and interact with the osteocytes. Osteoclasts attach to the bone and secrete enzymes to resorb the bone, releasing TGF-β that results in the differentiation and activation of the osteoblasts. The osteoblasts line the surface of the bone and secrete the organic matrix of bone. The osteocytes within the bone are thought to act as mechanosensory devices that determine the stress levels within the bone.

Adapted from (Johnstone and Foster, 2001).


Pre-osteoclast
Bone-lining cells
Osteoclast
Osteoblasts
osteoid
Osteocytes

or thirdly, they can be lost from the bone surface (Recker, 1992). Osteoblasts are responsive to PTH, which together with activity of local cytokines, results in stimulation of the osteoblasts to release mediators that activate osteoclasts (Rodan and Martin, 1981).

1.2.4.3 Osteocytes

Osteocytes are mature osteoblasts that are responsible for the maintenance of the bone matrix in which they reside. They account for more than 90% of bone cells, and are capable of synthesising and resorbing matrix (Cooper *et al.*, 1966). Osteocytes have a single nucleus and long, branching cytoplasmic extensions that project through the mineralised matrix to come in contact with the cytoplasmic extensions of other osteocytes and cells, including bone-lining cells. Due to the limited ability of nutrients and metabolites to diffuse through the mineralised matrix, these cytoplasmic extensions allow communication throughout the matrix (Reinholt *et al.*, 1990; Buckwalter *et al.*, 1995).

1.2.4.4 Osteoclasts

Osteoclasts are large multinucleated cells that arise from mononuclear precursors that are stimulated to proliferate and fuse (Buckwalter *et al.*, 1995). The distinctive feature of the osteoclast is a complex folding of the cytoplasmic membrane at the attachment site to the bone matrix (brush border). The brush border is completely surrounded by the "clear zone", a zone where the surface membrane of the osteoclast is smooth and lies directly against the underlying bone (Vaes, 1988). Osteoclasts resorb the bone by isolating a region of bone and attaching themselves to the bone surface via integrins. Endosomes containing membrane-bound proton pumps then insert into the cell membrane and transport protons into the space between the osteoclast and the bone surface to lower the local pH from approximately seven to four. Bone mineral is solubilised by the acidic pH and acid

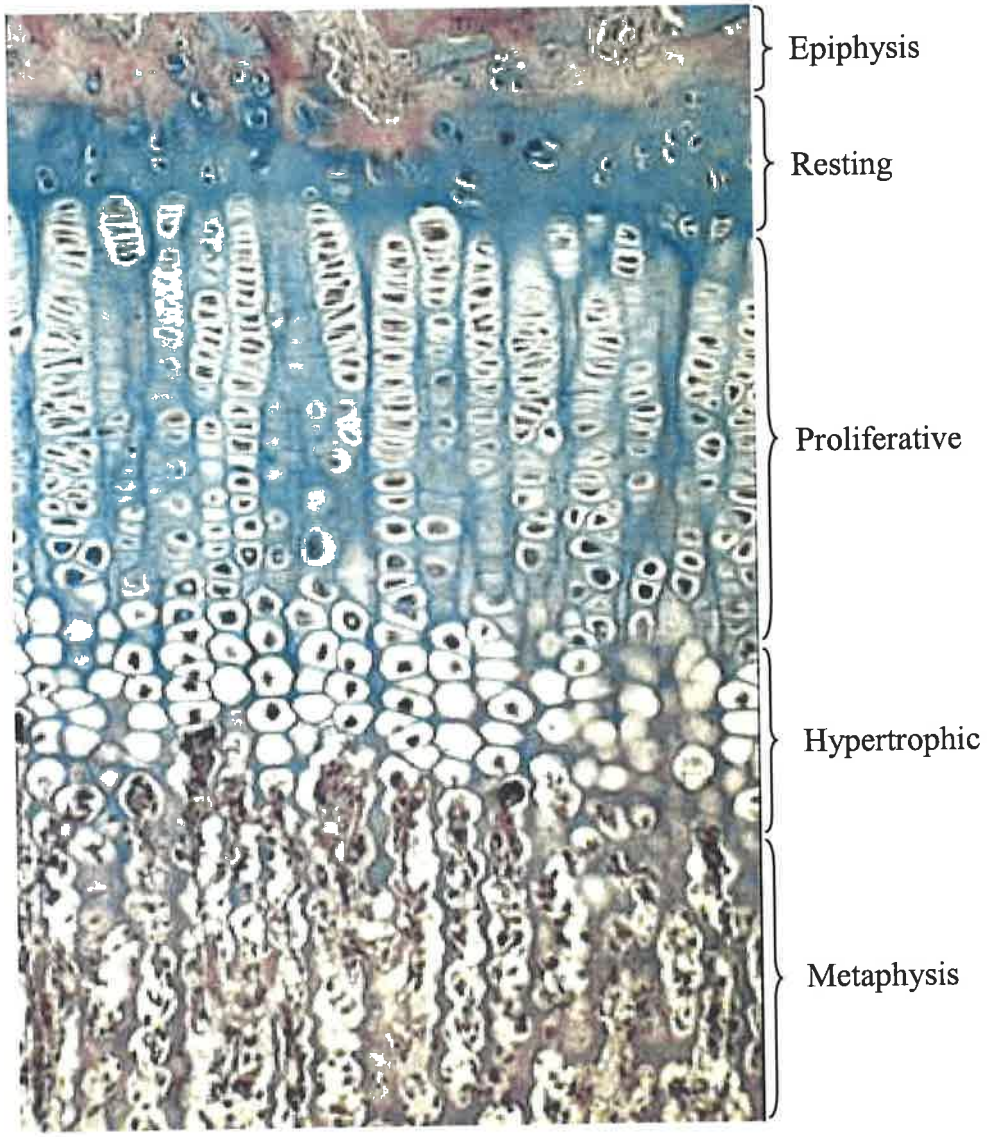
proteases secreted by the osteoclasts degrade the organic matrix (Baron *et al.*, 1985; Blair *et al.*, 1989). Once the osteoclasts have completed their resorptive activity, they can migrate to another resorption site, or divide into mononuclear cells that can be reactivated to form new osteoclasts (Buckwalter *et al.*, 1995).

1.3 Growth Plate Structure

The longitudinal growth of the developing long bones occurs at the cartilaginous growth plates located at either end of the long bones in children. Growth involves the establishment of new chondrocytes, the cells within growth plate cartilage that are responsible for the production and subsequent mineralisation of the matrix. The chondrocytes undergo several processes including cell division, matrix production, hypertrophy and matrix mineralisation (Wilsman *et al.*, 1996). Although the chondrocytes do not produce bone, they are responsible for the generation of a mineralised matrix, which serves as a scaffold for the laying down of bone. The growth plates continue functioning until skeletal maturity is reached and the growth plate undergoes closure.

The growth plate consists of three histologically distinct zones: the resting zone, proliferative zone and hypertrophic zone (Fig. 1.3). Growth plate that is removed and reversed maintains the same cellular orientation and continues to mature in the same manner as in the original growth plate, demonstrating absolute polarity of the growth plate structure (Abad *et al.*, 1999).

Figure 1.3: The growth plate from the proximal tibia of a sheep is shown indicating the three morphologically distinct zones. The resting zone lies immediately below the epiphysis and is characterised by a sparse distribution of single or paired cells in an abundant matrix. The proliferative zone is composed of chondrocytes that have taken on a flattened appearance and aligned themselves into columns. Within the hypertrophic zone the chondrocytes increase in volume prior to being engulfed by the invading mineralisation front of the metaphysis.


Epiphysis
Resting
Proliferative
Hypertrophic
Metaphysis

1.3.1 Resting Zone

The resting zone is located distal to the bone of the secondary ossification centre and is histologically characterised by a sparse distribution of single or paired cells in an abundant matrix (Iannotti, 1990). The cells are positioned randomly throughout the extracellular matrix, but the chondrocytes closer to the proliferative zone are aligned in the direction of growth (Johnstone *et al.*, 2000b). The function of the chondrocytes within the resting zone is unclear, as the cells are relatively inactive in both proliferation and matrix turnover. BrdU labelling, a measure of DNA synthesis, has shown that the cells within the resting zone have long cell cycle times compared to the proliferative zone cells (Farnum and Wilsman, 1993). The oxygen tension is low, showing that the blood vessels that supply the epiphysis lose little oxygen to the resting zone cells (Brighton and Heppenstall, 1971; Anderson *et al.*, 1975). Intracellular and ionised calcium levels are low within this zone (Iannotti and Brighton, 1989), and matrix proteoglycans are present in aggregate form, which are thought to inhibit mineralisation (Buckwalter, 1983). The major proteoglycan of cartilage, aggrecan, is present in a form comparable to that in the epiphyseal and articular cartilage, tissues that do not undergo mineralisation (Byers *et al.*, 1997). Some resting zone cells may migrate towards the periphery of the growth plate and provide cells for the growth in width of the growth plate and the growth in length and width of the periosteal bone (Langenskiöld, 1998).

1.3.2 Proliferative Zone

Located immediately below the resting zone, the proliferative zone contains cells from the time they begin to divide more frequently until the cells exit the cell cycle and begin terminal differentiation (Iannotti, 1990). The cells within this zone are arranged in longitudinal columns, with their role being matrix production and cell division

(Buckwalter, 1983), with 30% of longitudinal growth attributable to the cell division within this zone (Wilsman *et al.*, 1996). Confocal microscopy revealed that the chondrocytes within the proliferative zone are not aligned in continuous columns that span the growth plate but form distinct nests of cells (Johnstone *et al.*, 2000b). These nests of cells are eventually engulfed by the mineralisation front and give rise to the structure of the primary spongiosa. Controversy surrounds the origin of the uppermost cell in each column. In one hypothesis the first cell of a chondrocyte column is the progenitor cell. This cell divides giving rise to two daughter cells, one of which becomes incorporated into the proliferative zone while the other remains the progenitor cell for the column (Kember, 1971; Brighton, 1984). Another hypothesis is that the progenitor cell must be located above the proliferative zone within the resting zone (Johnstone *et al.*, 2000b). This is supported by the observation that the cells are arranged in nests, in which case the nests including the progenitor cells would be lost at the mineralisation front.

The oxygen tension in the proliferative zone is high due to the rich vascular supply from the epiphyseal artery (Brighton, 1978). The intracellular calcium levels are similar to that in the resting zone, but the ionised calcium levels are higher (Iannotti, 1990). Matrix proteoglycans are also present in aggregate form in this zone, preventing mineralisation (Buckwalter, 1983), and the rate of cellular proliferation within the proliferative zone is such that it will offset the cellular loss at the chondro-osseous junction (Wilsman *et al.*, 1996).

Before entering the hypertrophic zone, there is a transition zone that contains cells distal to the last chondrocytes undergoing cell division and proximal to those chondrocytes demonstrating the rapid increase in volume, characteristic of the hypertrophic

chondrocytes (Farnum and Wilsman, 1993). This transition is regulated by Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP), which function in a common feedback loop to control the rate of terminal differentiation (Lee *et al.*, 1996; Vortkamp *et al.*, 1996).

1.3.3 Hypertrophic Zone

As the chondrocytes proceed through the hypertrophic zone, they increase in volume by five to ten times and also increase their matrix production (Buckwalter, 1983; Iannotti, 1990), contributing towards 70% of longitudinal growth (Wilsman *et al.*, 1996). The metaphyseal artery does not penetrate the hypertrophic zone, resulting in an avascular environment and an oxygen tension that is extremely low (Brighton and Heppenstall, 1971). The intracellular calcium that was present in the proliferative zone is released into the hypertrophic zone matrix, and the matrix vesicles within the hypertrophic zone take up the calcium ions (Iannotti, 1990). Within the hypertrophic zone, the proportion of aggregated proteoglycans and the aggregate size decreases compared to the resting and proliferative zones, and these changes may be significant in the control of cartilage mineralisation (Buckwalter *et al.*, 1987). There is also a decrease in the sulphation of the major proteoglycan, aggrecan, within the hypertrophic zone (Byers *et al.*, 1997).

Although the exact mechanism is unknown, at the transition point between cartilage and bone, signalling events result in the breakdown of the last transverse septum and the endothelial cells of the metaphyseal blood supply move into the space previously occupied by the terminal chondrocyte. It is important that the chondrocytic turnover is offset by the cellular proliferation within the proliferative zone, or there will be a decrease in total cellular numbers ultimately leading to growth plate closure (Farnum and Wilsman, 1987).

At the present time, controversy remains as to the fate of the terminal hypertrophic chondrocyte. Evidence has been presented for two hypotheses, that (1) the chondrocyte continues to exist after the point of erosion of the last transverse septum and differentiates into an osteoblast (Hunziker *et al.*, 1983; Hunziker *et al.*, 1984; Roach *et al.*, 1995) and (2) the chondrocyte undergoes apoptosis (Gibson *et al.*, 1995; Zenmyo *et al.*, 1996). Hunziker *et al.* (Hunziker *et al.*, 1983; Hunziker *et al.*, 1984) have shown morphological evidence that the membrane integrity and cellular ultrastructural integrity of the chondrocytes is maintained to the level of the terminal chondrocyte. In comparison, studies by Gibson *et al.* (Gibson *et al.*, 1995) and Zenmyo *et al.* (Zenmyo *et al.*, 1996) provided evidence that the terminal cell dies via apoptosis. Roach *et al.* (Roach *et al.*, 1995) reported that there is an asymmetric cell division of the terminal cells, with one cell dying via apoptosis and the second differentiating into an osteoblast. Therefore, this report could reconcile the two opposing views of the fate of the hypertrophic chondrocytes.

1.3.4 Mineralisation

Mineralisation of the growth plate matrix is a tightly controlled process, both temporally and spatially, and is limited to a few layers of hypertrophic chondrocytes at the chondro-osseous border. The mineralisation process is crucial for longitudinal bone growth as only osteoblasts and osteoclasts remodel the calcified cartilage matrix. Although a key process, the exact mechanism of mineralisation is unknown. However, there are many possibilities currently under investigation.

As mineralisation occurs only within the hypertrophic zone of the growth plate, it is conceivable that there is a change in the composition or organization of the matrix that allows matrix calcification only within this zone. Several changes are apparent within the

hypertrophic zone of the growth plate. Firstly, type X collagen replaces type II collagen as the predominant collagen within the growth plate (Schmid and Linsenmayer, 1985). Although the exact function of type X collagen is unknown, it has been suggested that it is involved with the distribution of matrix vesicles and proteoglycans within the growth plate (Kwan *et al.*, 1997). Secondly, there is an increase in the concentration of several matrix metalloproteases (MMPs). Stromelysin (also known as MMP-3) has been implicated in cartilage destruction in osteoarthritis and may be important in altering the extracellular matrix of the hypertrophic zone prior to mineralisation (Armstrong *et al.*, 2002). A study by D'Angelo *et al.* (D'Angelo *et al.*, 2001) reported the presence of MMP-2,-9 and -13 in matrix vesicles isolated from the hypertrophic zone, suggesting that these MMPs have a role in matrix remodelling prior to mineralisation.

It is widely accepted that proteoglycans play a role in mineralisation (Howell and Pita, 1976; Buckwalter, 1983). The traditional view is that aggregated proteoglycans inhibit hydroxyapatite formation, and must be removed or modified for mineralisation to advance (Howell and Pita, 1976; Buckwalter, 1983). However, there are numerous studies that do not support this view, demonstrating that the concentration of proteoglycans remains constant as the mineralisation front approaches (Poole *et al.*, 1982a; Poole *et al.*, 1982b; Scherft and Moskalewski, 1984; Matsui *et al.*, 1991). Other evidence also suggests that there may be focal concentrations of proteoglycans at the mineralisation site (Shepard and Mitchell, 1985). A study by Byers *et al.* (Byers *et al.*, 1997) examined aggrecan, the major proteoglycan present within the growth plate, and showed that the size of the aggrecan monomer within the hypertrophic zone increased as a result of an increase in the length of the chondroitin sulphate side chains. However, this increase in length was accompanied by a decrease in sulphation. Studies using a homozygous brachymorphic mouse deficient in

the enzyme required for sulphating the side chains of proteoglycans demonstrated that an alteration in the sulphate content could accelerate the calcification process (Orkin *et al.*, 1976; Orkin *et al.*, 1977). It was suggested that a decrease in sulphation might alter the ability of the proteoglycans to bind calcium, therefore increasing the amount of free calcium ions within the hypertrophic zone. These ions may be at a concentration that is high enough to allow mineral formation when the phosphate ion concentration is also high (Byers *et al.*, 1997).

At the present time, controversy still remains as to the nucleation site for matrix mineralisation, with chondrocalcin and matrix vesicles both possible candidates (Anderson, 1969; Poole *et al.*, 1984; Alini *et al.*, 1992).

Chondrocalcin (the C-propeptide of type II collagen) is present in high concentrations in the extracellular matrix of the hypertrophic zone. It is a calcium binding protein with a strong affinity for hydroxyapatite. It is selectively concentrated in the longitudinal septae of the chondrocyte columns (Poole *et al.*, 1984; Alini *et al.*, 1992), suggesting that it may play a fundamental role in the creation of nucleation sites for the calcification of cartilage matrix.

Other studies have suggested that the matrix vesicles are the site for nucleation (Anderson, 1969). Matrix vesicles contain annexins II, V and VI, proteins that enable calcium ion influx into the matrix vesicles and the formation of the first crystal inside the vesicles (Kirsch *et al.*, 2000), and alkaline phosphatase, an enzyme that generates inorganic phosphate from organic phosphate compounds (Anderson, 1995). Annexin V plays major roles in the function of the vesicles, particularly during the onset of calcification when the

first mineral phase forms and grows inside the vesicle lumen. It also mediates the influx of calcium ions and binds directly to collagen types II and X, anchoring the vesicles to the extracellular matrix. Once the crystal reaches a certain size, it ruptures the matrix vesicle membrane and penetrates the extracellular matrix (Anderson, 1995).

1.3.5 Blood Supply to the Growth Plate

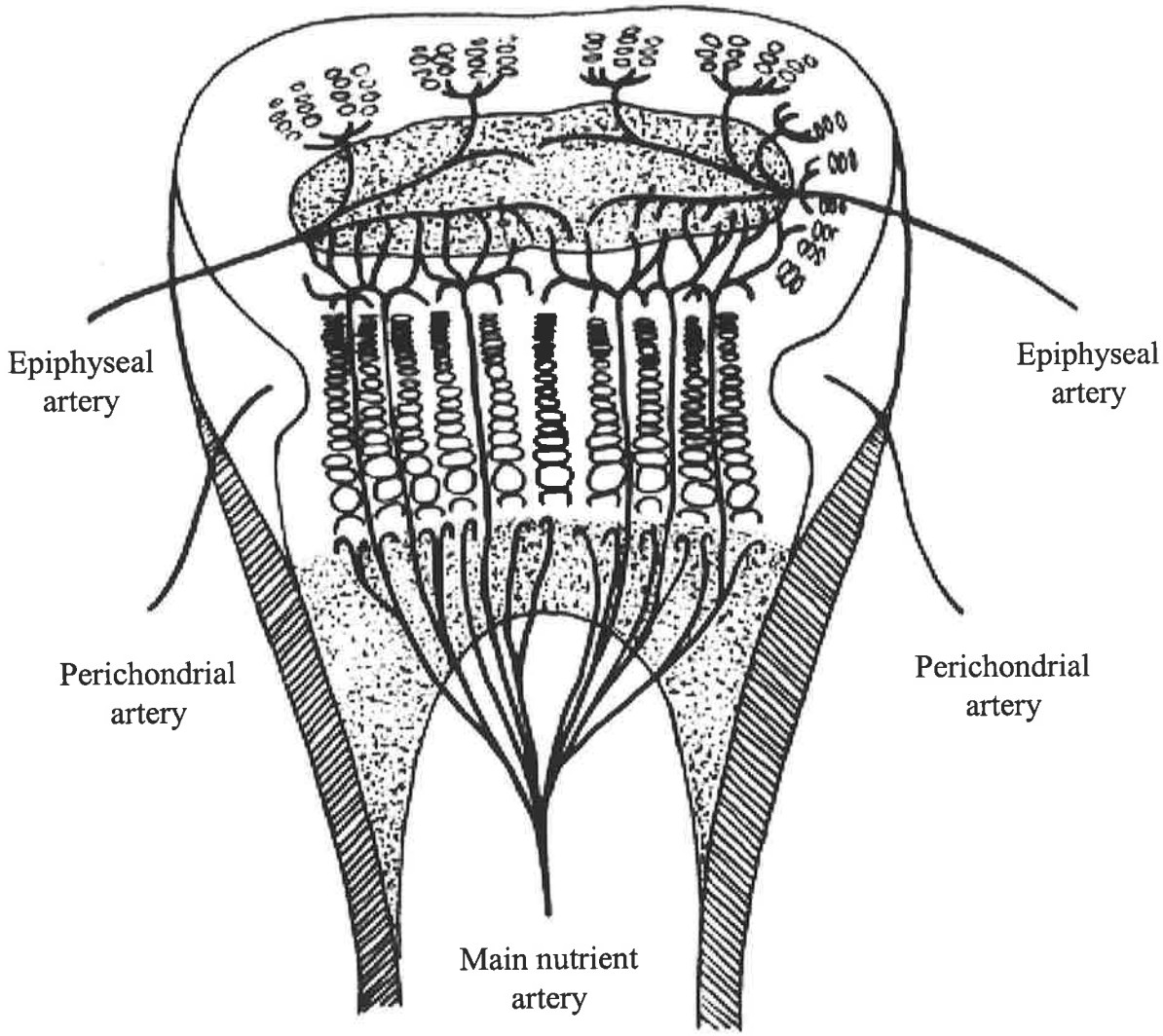
The supply of oxygen and nutrients to the growth plate is crucial for its continual development and function. The main arteries involved are the epiphyseal artery, the main nutrient artery and the perichondrial arteries (Fig. 1.4).

The epiphyseal artery enters the epiphysis and its terminal branches traverse the resting zone, terminating at the uppermost cells of the proliferative zone. The artery does not penetrate between cartilage columns and arteries never pass from the epiphyseal side to the metaphyseal side of the growth plate (Trueta and Amato, 1960). This artery supplies oxygen and nutrients to the chondrocytes of the proliferative zone (Iannotti, 1990). Disruption of the epiphyseal blood supply can lead to necrosis of the growth plate and the temporary or complete cessation of growth, resulting in differential rates of growth across the affected growth plate and significant contour changes (Peterson, 1984).

The main nutrient artery enters the metaphysis and supplies it with oxygen and nutrients. The artery continues to divide into smaller vessels until it reaches the growth plate, where it forms capillary loops at the last transverse septae at the bone-cartilage interface, which turn back on themselves to form a venous return. There is no direct contact between the vascular loop and the last intact septum (Trueta and Amato, 1960). Interruption of the metaphyseal blood supply has no effect on chondrogenesis within the resting zone or the

Figure 1.4: A schematic representation of the arterial blood supply to the growth plate. The epiphyseal arteries, main nutrient artery and perichondrial arteries are shown. The blood supply to the resting zone is partly accomplished by the transphyseal vessels, which traverse through the growth plate from the metaphysis to the epiphysis.

Adapted from (Wirth *et al.*, 2002).


Epiphyseal artery
Epiphyseal artery
Perichondrial artery
Perichondrial artery
Main nutrient artery

subsequent maturation of the chondrocytes within the proliferative and hypertrophic zones. However, the transformation from cartilage to bone (primary spongiosa) at the chondro-osseous boundary is blocked. Cartilage continues to be added to the cell columns, but it is not replaced by bone at the chondro-osseous boundary resulting in a widening of the growth plate. If the blood supply is re-established, the widened region quickly ossifies, blood vessels invade and the growth plate returns to its normal width (Trueta and Amato, 1960). This study suggested that a molecule from the metaphyseal blood supply was important for the mineralisation process. Chondrocytes co-cultured with crude cell isolates from the metaphysis showed a 93% increase in ^{45}Ca incorporation, compared to co-cultures with vascular endothelial cells and osteoblastic cells that showed no effect on ^{45}Ca incorporation. It was established that the factor responsible was a soluble molecule of 12-14 kDa in size that was stable to temperatures up to 90°C. Members of the TGF-β family were put forward as candidate molecules due to their thermostability properties (Johnstone *et al.*, 2000a). Vascular supply is clearly important not only for oxygen and nutrient supply to the metaphysis and growth plate, but also in delivering factors important in the process of matrix mineralisation.

A recent study has described the presence of transphyseal blood vessels that pass through the growth plate from the metaphysis to the epiphysis in 6 month old sheep (Wirth *et al.*, 2002). The role of these vessels is unclear and currently under investigation.

The perichondrial arteries supply oxygen and nutrients to the perichondrial ring of LaCroix (Trueta and Amato, 1960; Iannotti, 1990).

1.4 Constituents of the Extracellular Matrix

The biological functions of both growth plate cartilage and bone are dependant mostly on the properties of their extracellular matrices. The extracellular matrix of cartilage, whose major components are cartilage-specific collagens and sulphated proteoglycans, is synthesised by chondrocytes and is responsible for regulating growth factor interactions, cell-matrix interactions and collagen fibril size (Table 1.1). Osteoblasts are responsible for the synthesis of most of the extracellular matrix of bone, the main component of which is type I collagen, which imparts strength and provides the initial site for mineralisation. Several other molecules are involved in regulating collagen fibril size and the mineralisation process (Table 1.1). The identification of naturally occurring mouse models and generation of transgenic or knockout mouse models for cartilage and bone matrix proteins has improved our understanding of the functions of these proteins and are discussed in the following sections.

1.4.1 Type I Collagen

Type I collagen is the most abundant collagen in bone, contributing 90% of total collagens (Lind, 1996). It is synthesised by osteoblasts in newly forming bone, and appears in the diaphysis along with the formation of the bone marrow in areas of endochondral ossification (Mundlos *et al.*, 1990).

Mutations in either of the type I collagen genes (*COL1A1* or *COL1A2)* can result in osteogenesis imperfecta, a heritable disease characterised by osteopenia (decreased bone mass) and fragile bones prone to fracture (Byers, 1990) or osteoporosis, a disease also presenting with osteopenia resulting in increased fractures following minor trauma (Spotila *et al.*, 1991). A transgenic mouse was generated with a mutation in the *COL1A1* gene,

Table 1.1 **Molecules associated with bone and cartilage**

Molecule	Site of Expression	Proposed Function
Collagens		
Type I collagen	*Predominate collagen of bone*	*Provides strength, site of mineralisation (Sandberg and Vuorio, 1987; Mundlos et al., 1990)*
Type II collagen	*Predominate collagen of cartilage*	*Resists tensile stress (Sandberg and Vuorio, 1987; Mundlos et al., 1990)*
Type IX collagen	*All cartilage*	*Associates with the surface of type II collagen (Fassler et al., 1994; Vornehm et al., 1996)*
Type X collagen	*Hypertrophic cartilage*	*Matrix mineralisation, facilitates removal of type II collagen, foundation for deposition of bone matrix, vascular invasion (Wu et al., 1989; Schmid et al., 1990; Thomas et al., 1990; Price et al., 1994)*
Type XI collagen	*All cartilage*	*Controls type II collagen fibril size (Vornehm et al., 1996)*
Proteoglycans		
Aggrecan	*Throughout cartilage*	*Imparts resistance to compression. Forms aggregates with hyaluronan and link proteins (Muir, 1995)*
Decorin	*Cartilage, skin and bone*	*Regulates collagen fibril size, TGF-β activity and cell proliferation (Scott and Orford, 1981; Scott et al., 1986; Scott, 1988; Bianco et al., 1990; Santra et al., 1995; De Luca et al., 1996)*
Biglycan	*Cartilage, skin and bone*	*Binds collagen fibrils and regulates TGF-β activity (Bianco et al., 1990; Yamaguchi et al., 1990; Hildebrand et al., 1994; Schönherr et al., 1995b)*
Matrix Gla Protein	*Throughout cartilage*	*Inhibits mineralisation (Luo et al., 1997)*

Table 1.1 **Molecules associated with bone and cartilage**

Molecule	Site of Expression	Proposed Function
Osteopontin	*Osteoblasts in areas of cartilage-bone transition*	*Regulator of mineralisation, attracts and bonds osteoclasts, macrophage recruitment and regulates collagen fibril size (Reinholt et al., 1990; Hultenby et al., 1991; Giachelli et al., 1998; Liaw et al., 1998)*
Osteonectin	*Osteoblasts, osteocytes, osteoid, bone and cartilage*	*Regulatory role in mineralisation (Mundlos et al., 1992)*
Osteocalcin	*Osteoblasts*	*Attracts osteoclasts to the sites of bone resorption and limits mineralisation (Ikeda et al., 1992)*

which resulted in the synthesis of shortened collagen chains that could not fold correctly but could associate with the normal collagen chains (Pereira *et al.*, 1993). The transgenic mice showed various skeletal abnormalities consistent with osteogenesis imperfecta, including multiple fractures of the long bones and ribs and a reduced collagen and mineral content. Postmenopausal osteoporosis presents with similar manifestations to that of mild osteogenesis imperfecta, and it has been reported that there may be a genetic and phenotypic overlap between the two diseases (Spotila *et al.*, 1991). The phenotype resulting from a mutation in either of the type I collagen genes indicates the importance of type I collagen in imparting strength in the bones and providing a scaffold for the mineralisation process.

1.4.2 Type II Collagen

Type II collagen is found almost exclusively within cartilage, and is a marker of chondrocyte differentiation (Liu *et al.*, 1995; Muir, 1995). It contributes 30-60% of the dry weight of the cartilage matrix (Li *et al.*, 1995) and is present within all regions of the growth plate, but is down regulated in the hypertrophic zone where there is a switch from type II collagen to type X collagen (Iyama *et al.*, 1991). Mutations in the *Col2a1* gene responsible for type II collagen in cartilage 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; (Horton, 1992; Prockop *et al.*, 1993; Ritvaniemi *et al.*, 1995)). Transgenic mice to model this disease were generated by the inactivation of the *Col2a1* gene (Li *et al.*, 1995), and morphological and histological examination revealed that many of the organs and bones of the affected mice developed normally, including the 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 (Li *et al.*, 1995).

1.4.3 Type X Collagen

Type X collagen is found in the hypertrophic zone of the growth plate and is a marker for cartilage undergoing endochondral ossification (Lu Valle *et al.*, 1993). The proposed function of type X collagen is complicated by the fact that there are conflicting reports on the phenotype of type X collagen null mice (Rosati *et al.*, 1994; Kwan *et al.*, 1997). The first study reported that a type X collagen null mouse developed normally without type X collagen and displayed normal skeletal pathology, with no histological differences between the normal and mutant mice and no differences in the cells when analysed by electron microscopy (Rosati *et al.*, 1994). In contrast, the second study described alterations in trabecular architecture, matrix vesicle location and collagen-matrix interactions in two knockout models in different mouse strains (Kwan *et al.*, 1997). The differences seen between these two studies may be due to the different genetic backgrounds of the mice used, as differences in phenotypic severity were observed in the two strains used by Kwan *et al.* (Kwan *et al.*, 1997). Mutations within the type X collagen gene resulting in skeletal deformities have been reported in humans with Schmid metaphyseal chondrodysplasia by Warman *et al.* (Warman *et al.*, 1993) and in a dominant negative transgenic mouse model by Jacenko *et al.* (Jacenko *et al.*, 1993). It was suggested by Rosati *et al.* (Rosati *et al.*, 1994) that the differences seen between their type X collagen null mouse model and those

studies reporting mutations within the type X collagen gene might be due to the mutated type X collagen gene, rather than an absence of the protein. The abnormal type X collagen polypeptides may become incorporated into trimers and then disrupt the supramolecular assemblies in the matrix, thereby altering endochondral ossification. Two possible explanations were put forward for the normal phenotype described by Rosati *et al.* for the type X collagen null mouse. Firstly, type X collagen may have no function and secondly, other extracellular matrix molecules may be able to fulfil the function of type X collagen in its absence (Rosati *et al.*, 1994). Kwan *et al.* attribute the phenotype of their type X collagen null mouse to an abnormal distribution of proteoglycans and matrix vesicles to zones of the growth plate other than the hypertrophic zone (Kwan *et al.*, 1997).

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 (Wu *et al.*, 1989; Schmid *et al.*, 1990; Thomas *et al.*, 1990; Price *et al.*, 1994). 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 (Wu *et al.*, 1989; Schmid *et al.*, 1990; Thomas *et al.*, 1990; Kwan *et al.*, 1997).

1.4.4 Matrix Gla Protein

Matrix gla protein (MGP) is a 79 amino acid protein found in bone, cartilage and dentin that contains 5 residues of the vitamin K dependent amino acid γ-carboxyglutamic acid (gla; (Price and Williamson, 1985). MGP is expressed in the hypertrophic chondrocytes of the growth plate and within other soft tissues (Luo *et al.*, 1997).

In 1997, Luo *et al.* (Luo *et al.*, 1997) developed a mouse model deficient in MGP, and showed that these mice die within 2 months of birth due to arterial calcification, which leads to blood vessel rupture. These mice display short stature, osteopenia and fractures, due to disorganised chondrocyte columns within the growth plate, and show extensive calcification along the aorta and cartilaginous structures. Within the growth plate, calcification is normally restricted to the hypertrophic zone. However, in the mutant mice the calcification continued into the proliferative zone. These results indicate that MGP functions to inhibit the calcification of certain extracellular matrices (e.g. aorta, trachea) that do not normally calcify, possibly through its mineral-ion-binding ability, and regulates the calcification process in the growth plate cartilage (Luo *et al.*, 1997).

1.4.5 Osteopontin

Osteopontin is a non-collagenous matrix protein that is found in areas of cartilage to bone transition. As it is found in the osteoblasts at the mineralisation front, it was suggested that osteopontin might be a local regulator of the mineralisation process (Hultenby *et al.*, 1991). The protein contains a 5 amino acid sequence (Gly-Arg-Gly-Asp-Ser) that is essential for cell adhesion-promoting activity, and at the amino terminal end has a highly acidic region, which has been implicated in hydroxyapatite binding. It has also been suggested that osteopontin is important in the attachment of osteoclasts to bone and in their role in the process of bone resorption (Reinholt *et al.*, 1990; Ross *et al.*, 1993). Osteopontin binds to the osteoclasts through the $\alpha_v\beta_3$ integrin, which then attaches the osteoclasts to bone where they dissolute bone mineral, collagens and other bone proteins. In addition to its roles in bone cell recruitment, osteopontin has also been implicated in the wound healing process, in particular the influx of macrophages during the inflammatory response and as a regulator of collagen fibrillogenesis (Giachelli *et al.*, 1998; Liaw *et al.*, 1998). In the bone

healing process, osteopontin is secreted by macrophages for cell adhesion and phagocytosis, signifying that the protein can evoke immune activity (McKee and Nanci, 1996).

Two osteopontin null mice models have been generated, both of which show no obvious skeletal abnormalities (Liaw *et al.*, 1998; Rittling *et al.*, 1998). One of these models was used to investigate the role of osteopontin in bone resorption following ovariectomy (removal of the ovaries), a model for post-menopausal osteoporosis (Yoshitake *et al.*, 1999). The absence of osteopontin protected the mouse from the rapid bone loss seen in osteoporosis, possibly as a result of impaired osteoclast function. The second mouse model investigated the role of osteopontin in the healing of a skin wound (Liaw *et al.*, 1998). It was found that osteopontin was present in high concentrations in the infiltrating inflammatory cells and macrophages in the normal mice as early as 6 hours after injury. The absence of osteopontin resulted in an accumulation of debris within the cell site, probably due to compromised macrophage function, as well as a less organised wound matrix. The collagen fibrils in the wounds of the mutant mice were smaller in diameter, indicating osteopontin may affect the types of collagen synthesised or the turnover of matrix components involved in the regulation of collagen fibril formation (Liaw *et al.*, 1998).

1.4.6 Osteonectin

Osteonectin is a secreted calcium binding protein and is one of the most abundant non-collagenous proteins in developing bone. It is predominantly found in osteoblasts and their precursor osteocytes, and the mineralising cartilage of developing bone (Young *et al.*, 1992). The mRNA for osteonectin is found in cells of the osteoblastic lineage, and within

the proliferative and upper hypertrophic regions of the growth plate (Mundlos *et al.*, 1992; Young *et al.*, 1992). Osteonectin has the ability to bind calcium suggesting a role in the mineralisation process. It can mediate the binding of hydroxyapatite crystals to type I collagen and once bound to type I collagen it can initiate the deposition of calcium and phosphate from stable solutions. These observations indicate that osteonectin may be involved in the initial formation of mineral and the attachment of crystals to the type I collagen fibres (Termine *et al.*, 1981).

An osteonectin null mouse model was generated that displayed no gross skeletal abnormalities (Norose *et al.*, 1998). However, a comprehensive study using contact x-ray and histomorphometry revealed decreased bone remodelling resulting in profound osteopenia (Delany *et al.*, 2000). The mutant mice displayed a decreased trabecular bone formation rate due to a reduced number of osteoblasts, and compromised bone strength resulting from a reduced number of trabeculae and an increase in the spacing between them (Delany *et al.*, 2000). These results indicate that osteonectin is crucial in the support of bone remodelling and maintenance of bone mass. Additional studies in this mouse model have shown that a lack of osteonectin results in impaired wound healing of the skin, impeding the migration of fibroblasts and thus retarding granulation tissue formation (Basu *et al.*, 2001). Osteonectin has also been implicated in the maintainance of lens transparency in the eye (Gilmour *et al.*, 1998; Norose *et al.*, 1998).

1.4.7 Decorin

Decorin is a small proteoglycan that is heavily enriched in bone matrix (Kresse *et al.*, 1994). In this environment, it contains chondroitin sulphate chains at the amino terminal, but contains dermatan sulphate chains when in soft connective tissues (Robey, 1996). The

amino terminal is believed to play a role in binding the protein to cell surfaces and other extracellular matrix proteins (Danielson *et al.*, 1997). The central cysteine-free domain contains specific amino acid residues that allow binding of decorin to type I collagen (Schönherr *et al.*, 1995a; Svensson *et al.*, 1995). At the present time, the function of the carboxyl terminal remains unknown. Possible functions for decorin include protein-protein interactions to regulate orderly matrix assembly (Scott and Orford, 1981; Scott *et al.*, 1986; Scott, 1988), and control of cell proliferation (Santra *et al.*, 1995; De Luca *et al.*, 1996).

Danielson *et al.* (Danielson *et al.*, 1997) generated a null mouse model in which decorin expression was abolished. The homozygous mutant animals were viable and displayed no gross anatomical abnormalities. However, they did have laxity and significant fragility of the skin as a result of dermal thinning and loose connective tissue in the hypodermal region. These skin abnormalities also resulted in significantly reduced tensile strength. Ultra-structural analysis of the skin showed that type I collagen was less orderly packed and had a greater variability in size and shape. Dermatosparaxis ("torn skin") syndrome in animals and Ehlers-Danlos syndrome (EDS) in humans has a phenotype similar to that of the decorin null mouse (Lichtenstein *et al.*, 1973; Byers, 1994). However, these syndromes are caused by a mutation in the type I procollagen genes that ultimately leads to the accumulation of unprocessed collagen. The fragile skin condition is a result of an inability of decorin molecules to bind to the collagen, and it is speculated that these disorders may share a common pathogenetic mechanism with the decorin null mouse. Although not described, it is predicted that a disease could exist caused by a recessive deletion or mutation in the collagen-binding domain of the decorin gene.

1.4.8 Biglycan

Similar to decorin, biglycan is a small proteoglycan found in bone matrix (Kresse *et al.*, 1994). It has been proposed that biglycan may function in connective tissue metabolism by binding collagen fibrils (Schönherr *et al.*, 1995b) and TGF-β (Yamaguchi *et al.*, 1990; Hildebrand *et al.*, 1994), and may promote neuronal survival (Kappler *et al.*, 1997).

A mouse model was generated that was deficient in biglycan (Xu *et al.*, 1998), demonstrating a reduced growth rate and a decreased bone mass. The mutant mice appeared normal at birth, but after 6 months their growth rate and body weight were significantly lower than normal littermates. The length of the femur was shorter in the mutants, suggesting that biglycan may have a role in regulating postnatal skeletal growth. The trabeculae of the metaphysis were thinner and fewer in number, and were poorly connected to each other. There was also a decrease in the cortical bone thickness in the diaphysis. At the cellular level, the number of osteoblasts present on the surface of the bone was decreased in the mutant animals, whereas there was no difference in the number of osteoclasts. This indicates that the reduced bone mass seen in the mutant mice was a result of decreased bone formation and not an increase in bone resorption. Results from the biglycan-deficient mouse suggest that biglycan is a positive regulator of bone formation and bone mass.

1.4.9 Growth Factor Regulation

In addition to the molecules that constitute the extracellular matrix that are critical for bone formation, soluble messenger molecules such as growth factors have key roles in the regulation of synthesis and turnover of bone (Table 1.2). They can circulate in a free form, be bound to a carrier molecule, or bind to components of the extracellular matrix (Price *et*

Table 1.2: Growth Factors associated with Bone and Cartilage

Growth Factor	Proposed Function
TGF-β	*Regulates fracture repair, mediates normal skeletal embryonic development and skeletal remodelling in adults (Kimelman and Kirschner, 1987; Carrington et al., 1988; Sandberg et al., 1988; Bonewald and Mundy, 1990)*
FGFs	*Stimulates proliferation of osteoblasts, chondrocytes and periosteal cells. Important in fracture repair (Baron et al., 1994; Ornitz and Marie, 2002)*
IGFs	*Important in bone cell recruitment and differentiation (Conover, 1996)*
BMPs	*Stimulate bone formation (Urist, 1965; Knusten et al., 1993; Hentunen et al., 1995; Cheifetz et al., 1996) and chondrogenesis (Asahina et al., 1996; Grgic et al., 1997; Sellers et al., 1997; Katic et al., 2000; Louwerse et al., 2000; Sellers et al., 2000; Jelic et al., 2001; Cook et al., 2003)*
PDGF	*Stimulates cell replication, bone resorption and collagen degradation. Inhibits differentiated function of osteoblasts (Canalis and Rydziel, 1996)*
TNF-α	*Potent stimulator of bone resorption (Gravallese et al., 2001) and inhibits bone collagen synthesis (Daireaux et al., 1990)*
VEGF	*Mediates angiogenesis (Gerber et al., 1999)*

al., 1994). As with matrix molecules, there is often a degree of redundancy with growth factors, in that a gene knockout for a particular growth factor may have little or no effect on the phenotype.

1.4.9.1 Angiogenic Factors

Angiogenic factors are critical in bone formation as they promote neovascularisation. Direct angiogenic factors act on endothelial cells, promoting proliferation and/or the migration of cells into areas in which they are released. Examples of these factors include platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β) and fibroblast growth factors (FGFs). Indirect angiogenic factors act by recruiting macrophages and monocytes, which in turn release their own direct-acting angiogenic factors. An example of these factors is tumour necrosis factor alpha (TNF-α) (Price *et al.*, 1994).

Vascular endothelial growth factor (VEGF) is an essential mediator of angiogenesis. It has been shown to be necessary for embryonic development, as the loss of a single VEGF allele results in embryonic lethality (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). The expression of VEGF is stimulated by hypoxia, and the avascular nature of the hypertrophic zone of the growth plate provides an environment suited for VEGF expression (Ferrara and Davis-Smyth, 1997). The proposed role of VEGF is to recruit endothelial cells, thus inducing and maintaining blood vessels, which then bring nutrients, chondroblasts, osteoblasts and pro-apoptotic signals important in the process of angiogenesis (Gerber *et al.*, 1999)

Currently, there are 22 genes within the FGF family that are divided into 6 sub-families (Ornitz and Marie, 2002). The FGFs display both angiogenic and chemotactic properties,

and therefore it was hypothesised that they might serve to couple chondrogenesis and ossification within the mammalian growth plate (Baron *et al.*, 1994). Both FGF-1 and FGF-2 are expressed by the proliferative and upper hypertrophic chondrocytes of the growth plate, and FGF-2 also within the extracellular matrix of the hypertrophic zone (Baron *et al.*, 1994). When injected into the growth plate, FGF-2 was shown to increase vascular invasion, and in turn the rate of ossification. The signal was specific for the metaphyseal vessels as there was no abnormal ingrowth of the epiphyseal blood supply into the growth plate. This suggests that the role of FGF-2 is to attract vascular and bone cell invasion from the metaphyseal bone, thus coupling osteogenesis with chondrogenesis (Baron *et al.*, 1994).

1.4.9.2 Transforming Growth Factor Beta (TGF-β)

A role for TGF-β has been described in morphogenesis, the regulation of endochondral ossification and in bone remodelling (Kimelman and Kirschner, 1987; Carrington *et al.*, 1988; Bonewald and Mundy, 1990). Bone is considered to be a reservoir in the body for TGF-β, which has been co-localised in osteoblasts and osteoclasts that express high levels of type I procollagen mRNAs (Sandberg *et al.*, 1988). Immunohistochemical studies have also shown that TGF-β is not present within the growth plate (Ellingsworth *et al.*, 1986), suggesting that if TGF-β has a role within the final stages of chondrocyte differentiation, it may act via a paracrine route (from bone or perichondrium) to exert its effects on the chondrocytes (Sandberg *et al.*, 1988). The expression of TGF-β within osteoclasts, together with TGF-β having effects on the proliferation and differentiation of osteoblastic cells *in vitro,* is consistent with a role in the bone remodelling process (Sandberg *et al.*, 1988; Bonewald and Mundy, 1990).

1.4.9.3 Bone Morphogenetic Proteins (BMPs)

The BMPs are a large group of signalling molecules that are members of the TGF-β superfamily and are expressed in distinct spatial and temporal patterns in numerous embryonic organs and tissues, with multiple roles at different stages of development (Reddi, 1994; Hogan, 1996; Vortkamp, 1997). Redundancy of function exists between different BMPs, as demonstrated by the BMP-7 knockout mouse (Dudley *et al.*, 1995; Luo *et al.*, 1995), and the embryonic lethal BMP-5/BMP-7 double mutant mouse (Solloway and Robertson, 1999). Homozygous mutant BMP-7 mice have small kidneys and die shortly after birth due to renal failure. The kidneys appear to develop normally until approximately 11.5 days *post coitum*, when further development ceases (Dudley *et al.*, 1995; Luo *et al.*, 1995). This suggests that other BMPs may compensate for the absence of BMP-7 in the initial stages of kidney development, but at a certain time there is an absolute requirement for BMP-7 to maintain the continued proliferation, survival and/or differentiation of mesenchymal and/or epithelial cells (Hogan, 1996). BMP-5 mutant mice are viable as homozygotes, with defects in the development of specific bone elements and several soft tissues (King *et al.*, 1994). *In situ* hybridisation of BMP-5 and BMP-7 mRNA in mouse embryos demonstrated a wide overlap of expression in several tissues. In the double mutant mice, the phenotype observed displayed abnormalities in virtually all of the sites in which BMP-5 and BMP-7 were co-expressed (Solloway and Robertson, 1999). Therefore, the abolishment of both BMP-5 and BMP-7 does not allow for compensation in function, resulting in a more severe phenotype than that seen in either the BMP-5 or BMP-7 mutant mouse.

The osteogenic effects of the BMPs were first described when demineralised bone matrix was implanted into non-bony sites and induced new bone and bone marrow formation

(Urist, 1965). These molecules are important in stimulating cells of the osteoblastic lineage (Knusten *et al.*, 1993; Cheifetz *et al.*, 1996) and may also contribute to bone resorption by affecting the recruitment of osteoclasts (Hentunen *et al.*, 1995). Several of the BMPs have been utilised to heal segmental or non-union bone defects that would normally be unable to repair (Cook *et al.*, 1994a; Cook *et al.*, 1994b; Cook *et al.*, 1995; Geesink *et al.*, 1999; Friedlaender *et al.*, 2001).

In recent years, the chondrogenic potential of certain members of the BMP family has been discovered. Recombinant BMP-2 and recombinant BMP-7 have been incorporated into full thickness articular cartilage defects and thyroid cartilage defects (Grgic *et al.*, 1997; Sellers *et al.*, 1997; Katic *et al.*, 2000; Louwerse *et al.*, 2000; Sellers *et al.*, 2000; Jelic *et al.*, 2001; Cook *et al.*, 2003). The newly formed cartilage integrated well with the host cartilage, and there were no signs of deterioration of the new tissue.

1.5 Growth Plate Injuries and Treatments

1.5.1 Classification and Incidence of Growth Plate Injuries

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, with other causes of growth plate injury occurring as a consequence of radiation, infection, tumour, metabolic abnormality, frostbite and burns (Peterson, 2001). Fractures through the growth plate can be from either a physiological failure or a mechanical failure, with mechanical failure often leading to physiological failure. Growth plate injuries can occur when the mechanical demands placed on a bone exceed the mechanical strength of the epiphysis-growth plate-metaphysis complex. Accurate assessment of growth plate

injuries is required because the location of the damage determines the fate of the proliferative cell activity and epiphyseal blood supply, and therefore future growth (Peterson, 1984).

In 1892, Poland initially classified growth plate fractures into 4 classes based on observations made from compound fractures, dissections of patients with fatal injuries, or from amputated limbs (reviewed in (Peterson, 2001)). After the introduction of the x-ray in 1895, the classification of growth plate fractures was defined in a more scientific manner. Throughout the twentieth century, the description of growth plate injuries was continually refined, however a universal classification system has yet to be established. The most recognised classification is that of Salter and Harris (Salter and Harris, 1963), who described 5 classes of growth plate injury. The first 4 fracture types were previously described by Poland (Peterson, 2001) and Aitken (Aitken, 1936), however Salter and Harris introduced a compression injury (type V), which incorporated the death of the germinal layer cells of the growth plate (Fig. 1.5A).

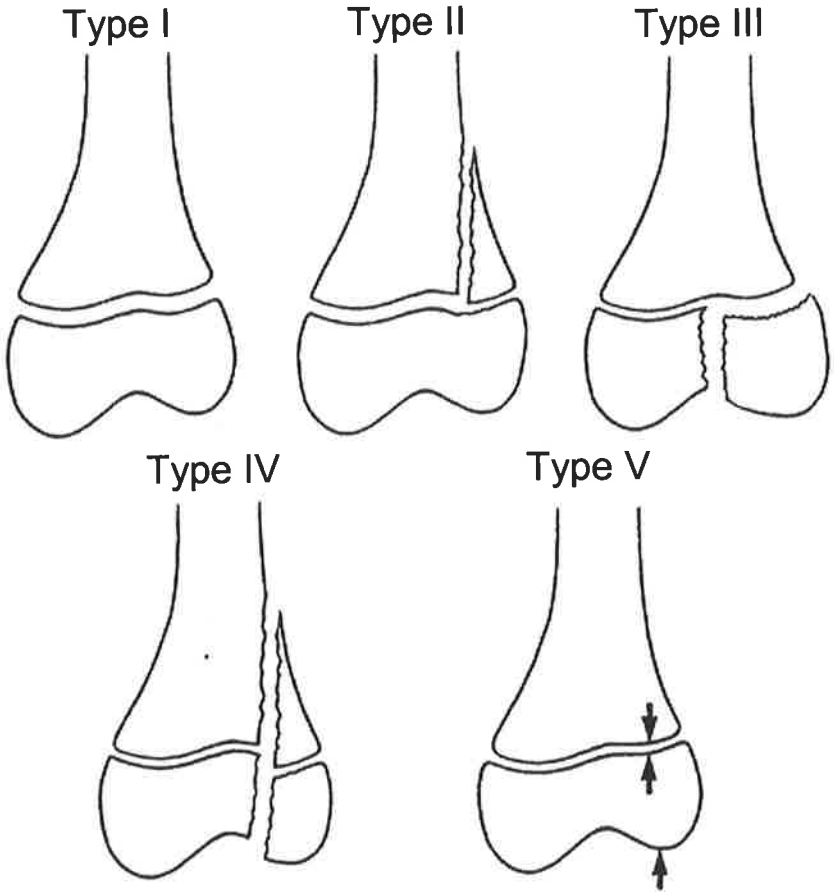
Limb length discrepancies occur when injury causes the arrest of an entire growth plate, often through the formation of a bone bridge. Several treatment strategies are available to resolve the difference in length of the arrested bone and the contralateral or companion bone, but treatment is further complicated if only a part of the growth plate is affected. In addition to the difference in limb length, the bone bridge formed at the site of the damaged growth plate may result in angular deformity of the limb.

Several case studies have reported the incidence of growth plate fractures to range from 18% (Worlock and Stower, 1986; Mizuta *et al.*, 1987) to 30% (Mann and Rajmaira, 1990)

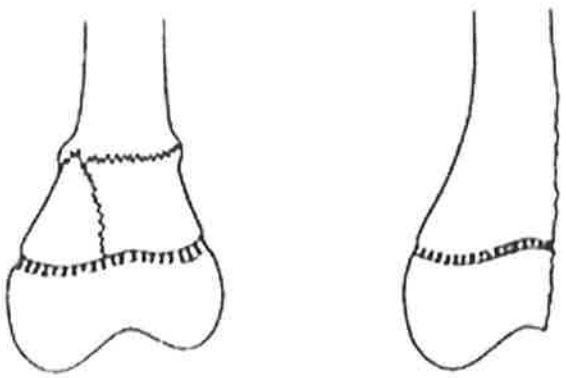
Figure 1.5: The classification system of growth plate fractures. (A) The original classification by Salter and Harris is shown. (B) Two additional fractures identified by Peterson during the Olmstead County study that were not described by Salter and Harris. The classification system of Peterson contains fractures type I-IV of the Salter-Harris system and the two additional fractures shown in (B). The type V fracture from the Salter-Harris classification was removed by Peterson as no injuries of this type were identified in the Olmstead County study.

Adapted from (Peterson, 1994b).

A



B



of children's fractures. Population based data is needed for epidemiological purposes, which will help with prognosis, elucidating etiology and planning interventions to prevent injuries. Recently, the first population based study was conducted in the Olmstead County, Minnesota, USA. (Peterson, 1994a, b; Peterson *et al.*, 1994). Throughout the 10-year study 951 growth plate fractures were identified and the incidence of growth plate fractures was calculated to be 279 per 100,000 person-years, with males undergoing nearly twice as many fractures as females. The distribution of fractures on the left and right sides was equal and most fractures were sustained through sport and recreational activities. Only 6.4% of the growth plate injuries underwent partial or complete premature growth plate closure.

Two unclassified types of growth plate injury were identified during the time of the Olmstead County study. The first was a fracture of the metaphysis that extended to but not along the growth plate, and the second was an injury in which part or all of the growth plate was missing. These injuries were seen as a result of either a lawnmower injury or gunshot wound (Peterson, 1994a).

As a result of this study, a new classification system was described, including types I to IV of the Salter-Harris classification and incorporating the two new fractures identified by Peterson *et al.* (Peterson, 1994a, b). The Salter-Harris type V injury was removed from the classification system as no injuries fitting that description were identified in the Olmstead County study (Fig. 1.5B).

1.5.2 Treatment of Growth Plate Injuries

The treatment of growth plate injuries largely depends on the age of the patient and the type of injury that has been sustained. If the patient is approaching skeletal maturity, surgical intervention may be unnecessary. However, a young patient with significant growth remaining will require surgical treatment to maximise the growth potential of the limb.

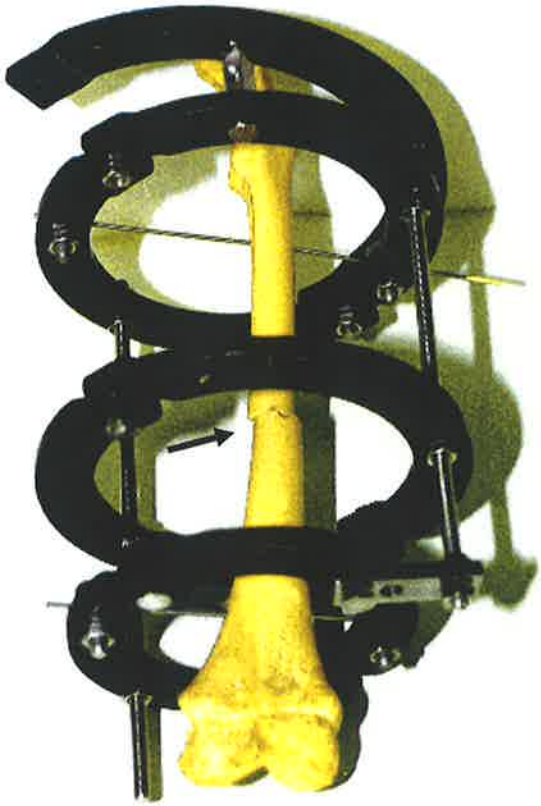
Several treatment modalities are available to correct both limb length and angular deformities, depending on the severity of the irregularity. A slight length discrepancy can be corrected using a shoe lift, however younger patients may require arrest of remaining limb growth in the affected bone to prevent angular deformity occurring. Established angular deformity is often corrected with a wedge osteotomy, while large limb length discrepancies may need a bone lengthening or shortening procedure (Peterson, 1984).

A common procedure for correcting large limb length discrepancies is to lengthen the affected bone to match the growth of the contralateral limb (Ilizarov, 1990). The procedure involves the external placement of a large frame around the affected bone that is anchored to either end of the bone using wires. The shortened bone is then broken within the diaphysis and lengthened using screws on the external frame (Fig. 1.6). This course of action is invasive, time consuming and painful, often resulting in complications including pin site infections, fractures, dislocations and more seriously, compartment syndromes. As only a limited increase in limb length can be achieved during the procedure, the patient often undergoes the procedure several times throughout adolescence until skeletal maturity is reached.

Figure 1.6: (A) The Ilizarov frame is commonly used to correct large limb length discrepancies by lengthening the affected bone to match the growth of the contralateral limb. Once the frame is attached to the bone, the bone is broken within the diaphysis to allow lengthening (arrow). (B) A patient with the Ilizarov frame fixed to the lower leg. The patient lengthens the bone by turning the screws on the outside of the frame (arrowheads). This patient also had the complication of a compartment syndrome.

Photos kindly provided by Assoc. Prof. B.K. Foster.

A



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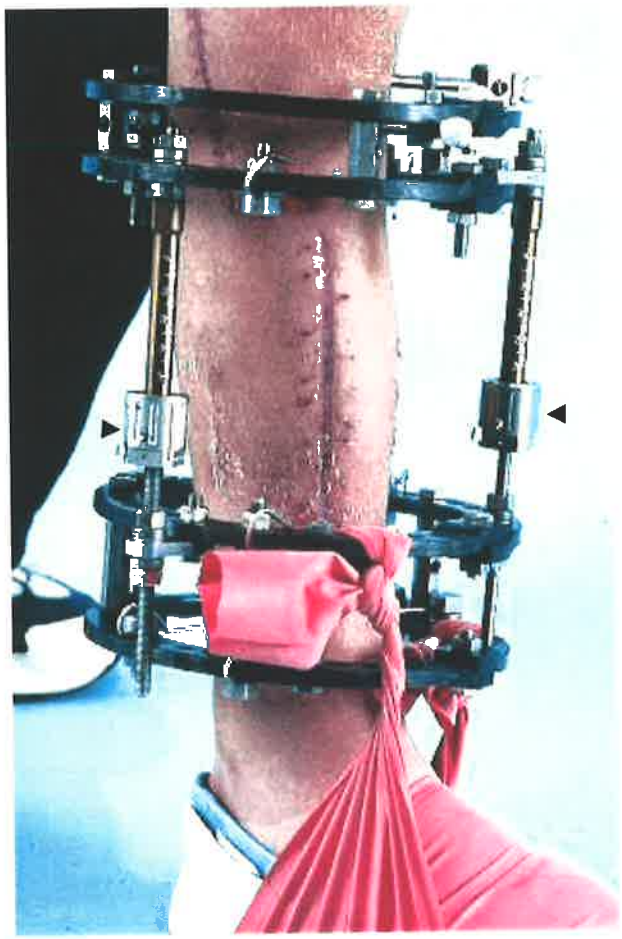


1.5.3 The Langenskiöld Procedure

One of the major causes of limb length discrepancy due to trauma is a result of damage to the growth plate (Moseley, 1987). A fracture through the growth plate can lead to the formation of a bone bridge spanning the growth plate, and the prevention of future growth.

In 1965, Andreas Langenskiöld performed the first clinical operation to remove a bone bridge spanning the growth plate. Based on the work of Lexer who used fat transplants for scar prevention, the hole was filled with autologous fat and the result was a correction of 10 degrees of recurvatum deformity and no reformation of the bone bridge (Langenskiöld, 1967). This operative procedure was based on an observation in a patient with Ollier's disease and subsequent research using a rabbit model that demonstrated that the presence of injured or dead growth plate cartilage prevented the formation of a bone bridge (Langenskiöld and Edgren, 1950). The presence of a material that prevented bone bridge formation also allowed the injured portion to regenerate from the adjacent parts of the growth plate (Langenskiöld and Edgren, 1950; Heikel, 1960). Österman (Österman, 1972) further investigated the operative technique used by Langenskiöld in a rabbit model, and determined that if a bone bridge could be removed without injuring the remaining growth plate the progression of deformity could be interrupted. He also concluded that the use of a suitable interpositional material could prevent the reformation of a bone bridge and the deformity could be corrected by future growth.

In 1975, the operative technique was published (Langenskiöld, 1975) and a description of its use in two cases was reported. A further 33 cases were reported in 1979 (Langenskiöld and Österman, 1979). In most of these cases, the angular deformity of patients improved, but there were variable results with regard to limb growth. Often some additional growth in

length occurred following removal of the bone bridge, but it was generally not sufficient to correct the discrepancy in length.

Despite continual review of the criteria for the Langenskiöld procedure based on improved preoperative imaging methods and knowledge of predictive factors, reviews of clinical Langenskiöld procedures have reported fair and poor results in 15% to 43% of patients (Langenskiöld, 1981; Peterson, 1984; Williamson and Staheli, 1990; Hasler and Foster, 2002). The most common causes of failure were incomplete resection or reformation of the bone bridge due to fat displacement or necrosis. Recently, an anticipatory Langenskiöld procedure has been described, in cases where bone bridge formation is likely to occur (Foster *et al.*, 2000). Long-term results from this procedure are not yet available, and thus it is unknown if it is more successful than the original Langenskiöld procedure.

1.6 Growth Plate Repair Mechanisms

Although fracture repair involving bone has been investigated in detail both at the molecular and structural level, very little is known about growth plate repair following fracture. As this is the site that contributes to the majority of longitudinal growth, it is important to know what is occurring, at both the macroscopic and molecular levels. As relatively little is known about growth plate repair, the repair mechanisms of bone and other cartilaginous tissues may provide useful information.

1.6.1 Growth Plate Repair

There are conflicting observations as to the regenerative potential of the growth plate following injury. In 1914 Obata and Heller observed almost complete regeneration of the

growth plate following transplantation of the cartilage (reviewed in (Langenskiöld *et al.*, 1989). However, Haas (in 1919; (Langenskiöld *et al.*, 1989)) and Banks and Compere (Banks and Compere, 1941) concluded that the growth plate cartilage had no capacity for regeneration following surgical attack. Since then the observation of growth plate cartilage regeneration following injury in rabbits, humans and minipigs has been reported within a single laboratory in Finland {Langenskiöld, 1950 #163;Heikel, 1960 #164;Osterman, 1972 #170;Langenskiöld, 1979 #168;Langenskiöld, 1986 #169}.

The process of growth plate repair is dependant on the type of injury sustained. If a fracture occurs in which the fracture plane remains within the growth plate (for example, Salter-Harris Type I), there is little disorganisation seen and healing occurs without major disruption to the function of the growth plate (Wattenbarger *et al.*, 2002).

When the fracture plane continues through the growth plate from the epiphysis to the metaphysis, formation of a bone bridge at the injury site (Gruber *et al.*, 2002), along with vertical septae, cellular debris and irregularity of growth plate organization (Wattenbarger *et al.*, 2002) are seen. The communication of the epiphysis and the metaphysis through the fracture line is believed to be the causative factor for the bone bridge formation. Studies in mouse, rat and sheep models have ascertained the sequence of events leading to bone bridge formation following ablation of a portion of the growth plate (Wirth *et al.*, 1994; Lee *et al.*, 2000; Xian *et al.*, 2003). Following injury, the defect fills with fibrous tissue and there is an infiltration of mesenchymal cells. Small trabeculae form within the defect, which mature with time, and the growth plate adjacent to the injury site degenerates and transforms into a fibrocartilage-like tissue that extends towards the metaphysis. The

process of bone formation is not via the endochondral pathway, as there is an absence of collagen types II and X (Lee *et al.*, 2000; Xian *et al.*, 2003).

1.6.2 Bone Fracture Repair

The fracture repair process of bone has three main phases: inflammation, repair and remodelling. Following fracture, the disruption of local blood flow results in haemorrhage and cell death. Coagulation and platelet activation stop the bleeding, resulting in the formation of a haematoma. The haematoma is a source of signalling molecules that initiate the cascades of cellular events that are critical to fracture healing. The platelets in the clot release inflammatory mediators and angiogenic factors such as TGF-β, FGF-2 and PDGF to promote neovascularisation. These growth factors may also play a role in regulating the proliferation and differentiation of committed mesenchymal cells (Bolander, 1992).

The first stage of the repair phase is the formation of a soft callus, which begins when the swelling subsides and continues until the bony fragments are united by a fibrous cartilaginous tissue and are no longer freely moveable (Bolander, 1992). New bone formation within the fracture callus can proceed via two pathways: intramembranous or endochondral ossification. The mesenchymal cells are stimulated by BMPs to differentiate into osteoblasts and produce bone in an intramembranous fashion, resulting in the soft callus being converted to a hard callus (Bolander, 1992; Sandberg *et al.*, 1993). In mechanically unstable fractures, some mesenchymal cells may become chondrogenic and produce bone via the endochondral pathway (Liu *et al.*, 1995). The new bone forms adjacent to the old bone in a fashion similar to that of intramembranous ossification. The new bone matrix consists mainly of collagen type I, and the production of osteonectin peaks during the ossification process. After the bony fragments are united, the stability

prevents any shortening of the limb, but angulation can still occur. During this time there is a great increase in the vascularity of the fracture site (Bolander, 1992) ready for the initiation of the remodelling phase.

Throughout the remodelling stage the osseous callus undergoes extensive remodelling into lamellar bone by the simultaneous removal and replacement of bone by the osteoblasts and osteoclasts. The callus is gradually resorbed and osteoblasts start to produce new transverse lamellae of bone to unite the ends of the bones (Sandberg *et al.*, 1993).

Various growth factors regulate the fracture repair process. TGF-β is a potent inducer of collagen production, as well as increasing the production of osteopontin, osteonectin and proteoglycans (Massague, 1990). It also has the ability to inhibit the formation and activation of osteoclasts, resulting in a net effect of extracellular matrix accumulation (Pfeilschifter *et al.*, 1988). Within the fracture repair process, TGF-β is initially released from platelets into the hematoma where it stimulates synthesis of itself and other growth factors by the mononuclear cells present at the injury site. The growth factors released from the mononuclear cells may then influence the inflammatory response (Joyce *et al.*, 1990). TGF-β also regulates the progression of intramembranous ossification, playing a role in the stimulation of bone matrix synthesis, as well as regulating chondrocyte proliferation and differentiation, and cartilage matrix synthesis. Taken together, these observations suggest that TGF-β may regulate the transition from cartilage to bone production during the endochondral ossification stage of fracture healing (Joyce *et al.*, 1990).

1.6.3 Articular Cartilage Repair

Articular cartilage is remarkably durable, but once injured, has a very limited ability to self-repair. Following injury there are two requirements for effective healing: the presence of specific cells to remove necrotic material and synthesise new tissue, and a vascular supply to provide the specific cells and bioactive molecules required (Newman, 1998). Articular cartilage is limited in its ability to undergo repair as it is avascular, preventing any inflammatory or reparative response, and the chondrocytes are embedded in a matrix of collagens and proteoglycans that prevents cell migration to the injury site. The density and avascular nature of articular cartilage allows it to effectively protect the surfaces of bones, but also contributes to its inability to regenerate (Newman, 1998; Ghivizzani *et al.*, 2000).

At the present time, the main treatment strategies for repair of articular cartilage injuries focus on correcting the two deficiencies of articular cartilage: bringing in new cells for chondrogenesis and facilitating access to the vascular system (Newman, 1998). Shaving of the damaged cartilage, penetration of the subchondral bone, tissue transplantation, chondrocyte or mesenchymal stem cell implantation and the use of synthetic and biological matrices are some of the methods that are currently used to promote healing or are under investigation as potential therapies (Buckwalter and Mankin, 1998; Newman, 1998).

As cartilaginous structures have a limited ability to repair, it leaves them vulnerable to deformity once injured. In the case of the growth plate, an injury early in childhood can destroy the mechanism for growth, with patients relying on mechanical intervention to reverse the pathology.

1.6.4 The Use of Osteogenic Protein-1 in Cartilage Repair

Osteogenic protein-1 (OP-1; also known as BMP-7) was originally identified as a potent inducer of bone in ectopic assays (Urist, 1965), and is now used to facilitate the healing of non-union bone fractures (Friedlaender *et al.*, 2001).

In addition to its powerful bone healing properties, the chondrogenic potential of OP-1 has been well documented (Chen *et al.*, 1993; Flechtenmacher *et al.*, 1996; Grgic *et al.*, 1997; Louwerse *et al.*, 2000; Hidaka *et al.*, 2001; Jelic *et al.*, 2001). *In vitro* studies in rat calvarial cells, ATDC5 cells, mouse embryonic long bone cultures, transplanted perichondrium cultures and articular cartilage cultures have reported an increase in the synthesis of type II collagen and proteoglycans following the administration of rhOP-1 (Asahina *et al.*, 1993; Chen *et al.*, 1993; Dieudonne *et al.*, 1994; Asahina *et al.*, 1996; Flechtenmacher *et al.*, 1996; Haaijman *et al.*, 1997; Klein-Nulend *et al.*, 1998a; Klein-Nulend *et al.*, 1998b). Additional studies have determined that the composition of the proteoglycans produced is the same as those produced by normal untreated chondrocytes (Lietman *et al.*, 1997). The increase in type II collagen and proteoglycan synthesis is paralleled with an increase in the mRNA expression for aggrecan, hyaluronan synthase-2 and CD44, molecules necessary for matrix retention (Nishida *et al.*, 2000a).

Recently, the OP-1 mRNA has been cloned into an adenovirus vector and used to modify bovine articular chondrocytes (Hidaka *et al.*, 2001). The adenovirus-modified chondrocytes induced OP-1 mRNA expression and bioactive protein, resulting in increased cartilage-specific matrix gene expression and matrix synthesis (Hidaka *et al.*, 2001).

The repair of articular cartilage defects *in vivo* using rhOP-1 has been reported in rabbits, goats, sheep and dogs (Grgic *et al.*, 1997; Louwerse *et al.*, 2000; Mason *et al.*, 2000; Jelic *et al.*, 2001; Cook *et al.*, 2003). The defect is repaired through the infiltration of mesenchymal cells that differentiate into cells resembling articular cartilage chondrocytes. In thyroid defects in dogs, bone, cartilage and ligament-like tissue have all been formed following the administration of recombinant OP-1 (Katic *et al.*, 2000). The newly formed tissues integrated well with the host cartilage, indicating that the remodelling of the cartilage matrix is stimulated by the recombinant OP-1 (Katic *et al.*, 2000; Jelic *et al.*, 2001). A study by Mason *et al.* reported the use of a combined gene therapy and tissue engineering approach to repair articular cartilage defects in rabbits (Mason *et al.*, 2000). A retroviral vector incorporating the OP-1 gene was introduced into mesenchymal stem cells and seeded onto a polyglycolic acid graft which was implanted into an osteochondral defect in rabbits. Complete or near complete regeneration of the bone and cartilage was seen in the grafts containing the OP-1 modified cells, compared to poor repair by the control grafts (Mason *et al.*, 2000).

Recently, rhOP-1 has been inserted into growth plate defects in sheep to promote regeneration of the growth plate cartilage adjacent to the introduced defect (Johnstone *et al.*, 2002). The results showed an increase of the growth plate width until the expansion of the growth plate cartilage ceased due to the formation of a bone bridge within the defect. The rhOP-1 was administered in a type I collagen paste, which provided a template on which bone formation occurred. If the interpositional material was changed to a substance that reduced the likelihood of bone formation, the use of rhOP-1 to repair and regenerate the growth plate may prove successful.

1.7 Significance of the Project, Aims and Hypothesis

The current treatments for limb length abnormalities resulting from growth plate fracture aim to correct the abnormality after it has occurred, rather than preventing it from arising. The treatments are traumatic to both the patient and their families, often need repeating throughout the adolescent period until skeletal maturity is reached, and do not restore the growth plate with normal function. This project investigates the histological and molecular changes to the growth plate and defect following the Langenskiöld procedure, and the use of the growth factor rhOP-1 in conjunction with this procedure to regenerate growth plate cartilage.

The aims of this project were to:

1. Use histological analysis to determine the effects the Langenskiöld procedure has on the remaining growth plate and within the defect area.
2. Use immunohistochemistry and *in situ* hybridisation to establish the molecular changes that occur within the remaining growth plate and the defect following the Langenskiöld procedure.
3. Determine the histological and molecular changes that occur following the administration of rhOP-1 in conjunction with the Langenskiöld procedure.

It is hypothesised that addition of rhOP-1 will promote repair of the growth plate through the synthesis of cartilage-specific molecules. The interpositional fat used in the Langenskiöld procedure will prevent the formation of bone within the defect site.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Surgical Reagents

The following antibiotics and analgesics were obtained from:

Benzyl penicillin	CSL Ltd. (Victoria, Australia)
Gentomycin	David Bull Laboratories (Victoria, Australia)
Lethabarb (sodium pentobarbitone)	Virbac (New South Wales, Australia)
Xylazene	Troy Laboratories Pty Ltd. (New South Wales, Australia)

2.1.2 Histochemical Reagents

The following reagents were obtained from:

APES (3'-aminopropyletriethoxysilane)	Sigma Chemical Co. (USA)
Eosin Y C.I. 45380	BDH Laboratory Supplies (Poole, England)
Haematoxylin C.I. 75290	Surgipath Medical Industries, Inc. (Richmond, Illinois, USA)

2.1.3 Histochemical Solutions

The solutions routinely used were kindly donated by the Department of Histopathology at the Women's and Children's Hospital (South Australia, Australia). They were made using distilled water and were as follows:

Lithium carbonate	10 mls of saturated stock solution and 40 mls of distilled water

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	50 mls of stock eosin and 50 mls of water

2.1.4 Antiserum

The following antisera were kindly provided by:

Deer type X collagen	Dr Gary Gibson (Henry Ford Clinic, Detroit, USA)
Human αI (I) collagen (LF-39)	Dr Larry Fisher (National Institutes of Health, Maryland,USA)
Human biglycan	Dr Larry Fisher (National Institutes of Health, Maryland,USA)
Human decorin	Dr Larry Fisher (National Institutes of Health, Maryland,USA)
Mouse osteogenic protein-1	Dr David Reuger (Stryker Biotech, Mass., USA)
Mouse osteonectin (LF-23)	Dr Larry Fisher (National Institutes of Health, Maryland,USA)

Human osteopontin (LF-124)	Dr Larry Fisher (National Institutes of Health, Maryland,USA)
2B6	ICN Immunobiologicals (Costa Mesa, Ca., USA)
5D4	ICN Immunobiologicals (Costa Mesa, Ca., USA)
7D4	Dr Bruce Caterson (University of Cardiff, Wales)

The following antisera were produced within our laboratory:

Matrix Gla Protein

Osteocalcin

The following antisera were purchased from:

Horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin	Silenus Labs (Melbourne, VIC, Australia)
Horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulin	Silenus Labs (Melbourne, VIC, Australia)
Biotin-conjugated goat anti-rabbit immunoglobulin	DAKO (Australia) Pty. Ltd. (NSW, Australia)
Biotin-conjugated rabbit anti-mouse immunoglobulin	DAKO (Australia) Pty. Ltd. (NSW, Australia)
Streptavidin	DAKO (Australia) Pty. Ltd. (NSW, Australia)

2.1.5 RNA Chemicals

The following reagents were obtained from:

10 x digoxigenin labelling mix	Boehringer Mannheim (Germany)
10 x Transcription buffer	Boehringer Mannheim (Germany)
T3, T7, Sp6 RNA polymerases	Boehringer Mannheim (Germany)
Anti digoxigenin conjugated alkaline phosphatase (Fab fragments)	Boehringer Mannheim (Germany)
Control RNA	Boehringer Mannheim (Germany)
Diethyl pyrocarbonate (DEPC)	Sigma Chemical Co. (USA)
Levamisole	Sigma Chemical Co. (USA)
NBT/BCIP	Boehringer Mannheim (Germany)
RNasin	New England Biolabs (Beverly, Mass., USA)
RNeasy Mini Kit	Qiagen (Hilden, Germany)
ULTRAhyb	Ambion Inc. (Texas, USA)

2.1.6 Plasmids and cDNAs for Riboprobes

The following plasmids and cDNAs were kind gifts from the following people:

Human osteopontin (HOP)	Dr. Larry Fisher (National Institutes of Health, Maryland, USA)
Human osteonectin (hon-2)	Dr. Larry Fisher (National Institutes of Health, Maryland, USA)
Human type II collagen (HC22)	Dr. Shireen Lamandè (University of Melbourne, Victoria, Australia)
Human type X collagen (pGEMhCOLX)	Dr. Shireen Lamandè (University of Melbourne, Victoria, Australia)

Mouse matrix gla protein cDNA	Dr. Thorston Schinke (Baylor College of Medicine, Texas, USA)
Rat pro-α1(I) collagen cDNA	Prof. David Findlay (Royal Adelaide Hospital, South Australia, Australia)

2.1.7 Electrophoresis

The following materials were obtained from:

Acrylamide solution (LiquiGel)	Gradipore (Pyrmont, NSW, Australia)
Agarose, DNA grade	Progen Industries Ltd. (Darra, Qld, Australia)
Ammonium persulphate	Bio-Rad Life Science Group (Hercules, California, USA)
Bromophenol blue	BDH Chemicals Ltd. (Poole, Dorset, England)
Ethidium bromide	Amresco (Solon, Ohio, USA)
Formaldehyde	Ajax Chemicals (Australia)
Formamide	Ajax Chemicals (Australia)
TEMED (N,N,N^1,N^1-tetramethyl-ethylenediamine)	Bio-Rad Laboratories (Hercules, Calif., USA)
SPP-1 *Eco*RI, pUC19 *Hpa*II size markers	Geneworks (Adelaide, SA, Australia)
Trans-Blot® Transfer Medium	Bio-Rad Laboratories (Hercules, Calif., USA)

2.1.8 Radiochemicals

[α^{32}P]-dUTP (10mCi/ml)	DuPont NEN Research Products (Boston, Mass. USA)

2.1.9 Enzymes

The following enzymes were obtained from:

Alkaline phosphatase	Boehringer Mannheim (Germany)
Restriction endonucleases	Boehringer Mannheim (Germany) and New England Biolabs (Beverly, Mass., USA)
Proteinase K	Boehringer Mannheim (Germany)
T4 DNA ligase	Boehringer Mannheim (Germany)
DNase I, RNase free	Boehringer Mannheim (Germany)

2.1.10 Buffers and Solutions

Unless otherwise stated, all buffers were made in milli Q filtered water. The buffers and solutions routinely used were as follows:

10 x DNA loading buffer	50% (v/v) glycerol, 1 mM EDTA pH 8.0, 0.25% (w/v)bromophenol blue
10% buffered formalin	40% (w/v) formaldehyde solution was diluted 1/10 in PBS pH 7.2
Decalcification solution (EDTA/CPC)	5% (w/v) EDTA, 2% (w/v) cetylpyridinium chloride in 10% buffered formalin
Decalcification solution (formic acid)	10% (v/v) formic acid, 5% (v/v) formaldehyde

Detection buffer (for *in situ* hybridisation)	0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5
LiCl lysis buffer	2.5 M LiCl, 50 mM Tris-HCl, 62.5 mM EDTA, 4% (v/v) Triton X-100, pH 8.0
Phosphate buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2
SDS-PAGE electrophoresis buffer	25 mM Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3
SDS-PAGE sample buffer	1% (w/v) SDS, 4 M urea, 80 mM Tris-HCl pH 6.8, 0.1% (w/v) bromophenol blue
SDS-PAGE transfer buffer	10 mM CAPS pH 11.0, 10% methanol
SDS-PAGE wash buffer	0.25 M NaCl, 0.02 M Tris, pH 7.0
20 x SSC	3 M NaCl, 0.3 M tri-sodium citrate, pH 7.0
TAE	40 mM Tris-acetate, 2mM EDTA, pH 8.5
TE	10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5

2.1.11 Bacterial Media

L-broth	1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl, pH 7.5
L-Agar	L-broth, 1.5% (w/v) Bacto agar

All bacterial media was autoclaved at 121 ℃ for 20 minutes.

Bacto tryptone, Bacto yeast extract and Bacto agar were obtained from DIFCO Laboratories (Detroit, Mich., USA).

2.1.12 Antibiotics

Ampicillin was obtained from Boehringer Mannheim (Germany). A 1000x stock solution was made by dissolving 1 gram of ampicillin in 10 mls of sterile water. The solution was stored at -20°C in 1 ml aliquots and used at a final concentration of 100 µg/ml.

2.1.13 Miscellaneous Materials, Chemicals and Kits

The following reagents were obtained from:

4-chloro-1-napthol	Sigma Chemical Co. (USA)
ABTS (2,2 azine-di(3-ethylbenzhiazoline sulfonic acid))	Sigma Chemical Co. (USA)
Bio-Rad Dye Reagent Concentrate	Bio-Rad Life Science Group (Hercules, California, USA)
Bovine serum albumin (BSA)	Sigma Chemical Co. (USA)

Chondroitinase ABC Lyase	ICN Immunobiologicals (Costa Mesa, Ca., USA)
Diaminobenzidine (DAB)	Sigma Chemical Co. (USA)
GeneClean II kit	BIO 101 (Vista, California, USA)
Herring sperm DNA	Sigma Chemical Co. (USA)
Nylon filters (GeneScreen*Plus*™)	DuPont NEN Research Products (Boston, Mass., USA)
Optiphase Hisafe scintillation fluid	Fisons Chemicals (Homebush, NSW, Australia)
Plasmid purification kits	Qiagen (Hilden, Germany) and Geneworks (Adelaide, SA, Australia)
Sephadex® G-50 beads	Pharmacia Biotech AP (Uppsala, Sweden)
SuperFrost® Plus slides	Menzel-Glaser (Germany)
Triton X-100	Ajax Chemicals (Australia)
Whatman GF/C filters	Whatman International Ltd (England)

All other chemicals not listed were of analytical grade and purchased from Ajax Chemicals, BDH Chemicals Ltd., Bio-Rad Life Science Group, Boehringer Mannheim or Sigma Chemical Co..

2.2 Surgical Procedures

Merino cross sheep were 12 – 16 weeks of age. All procedures were sanctioned by the institutional animal ethics committee and performed under sterile conditions. The animal ID numbers and the treatment they received are listed in Table 2.1.

2.2.1 Creation of the Growth Plate Defect

Animals were sedated by intravenous injection of thiopentone sodium BP (25 mg/kg) into the external jugular vein. After intubation (12.2 mm outside diameter endotracheal tube) general anaesthesia was induced with halothane (1.5% in 2:1 oxygen:air, 12 L/min). A longitudinal incision was made on the medial side of the left leg, exposing the proximal tibia. The periosteum of the bone was separated, exposing the growth plate. A 2 mm dental burr was used to create a defect 10 mm deep x 10 mm wide x 2 mm high. Bone fragments were removed using irrigation (sterile water) to minimise heat damage to the growth plate during drilling. Titanium Kirschner wires (K-wires) were inserted proximally and distally to the defect, 20 mm apart.

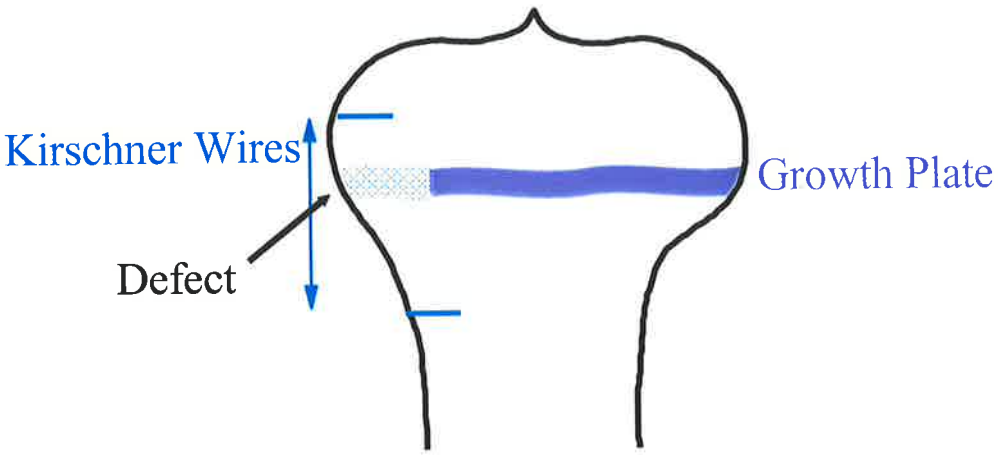
Fat was taken from the knee fat pad and placed in the defect. The periosteum was replaced and sutured to enclose the fat in the defect. Nine animals had rhOP-1 (350 μg/100 μl PBS) injected at the interface of the healthy growth plate and the interpositional fat. Nine additional animals had 100 μl PBS injected without rhOP-1. The selection of animals to receive rhOP-1 was blinded, with the contents of the vials not revealed until after all the data was collected. Three sheep had only Kirschner wires inserted to measure the normal rate of growth, as controls. The subcutaneous tissue was closed and sutured and the wound cleaned with Betadine to prevent infection. Intramuscular injections of gentomycin (1.6

Table 2.1 Animal ID Numbers and Treatment Conditions

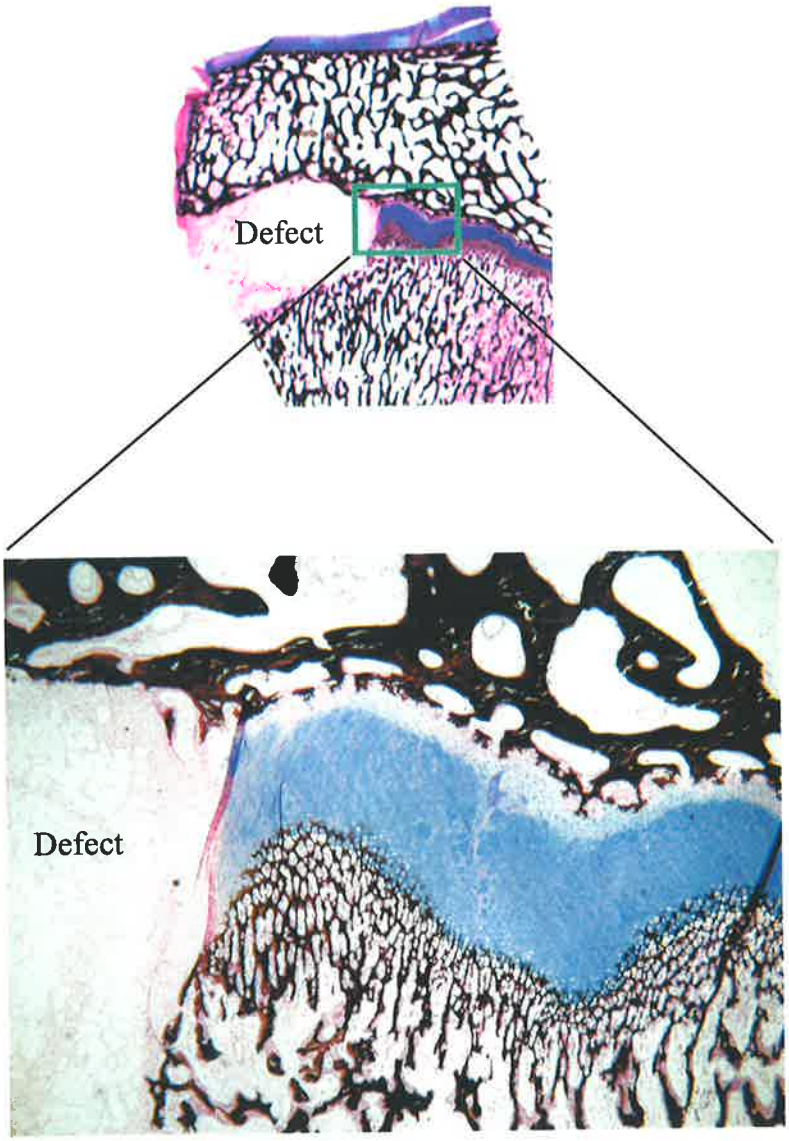
Animal ID Number	Treatment	Day of Sacrifice
102	Defect + rhOP-1	7
103	Defect + PBS	7
104	Defect + rhOP-1	14
105	Defect + PBS	14
107	Defect + PBS	56
108	Defect + rhOP-1	56
111	K-wires only	56
112	K-wires only	56
113	Defect + PBS	56
114	Defect + rhOP-1	56
115	K-wires only	56
116	Defect + PBS	56
117	Defect + rhOP-1	56
118	Defect + PBS	14
119	Defect + rhOP-1	14
120	Defect + rhOP-1	14
121	Defect + PBS	14
122	Defect + rhOP-1	7
123	Defect + PBS	7
124	Defect + PBS	7
139	Defect + rhOP-1	7

Figure 2.1: Schematic (A) and histological (B) representation of the surgical procedure. (A) The proximal medial growth plate in the tibia of the sheep was exposed and a 2 mm dental burr was used to ablate a 10 mm deep x 10 mm wide x 2 mm high area of the growth plate. Kirschner wires were inserted proximally and distally to the defect, 20 mm apart. (B) A von Kossa/ haematoxylin and eosin stained section showing the defect area and the remaining growth plate. The area within the green box is shown at higher magnification below the section. This orientation is used in all figures throughout this thesis.

A



B



mg/kg) and benzyl penicillin (0.4 mg/kg) were administered at the conclusion of the surgical procedure and for 4 days post surgery. Xylazene (20 mg/kg at 0.25 ml/day) was administered intravenously via syringe driver for three days post surgery.

2.2.2 *Euthanasia, Fixation and Collection of Tissue*

Animals that received rhOP-1 or PBS alone were sacrificed at days 7 (n=3), 14 (n=3) and 56 (n=3). The control animals were sacrificed at day 56. The animals were sedated and general anaesthesia was induced as described in 2.2.1. The femoral artery and vein were exposed and the vein clamped. The femoral artery was catheterised with a 0.8 mm intravenous catheter and 1000 units of heparin injected. The femoral vein was catheterised to allow flow through of fluid during fixation. The leg was perfused with 200 mls of 0.9% sodium chloride followed by 200 mls of 10% buffered formalin. The animal was sacrificed by an overdose of Lethabarb (Virbac (Australia), Peakhurst, NSW, Australia). The hind limb was removed and the proximal tibia excised using a bone saw. The excised portion was then cut longitudinally into 2 mm thick sections using an Isomet Low Speed Saw (Buehler Ltd., Lake Bluff, Il., USA) and placed in 10% buffered formalin for a period of 2 days. One section was placed in decalcification solution (5% (w/v) EDTA/ 2% (w/v) CPC or 10% (v/v) formic acid/ 5% (v/v) formaldehyde) for one week, with the solution changed daily. A second undecalcified section was kept for embedding in methylmethacrylate resin. The remaining sections were stored in 70% (v/v) ethanol until used.

2.3 Histopathology Methods

2.3.1 Processing of Tissue for Paraffin Embedding

After decalcification, the bone sections to be embedded in paraffin were placed in plastic embedding cassettes. The sections were processed by a Tissue Tek VIP processor overnight under pressure and vacuum. The conditions are outlined in Table 2.2. After processing the bone sections were embedded in paraffin wax.

2.3.2 Processing of Tissue for Methylmethacrylate Embedding

After fixation in 10% buffered formalin for 2 days, the sections were cut to size and placed in polypropylene vials for processing. All processing was performed at 4°C unless otherwise stated. The bone was incubated in 70% (v/v) acetone for 4 hours, 95% acetone for 4 hours, then absolute acetone for 3 days, with the acetone changed daily. Methylmethacrylate was washed with 5% NaOH, followed by 3 changes of distilled water and filtering through calcium chloride. The bone sections were infiltrated with 4 mls of methylmethacrylate and 0.4 mls of polyethylene glycol 400 and incubated overnight. The bone sections were polymerised in 4 mls of methylmethacrylate, 0.4 mls of polyethylene glycol 400 and 0.044 mls of K-Plast. The tubes were placed in a petri dish of water and incubated overnight at 37°C. The tubes were cut open, the blocks flattened using a coarse file, then attached to metal block holders with Araldite.

2.3.3 Preparation of APES Coated Slides

Clean microscope slides were loaded into racks and immersed in ethanol for 5 minutes at room temperature. The slides were transferred to ethanol containing 2% (v/v) APES

Table 2.2: Processing conditions for bone samples

Solution	Time	Temperature
70% ethanol	45 minutes	35°C
85% ethanol	1 hour	35°C
95% ethanol	45 minutes	35°C
95% ethanol	1 hour 15 minutes	35°C
100% ethanol	45 minutes	35°C
100% ethanol	1 hour	35°C
100% ethanol	1 hour 30 minutes	35°C
Chloroform	1 hour 30 minutes	35°C
Chloroform	1 hour 15 minutes	35°C
Wax	1 hour 15 minutes	60°C
Wax	1 hour 45 minutes	60°C

solution for 10 seconds, rinsed in ethanol and washed in distilled water. Slides were dried in an oven at 60°C overnight.

2.3.4 Sectioning of Tissue

Paraffin sections (5 μm) were cut on a Leitz 1512 microtome. The sections were floated on a 50°C waterbath and transferred to APES coated or SuperFrost Plus™ slides. The slides were left to dry overnight at room temperature.

Samples embedded in methylmethacrylate resin were cut by Peter McNeil at the Adelaide Spinal Research Centre located within the Institute for Medical and Veterinary Sciences in Adelaide.

2.3.5 Staining of Paraffin Embedded Tissue

2.3.5.1 Haematoxylin and Eosin

Sections were dewaxed in xylene and rehydrated through a series of ethanol washes (2 x 100% and 70%) to water. Sections were stained in haematoxylin for 2 minutes, rinsed in water and differentiated in lithium carbonate. After rinsing in water, the sections were stained in eosin for 2 minutes and again rinsed in water. The sections were dehydrated through ethanol and xylene and mounted using DPX mounting solution.

2.3.5.2 Sirius Red

Sections were dewaxed in xylene and rehydrated through a series of ethanol washes (2 x 100% and 70%) to water. After staining in Mayer's haematoxylin for 3 minutes, the sections were rinsed in water and differentiated in lithium carbonate. The sections were

stained in Sirius Red (1% (v/v) in saturated aqueous picric acid) solution for 30 minutes, rinsed in water, dehydrated in ethanol, cleared in xylene and mounted using DPX mounting solution.

2.3.6 *Staining of Methylmethacrylate Resin Embedded Tissue*

Haematoxylin and eosin, von Kossa, and haematoxylin and eosin/von Kossa staining was performed by Peter McNeil at the Adelaide Spinal Research Centre located within the Institute for Medical and Veterinary Sciences in Adelaide, using standard techniques.

2.4 Protein Methods

2.4.1 *Immunohistochemistry*

Paraffin embedded sections were dewaxed in xylene and rehydrated through a series of ethanol washes (2 x 100%, 90% and 70%) to water. The sections were incubated in 3% hydrogen peroxide for 1 hour at room temperature to inhibit the endogenous peroxidase activity. The sections were blocked in 20% horse serum for 1 hour at room temperature followed by 1 hour at 37°C in Chondroitenase ABC (0.05U/ml in 0.1 M Tris acetate, pH 8.0). The primary antibody was diluted in 20% horse serum (dilutions shown in Table 2.3) and incubated on sections overnight at 4°C in a humid chamber. The sections were washed twice in 1 x PBS for 10 minutes at room temperature. The secondary antibody was diluted in 20% horse serum and incubated on sections for 1 hour at room temperature in a humid chamber. The sections were washed twice in 1 x PBS for 10 minutes at room temperature and streptavidin (diluted 1:1000 in 20% horse serum) incubated on sections for 1 hour at room temperature in a humid chamber. The sections were washed twice in 1 x PBS for 10

Table 2.3 Dilution Factors of Antibody Sera used in Immunohistochemistry

Antibody Against	Monoclonal or Polyclonal	Dilution Factor
Type I Collagen	Polyclonal	1:1200
Type II Collagen	Monoclonal	1:200
Type X Collagen	Polyclonal	1:10000
Osteopontin	Polyclonal	1:800
Osteonectin	Polyclonal	1:1200
Decorin	Polyclonal	1:600
Biglycan	Polyclonal	1:400
Osteogenic Protein-1	Monoclonal	1:400
2B6	Monoclonal	1:1000
7D4	Monoclonal	1:10000
5D4	Monoclonal	1:5000

minutes at room temperature. DAB substrate was added to sections and left at room temperature until colour developed. The sections were rinsed in water, counterstained in Mayer's haematoxylin, differentiated in lithium carbonate, dehydrated and coverslipped.

2.4.2 Determination of Antibody Cross-Reactivity

2.4.2.1 Western Blot Analysis

Protein samples (100 μg) were mixed with 25% sample buffer and 5% β-mercaptoethanol. The samples were separated on a SDS-PAGE gel containing 12.5% polyacrylamide for approximately 3 hours at 200 V or until the bromophenol blue had reached the bottom of the gel. The gel was equilibrated in transfer buffer then transferred onto nitrocellulose membrane (Trans-Blot® Transfer Medium) for 1 hour at 0.5 V. Non-specific protein binding sites were masked by incubating the membrane in 5% BSA for 1 hour at room temperature. The membrane was washed three times in wash buffer and then incubated overnight at 4°C with an appropriate dilution of the required antibody (Table 2.3). The membrane was again washed three times in wash buffer and incubated with a secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin or horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulin) for 1 hour at room temperature. The blot was washed three times in wash buffer and developed using 4-chloro-1-napthol substrate (18 mg dissolved in 20 mls of methanol, then 5.1 mls combined with 24.9 mls wash buffer and 9.9 μl hydrogen peroxide). After sufficient colour development, the reaction was stopped by washing the membrane in several changes of water.

2.4.2.2 ELISA

A 96 well ELISA plate was coated with 100 µg of antigen per well (diluted in 0.1 M sodium bicarbonate) and incubated overnight at 4°C. The plate was washed three times with 0.02 M Tris, 0.25 M NaCl then blocked with 1% BSA, 0.02 M Tris, 0.25 M NaCl (100 µl/well) for 1 hour at room temperature. After three washes in 0.02 M Tris, 0.25 M NaCl, a serial dilution of the primary antibody was added and incubated at room temperature for 4 hours. The plate was washed in 0.02 M Tris, 0.25 M NaCl three times and 100 µl of secondary antibody (diluted 1:1000 in 1% BSA) added and incubated at room temperature for 1 hour. The plate was washed three times in 0.02 M Tris, 0.25 M NaCl and 150 µl of ABTS added. The plate was incubated for 20 minutes at room temperature, then read at 414 nm on a Ceres 900 Autoreader and Microplate Workstation (Bio-Tek Instruments Inc., Winooski, Vt., USA).

2.5 RNA Methods

All solutions for RNA methods were prepared using DEPC treated water. One litre of ddH_2O was incubated overnight at 37°C following the addition of 1 ml of DEPC. The DEPC was inactivated through autoclaving at 121°C for 20 minutes.

2.5.1 Northern Blot Hybridisation

2.5.1.1 Northern transfer

Samples containing 5 –10 µg of RNA were mixed with sample buffer (formamide, formaldehyde, 10 x MOPS) and denatured at 60°C for 5 minutes. After quenching on ice, 10 x loading dye was added and the samples were separated at 80 volts on a 1.2% formaldehyde gel in 1 x MOPS buffer until the bromophenol blue had migrated a sufficient

distance to allow separation of the RNA fragments. The gel was rinsed in 0.1 M ammonium acetate for 30 minutes followed by staining for 30 minutes in ethidium bromide, β-mercaptoethanol. The gel was destained in 0.1 M ammonium acetate for 30 minutes and visualised and photographed using an ultraviolet transilluminator (ULTRA-LUM Inc, Carson, Calif., USA) with a Polaroid MP-4 Land Camera containing 667 film. The gel was rinsed in water for five minutes prior to setting up the blot. The RNA was transferred to the nylon filter (GeneScreen *Plus*™) by capillary blotting overnight using 10 x SSC.

2.5.1.2 Labelling of probes

Probes were synthesised using the Strip-EZ™ RNA T7/T3 kit (Ambion), according to the maunfacturer's instructions.

2.5.1.3 Separation of unincorporated nucleotides

G50 columns were prepared by placing 2 pieces of Whatman GF/C glass microfibre filters into the bottom of a 1 ml syringe. The syringe was placed in a 10 ml screw cap tube and filled with Sepharose G50 beads. The tube was centrifuged at 1500 rpm for 2 minutes. The column was washed with 200 µl of TE and centrifuged and 1500 rpm for a further 2 minutes. The end of the syringe was placed in a screw cap eppendorf tube, and then both placed into the 10 ml screw cap tube. This in turn was placed in a 50 ml falcon tube with the lid on. The labelled riboprobe was added to the column and the total volume made up to 50 µl with TE. The column was centrifuged at 1500 rpm for 3 minutes and the labelled probe collected.

2.5.1.4 Scintillation counting

The sample containing radioisotopes (1 µl) was counted following the addition of 4 mls of OptiPhase HiSafe scintillation fluid in a Wallac 1409 scintillation counter.

2.5.1.5 Hybridisation

The nylon filter was placed in a hybridisation bottle with 6 mls of ULTRAhyb and incubated at 68°C for 1 hour. The probe (3.4 µCi) was added to the hybridisation tube and incubated at 68°C overnight.

2.5.1.6 Washing of Filter and Autoradiography

The filter was washed twice in 2 x SSC, 0.1% (v/v) SDS for 5 minutes at 68°C. Two washes in 0.1 x SSC, 0.1% SDS for 15 minutes at 68°C followed if high levels of non-specific binding were present. The filter was sealed in plastic and exposed to x-ray film for 1 day to 1 week at -80°C in an autoradiography cassette with enhancing screens.

2.5.1.7 Stripping of Filter

When required, the filter was stripped of hybridised probe using the Strip-EZ RNA T7/T3 kit as per the manufacturer's instructions.

2.5.2 Riboprobe Construction

Riboprobes were constructed from clones that were generously provided from researchers listed in section 2.1.6. The mRNA sequences from available species were aligned using the multiple sequence analysis program, eclustalw (www.angis.org.au). A region of sequence showing homology between the species was selected and exposed to a FASTA search

(www.angis.org.au) to ensure it was not homologous to any other known genes. The sequence was excised from the clone provided using restriction endonucleases and ligated into the pBlueScript II SK(+) vector (section 2.6.3 and 2.6.4).

As the riboprobes were constructed from mRNA from species other than sheep, Northern hybridisation (section 2.5.1) was used to check that the riboprobes were cross-reactive with sheep mRNA. RNA was isolated from resting, proliferative and hypertrophic chondrocytes of sheep using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

2.5.3 *Riboprobe Synthesis for* in situ *Hybridisation*

Plasmid (1 μg) was linearised by restriction endonuclease digestion (as described in 2.6.1). The eppendorf tube containing the linearised plasmid was placed on ice, and the following chemicals added: 2 μl 10 x DIG labelling mix, 2 μl 10 x transcription buffer, 1 μl RNasin and 2 μl of RNA polymerase. The total volume was made up to 20 μl with water. The solution was mixed, then incubated at 37°C for 2 hours. The tube was chilled on ice and 1 μl of 0.5 M EDTA pH 8.0 was added to stop the reaction. The riboprobe was precipitated overnight at -20°C after the addition of 2.5 μl 3 M sodium acetate pH 5.2, 75 μl 100% ethanol and 2 μl 10 mg/ml tRNA. The riboprobe was centrifuged at 13000 rpm for 30 minutes at room temperature and the supernatant removed. The tube was inverted, air dried, and the pellet resuspended in 100 μl of water. The riboprobe was stored at –20°C. The riboprobe was electrophoresed (as described in section 2.6.2) to check the riboprobe size.

A spot test was used to estimate the yield of the DIG-labelled riboprobes. DIG-labelled control RNA and the synthesised riboprobe were prediluted to a concentration of 20 ng/µl. Serial dilutions were made to a concentration of 0.01 pg/µl. Each dilution (1 µl) was spotted onto a nylon membrane and air dried. The membrane was blocked in 1% BSA for 30 minutes at room temperature and incubated in the α-DIG-alkaline phosphatase (Fab fragments) antibody (1:5000 in 1% BSA) for 30 minutes at room temperature. The membrane was washed in 1% BSA for 10 minutes at room temperature and then blocked in detection buffer for 2 minutes at room temperature. NBT/BCIP substrate (200 µl/10 mls) was added to the membrane and incubated in the dark until colour developed. The spot intensities of the control and experimental dilutions were compared to estimate the concentration of the riboprobes.

2.5.4 In situ *Hybridisation*

Paraffin embedded sections were baked in a dry oven at 60°C for 72 hours. Sections were dewaxed in xylene and rehydrated through a series of ethanol washes (2 x 100% and 70%) to water. The sections were incubated in 0.2 M HCl for 20 minutes at room temperature. Proteinase K (10 µg/ml) was added to the sections and incubated at 37°C for 1 hour in a humid chamber followed by 8U/ml DNase I for 1 hour at 37°C in a humid chamber. The prehybridisation solution containing 50% formamide, 5 x SSC, 10 µg/ml herring sperm DNA was added to the sections and incubated for 2 hours at 42°C in a humid chamber. Riboprobes (400 ng/ml) were added to the hybridisation mix of 50% formamide, 5 x SSC, 10 µg/ml herring sperm DNA and denatured at 80°C for 5 minutes. The prehybridisation solution was washed from the sections with 5 x SSC for 5 minutes at room temperature. The hybridisation solution was cooled to 50°C, added to the sections and incubated in a

humid chamber overnight at 42°C. The sections were washed in 0.5 x SSC, 1 mM EDTA for 5 minutes at 42°C followed by 15 minutes at room temperature in 50% formamide, 0.15 M NaCl, 5 mM Tris-HCl pH 7.4, 0.5 mM EDTA. The sections were washed in 0.5 x SSC for 20 minutes at 42°C then 0.5 x SSC for 5 minutes at room temperature. The sections were blocked in BSA (1%) in 1 x PBS for 30 minutes to 1 hour at room temperature. The α-DIG-alkaline phophatase (Fab fragments) antibody was diluted 1:1000 (in 1% BSA in 1 x PBS) and incubated on the sections in a humid chamber for 2 hours at room temperature. The sections were washed twice in 1 x PBS for 10 minutes at room temperature and blocked in detection buffer for 2 minutes at room temperature. NBT/BCIP solution (200 µl/ml) and 5 µl/ml 1 M levamisole was added to the sections. The sections were placed in a dark humid chamber and incubated overnight at room temperature. The sections were washed in 1 x PBS for 1 hour at room temperature, rinsed in water and counterstained for 30 seconds with 1% neutral red. After washing in water for 10 minutes, the sections were air dried and coverslipped using Loctite® 349 adhesive.

If insufficient staining was observed, additional matrix degradation was performed using 0.05U/ml Chondroitenase ABC (diluted in 0.1 M Tris acetate, pH 8.0) for 1 hour at 37°C. This step was performed immediately after the rehydration of the slides.

2.6 General Methods

2.6.1 Restriction Endonuclease Digestion of DNA

Restriction endonuclease digestion of DNA preparations was performed using the conditions specified for each enzyme as detailed by the manufacturer.

2.6.2 Agarose Gel Electrophoresis of DNA

Electrophoresis of DNA was performed in Kodak Biomax QS710 tanks for horizontal submerged gel electrophoresis. Samples were mixed with 10 x loading buffer and separated on agarose gels of 0.8 – 1.5% (w/v), depending on the size of the DNA fragments, until the bromophenol blue had migrated sufficient distance to allow appropriate separation of the desired DNA fragments. The gel was stained in ethidium bromide (0.5 μg/ml), rinsed in deionised water and visualised and photographed using an ultraviolet transilluminator (ULTRA-LUM Inc, Carson, Calif., USA) with a Polaroid MP-4 Land Camera containing 667 film. For preparative gels, the required DNA fragment was excised from the gel and purified using the GeneClean II kit, following the manufacturer's instructions.

2.6.3 Plasmid Vector Preparation for DNA Ligation

The plasmid was digested with the desired restriction endonuclease(s) to completion. Digestion was checked by agarose gel electrophoresis and stained with ethidium bromide (0.5 μg/ml). To prevent re-circularisation of the plasmid during ligation, the ends were dephosphorylated following restriction endonuclease digestion. Dephosphorylation was accomplished by adding alkaline phosphatase (10 U) to the reaction. The reaction mix was incubated at 37°C for 30 minutes. The plasmid was then extracted with TE saturated phenol/chloroform and precipitated with 2.5 volumes of absolute ethanol in the presence of 0.3 M sodium acetate (pH 5.2). The sample was stored at -20°C until required.

2.6.4 DNA Ligation

Ligations were performed in the presence of 1 x ligation buffer (Boehringer Mannheim) and T4 DNA ligase (1 unit, Boehringer Mannheim) in a total reaction volume of 20µl. The linearised plasmid DNA and the insert DNA preparations were semi-quantitated via agarose gel electrophoresis, then combined in plasmid:insert molar ratios of 1:1 and 1:3. A control ligation reaction containing the linearised, dephophorylated vector, T4 DNA ligase and no insert was included. Ligation reactions were performed for 4 – 6 hours at room temperature or overnight at 4°C. Ligation reactions were stored at -20°C until transformation.

2.6.5 Preparation of MC1061 Competent Cells

MC1061 *E. coli* cells were made competent by inoculating pre-warmed L-broth 1/100 with a fresh MC1061 overnight culture. The culture was incubated at 37°C with aeration until the cells had grown to an OD_{600} of 0.2. The cells were pelleted at 2500 x g for 10 minutes at 4°C, then resuspended in 0.5 volume of ice-cold transformation buffer (50 mM $CaCl_2$, 10 mM PIPES-HCl pH 6.6, 15% glycerol) and incubated on ice for 30 minutes. The cells were pelleted and resuspended in 0.05 volume of ice-cold transformation buffer. The cells were aliquoted, snap frozen in liquid N_2 and stored at -80°C until used.

2.6.6 Transformation of MC1061 Competent Cells

Competent MC1061 cells (100 µl, section 2.6.5) were thawed on ice. Ligated plasmid (5 µl, section 2.6.4) was added and mixed by pipetting, then incubated on ice for 10 minutes. Following heat shock at 37°C for 5 minutes, an equal volume of L-broth was added and the mixture incubated for 30 – 60 minutes at 37°C. The mixture was spread onto a 10 cm L-

agar plate containing 100 μg/ml ampicillin, allowed to dry, inverted and incubated overnight at 37°C for colony development.

2.6.7 Plasmid Preparations

Minipreps:

Single colonies were inoculated into 2 mls of L-broth containing 100 μg/ml ampicillin and grown overnight at 37°C with aeration. A 500 μl aliquot of the culture was pelleted by a 1 minute microcentrifugation, and resuspended in 100 μl of LiCl lysis buffer. The solution was extracted with an equal volume of TE saturated phenol/chloroform (1:1) and the aqueous layer (95 μl) transferred to an eppendorf tube containing 60 μl of isopropanol. The DNA was vortexed and precipitated by microcentrifugation at maximum speed for 10 minutes at room temperature. The pellet was washed with 70% (v/v) ethanol, air dried and resuspended in 20 μl of TE buffer.

Large Scale Plasmid Preparations:

Single colonies were inoculated into 2 mls of L-broth containing 100 μg/ml ampicillin and grown 6 – 8 hours at 37°C with aeration. A 500 μl aliquot of this culture was transferred into 50 – 100 mls of L-broth containing 100 μg/ml ampicillin and grown overnight at 37°C with aeration. The culture was centrifuged to pellet the bacterial cells (6000 rpm for 15 minutes at room temperature) and the plasmid DNA purified using plasmid preparation kits (QIAGEN or Geneworks) according to the manufacturer's instructions.

2.7 Statistical Analysis

2.7.1 Growth Plate Measurements

The height of the growth plate was measured using the Image-Pro® PLUS software (Media Cybernetics). The measurements were taken in the area of expansion adjacent to the defect, but did not include an area if it had formed a medial spur. An area of the growth plate at the most distant region of the growth plate was also measured to represent normal growth plate height. The Image-Pro® PLUS software measures and averages 150 measurements in a defined area. The measurements were compared for statistical significance using a one-way ANOVA with a significance level of $p < 0.05$.

2.7.2 Bone Measurements

Bone volume and surface area was calculated using the Quantimet system at the Institute of Medical and Veterinary Science, Adelaide, Australia under the guidance of Mr. Ian Parkinson and Associate Professor Nick Fazzalari. The measurements were compared for statistical significance using a one-way ANOVA with a significance level of $p < 0.05$.

Chapter 3

Histological Analysis Following the Langenskiöld Procedure

Introduction

The analysis of pathology following growth plate injury and the development of strategies to promote growth plate repair relies on the availability of good animal models. The requirements for an animal model to evaluate limb length and angular deformities are (1) a large skeleton that undergoes sufficient growth post injury and (2) an animal with similar morphological and histological characteristics to humans.

Several animal models of growth plate injury have been described, including rats (Phieffer *et al.*, 2000), mice (Cundy *et al.*, 1991), rabbits (Langenskiöld and Edgren, 1950; Österman, 1972) and sheep (Peltonen *et al.*, 1984; de Pablos *et al.*, 1986; Foster, 1989; Foster *et al.*, 1990; Hansen *et al.*, 1990; Wirth *et al.*, 1994; Johnstone *et al.*, 2002). In general, studies using small animal models have failed due to additional fractures of the bones following surgical treatment (Sledge and Noble, 1978), and the smaller animals do not undergo sufficient growth to allow observation of deformity. The sheep is an animal of generous size and allows for growth following the surgical procedure. It matures rapidly to adult height and its size allows the surgical procedure to be performed easily. Also, the bones of sheep have histological characteristics similar to that of human (Peltonen *et al.*, 1984; de Pablos *et al.*, 1986). The hind limb of the sheep is ideal to study angulation and shortening deformities, as there is only one long bone present, the tibia. Therefore, growth occurs from a single bone and any abnormalities that develop are not complicated by the presence of an adjacent bone (Sisson, 1975).

If an injury to the growth plate is sustained, it will heal without major disruption to future growth provided the fracture plane remains within the growth plate (Wattenbarger *et al.*,

2002). However, if the fracture line is through the entire growth plate, osteoclasts and osteoblasts infiltrate from the epiphysis and metaphysis and bone formation occurs across the growth plate (Wattenbarger *et al.*, 2002). It is this bone formation that contributes to limb shortening and angulation deformities. For normal limb growth to continue, the bone that has formed at the injury site must be removed. The Langenskiöld procedure removes the bone at the injury site and replaces it with an interpositional material that prevents subsequent bone formation. Various materials have been investigated for this purpose, including fat (Langenskiöld and Österman, 1979; Langenskiöld, 1981; Foster, 1989; Foster *et al.*, 1990; Foster *et al.*, 2000), muscle (Martiana *et al.*, 1996), silastic rubber (Lee *et al.*, 1993), methyl methacrylate (Cabanela *et al.*, 1972; Bueche *et al.*, 1990), periosteum (Wirth *et al.*, 1994), hyaline cartilage (Lennox *et al.*, 1983), physeal graft (Lee *et al.*, 1993) and chondrocyte transplants (Foster *et al.*, 1990; Lee *et al.*, 1998). Indomethacin has also been used to prevent bone bridge formation within growth plate defects (Sudmann *et al.*, 1982). For clinical applications, fat appears to be the most widely used material for this purpose (Langenskiöld, 1975; Langenskiöld and Österman, 1979; Langenskiöld, 1981; Langenskiöld *et al.*, 1986; Foster, 1989; Foster *et al.*, 2000; Hasler and Foster, 2002). It is readily available subcutaneously and has the advantage of being autologous (Foster, 1989).

Several studies conducted within our laboratory have used a sheep model to investigate growth plate repair mechanisms and treatment options to prevent or reverse skeletal deformity (Foster, 1989; Foster *et al.*, 1990; Hansen *et al.*, 1990; Wirth *et al.*, 1994; Johnstone *et al.*, 2002). Various interpositional materials were inserted to prevent bone bridge formation and promote growth plate regeneration, including fat (Foster, 1989), chondrocyte cultures (Foster *et al.*, 1990; Hansen *et al.*, 1990), cartilage (Wirth *et al.*, 1994), periosteum (Wirth *et al.*, 1994) and a type I collagen paste (Johnstone *et al.*, 2002).

Interposition of fat, chondrocyte cultures and cartilage prevented the reformation of bone at the defect site, however growth plate regeneration was not achieved (Foster, 1989; Foster *et al.*, 1990; Hansen *et al.*, 1990; Wirth *et al.*, 1994). Periosteum interposition resulted in bone bridge formation, along with disorganisation of the growth plate adjacent to the defect (Wirth *et al.*, 1994). The type I collagen paste was used in conjunction with recombinant human OP-1 (rhOP-1), a growth factor shown to induce chondrogenesis, both *in vitro* (Chen *et al.*, 1993; Dieudonne *et al.*, 1994; Flechtenmacher *et al.*, 1996; Klein-Nulend *et al.*, 1998a; Klein-Nulend *et al.*, 1998b; Hidaka *et al.*, 2001) and *in vivo* (Grgic *et al.*, 1997; Louwerse *et al.*, 2000; Jelic *et al.*, 2001; Cook *et al.*, 2003). In the sheep model, 14 days after surgery there was an increase in growth plate height adjacent to the defect, suggesting a chondrogenic response to the administration of rhOP-1. However, the type I collagen paste acted as a template for bone formation, and the defects of all animals, both rhOP-1 treated and untreated, filled with bone (Johnstone *et al.*, 2002).

For this study, the requirements for an interpositional material were that it was an inert substance, readily available and could prevent the formation of bone within the defect area. Previous studies have shown that the use of fat successfully inhibits bone bridge formation (Langenskiöld and Österman, 1979; Langenskiöld *et al.*, 1986; Foster, 1989; Foster *et al.*, 2000). Furthermore, the study by Foster (Foster, 1989) using the sheep model demonstrated that there is sufficient fat in the knee pad behind the proximal growth plate of the tibia to fill the created defect, preventing the need for a second incision, and thereby simplifying the procedure.

The purpose of this study was to investigate the mechanisms of growth plate repair and regeneration following the Langenskiöld procedure, and evaluate the effect the addition of

rhOP-1 has on the repair process. Histological analysis of the remaining growth plate, defect site and surrounding bone was conducted to examine the molecular events in the growth plate repair pathway, and the effect of rhOP-1 on this process.

Results

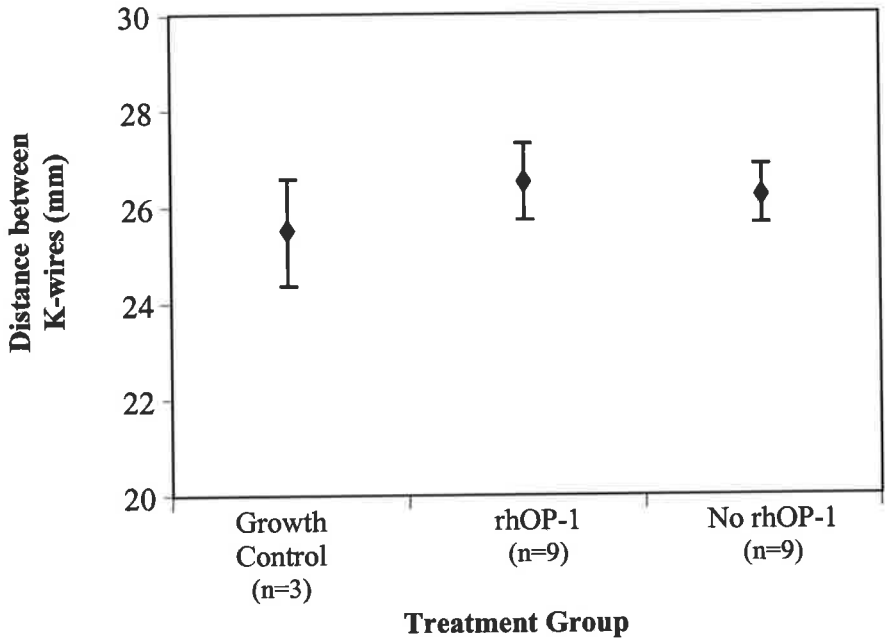
The surgical method developed by Foster (Foster, 1989) based on the original procedure by Langenskiöld (Langenskiöld, 1975) with some modifications was used to examine growth plate repair (see section 2.2 for details). In similar growth plate injury studies, complete bone bridge formation has been seen in mice (Lee *et al.*, 2000), rats (Garces *et al.*, 1994; Xian *et al.*, 2003), rabbits (Jaramillo *et al.*, 1990) and dogs {Staelmaier, 1995 #269} 2 or 3 weeks after surgery. Previous studies in sheep by Johnstone *et al.* and Wirth *et al.* reported an infiltration of inflammatory cells 4 days after the surgical procedure, and intramembranous ossification within the defect was seen as early as 14 days post surgery (Wirth *et al.*, 1994; Johnstone *et al.*, 2002). Using these observations as a guide, the time points chosen for this study were to represent the three main stages of fracture repair; inflammation (day 7), repair (day 14) and remodelling (day 56).

3.1 Limb Growth

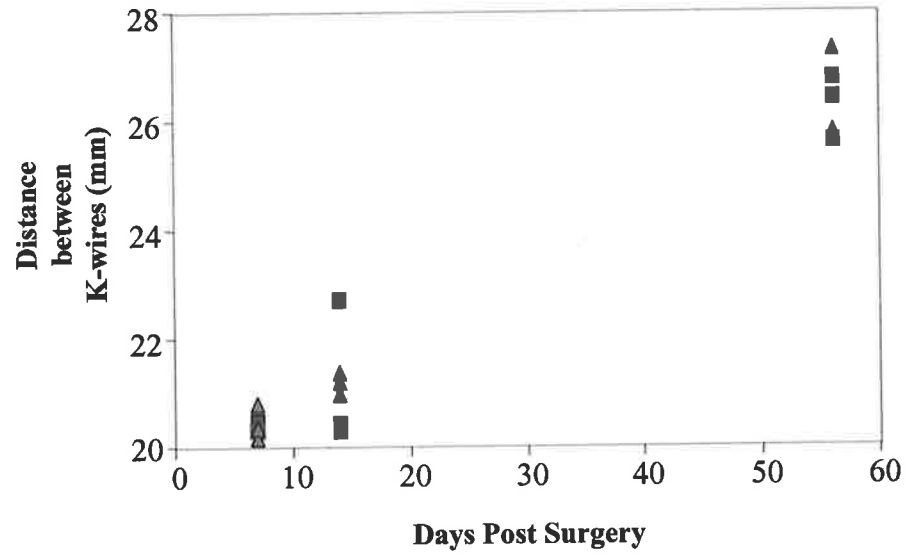
The positioning of K-wires either side of the growth plate at the time of surgery allowed accurate measurement of limb growth. The limbs of the normal animals continued to grow throughout the experimental period, with the K-wires separating an additional 5.46 ± 1.11 mm (mean ± standard deviation) at 56 days post surgery (Fig. 3.1A). A similar distance was recorded for the animals that had the Langenskiöld procedure performed, both with

Figure 3.1: Comparison of average limb growth rates of the treatment groups. K-wires were inserted 20 mm apart proximally and distally to the growth plate at the time of surgery. (A) The total limb growth (mean ± standard deviation) at day 56 for the different treatment groups. (B) Comparison of limb growth rates at 7, 14 and 56 days post surgery with (▲) and without (■) rhOP-1 treatment.

A


30
28
26
24
22
20
Distance between K-wires (mm)
Growth Control (n=3)
rhOP-1 (n=9)
No rhOP-1 (n=9)
Treatment Group

B


28
26
24
22
20
Distance between K-wires (mm)
0
10
20
30
40
50
60
Days Post Surgery

and without rhOP-1 treatment (Fig. 3.1A). When the limb length of the rhOP-1 treated and untreated animals was compared at days 7, 14 and 56, there was no significant difference between those animals treated with rhOP-1 and those that received phosphate buffered saline (PBS) alone at any of the three time points (Fig. 3.1B). The growth of the limbs proceeded in a linear manner throughout the time course, averaging 0.1 mm of growth per day.

3.2 Histological Observations

A histological assessment of the model was performed to establish any differences between the rhOP-1 treated and untreated animals following the Langenskiöld procedure, and to examine the changes that occur at 7, 14 and 56 days after surgery. Methylmethacrylate-embedded sections were stained with von Kossa/haematoxylin and eosin and paraffin-embedded sections were stained with haematoxylin and eosin and viewed under a light microscope (section 2.3.6 and 2.3.5.1). The degree of bone bridge formation, growth plate expansion, the response of the growth plate, a description of the defect site and the response of the surrounding bone were included in the histological evaluation of the surgical treatment. The sections were evaluated based on the method described by Foster (Foster, 1989), as outlined in the following paragraphs.

3.2.1 Bone Bridge Formation

Following the ablation of the growth plate, the formation of a bone bridge was characterised based upon the site and degree of bone formation. The site of bone formation was classified as either central (in the defect immediately adjacent to the remaining growth plate) or peripheral (in the defect adjacent to the periosteum). The degree of bone

formation was categorised based on the amount of bone that was present in the defect adjacent to either the growth plate or the periosteum (Fig. 3.2). The absence of bone within the defect was classified as no response (0; Fig. 3.2A), small islands of bone in the defect represented a weak response (+; Fig. 3.2B), a thin spicule of bone that would micro-fracture and not prevent future growth was an intermediate response (++; Fig. 3.2C) and a complete bone bridge spanning the defect from the epiphysis to the metaphysis was a strong response (+++; Fig. 3.2D).

At day 7, only one untreated animal (ID #124) showed evidence of bone within the defect (Fig. 3.2B, Table 3.1). It was a weak response, with a small island of bone present within the defect adjacent to the growth plate. Four animals at day 14 (two treated with rhOP-1 and two untreated) had central trabecular bone formation ranging in severity from small islands of bone to a thin spicule spanning the growth plate (Fig. 3.2B,C, Table 3.1). At day 56, two rhOP-1 treated animals and one untreated animal exhibited bone formation (Table 3.1). One rhOP-1 treated animal (ID #117) demonstrated a complete bone bridge spanning the defect from the epiphysis to the metaphysis (Fig. 3.2D). It is uncertain whether this bone bridge formed as a result of fat displacement and subsequent hematoma formation within the defect, or as a result of rhOP-1 treatment. The other two bone responses were thin spicules of bone that would micro-fracture throughout growth, and therefore would not result in the cessation of future growth plate function.

Treatment with rhOP-1 did not increase the likelihood of bone formation, as the untreated animals developed bone within the defect in as many cases as the rhOP-1 treated animals. The severity of bone formation between the animals treated with rhOP-1 compared to the

Figure 3.2: Bone formation within the defect. The degree of bone formation within the defect was characterised based upon the amount of bone present adjacent to the remaining growth plate. (A) A day 7 untreated animal with no bone formation within the defect, (B) a day 14 untreated animal with small islands of bone representing a weak response (+), (C) a day 56 untreated animal with thin spicules of bone that would micro-fracture and not prevent future growth (++), and (D) a day 56 rhOP-1 treated animal with a complete bone bridge spanning the growth plate from the epiphysis to the metaphysis (+++). The defect is represented by (*) and the bar represents 200 μm. Methyl methacrylate sections stained with von Kossa/haematoxylin and eosin.

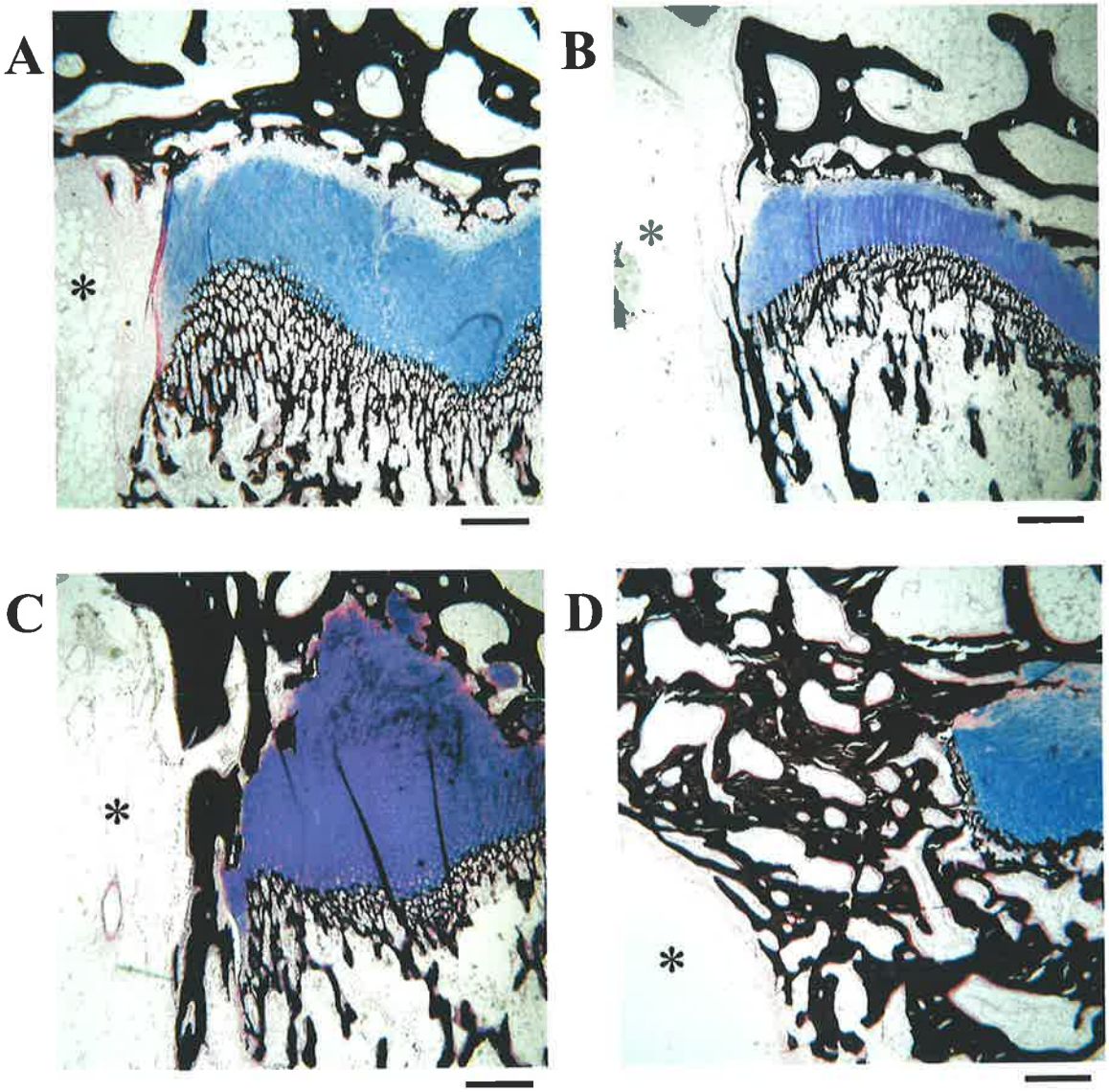

A
B
C
D
*
*
*
*

Table 3.1: Bone Bridge Formation

Day 7

	#103	#123	#124	#102	#122	#139
rhOP-1 Treatment	-	-	-	+	+	+
Site	-	-	C	-	-	-
Degree of Formation	-	-	+	-	-	-

Day 14

	#105	#118	#121	#104	#119	#120
rhOP-1 Treatment	-	-	-	+	+	+
Site	C	C	-	C	C	-
Degree of Formation	++	+	-	++	++	-

Day 56

	#107	#113	#116	#108	#114	#117
rhOP-1 Treatment	-	-	-	+	+	+
Site	C	-	-	C	-	C
Degree of Formation	++	-	-	++	-	+++

Animals were rhOP-1 treated (+) or untreated (-). The site of the bone bridge was described as either central (C) or peripheral (P). The degree of formation of the bone bridge was (+) for small spicules of bone adjacent to the growth plate, (++) for a thin spicule of bone that grew across the growth plate but would fracture during growth and (+++) was a complete bone bridge which spanned the epiphysis to the metaphysis.

untreated animals was similar, apart from the complete bone bridge seen in a the rhOP-1 treated animal at day 56 (ID #117).

3.2.2 Expansion of the Host Growth Plate

To establish whether rhOP-1 promotes cartilage outgrowth in the defect, measurements of growth plate height were taken adjacent and distal to the injury site. The distal region of the growth plate was used as a control to denote normal height. An increase in growth plate height adjacent to the injury was taken as a measure of growth plate outgrowth, since lateral outgrowth could not be measured accurately due to a lack of landmarks for the original boundary of the defect. Methylmethacrylate-embedded sections of the growth plate, defect and surrounding bone were stained with von Kossa/ haematoxylin and eosin (section 2.3.6). The average growth plate height adjacent and distal to the defect was measured using Image-Pro® PLUS software (section 2.7.1). Several sections from each animal were measured to allow for section variation. In the case of medial spur formation, growth plate height measurements were taken from a region of normal chondrocyte architecture and not from areas that resembled fibro-cartilage. Statistical significance was determined by one-way ANOVA with Tukey's post hoc tests using the statistical package SPSS for Windows, Version 10.0.5.

3.2.2.1 Total Height of the Growth Plate

Post injury a gradual increase in growth plate height adjacent to the defect in rhOP-1 treated animals was observed (Table 3.2), with the growth plate height observed at day 56 (875.2 μm ± 188.7 μm) significantly greater than at day 7 (572.0 μm ± 72.5 μm; $p < 0.01$). The untreated animals remained the same height at days 7 and 14, and then underwent a significant increase in growth plate height by day 56 (538.5 μm ± 71.5 μm to 744.5 μm ±

Table 3.2: Growth plate height adjacent to the defect in rhOP-1 treated and untreated groups

Days Post Surgery	Untreated Group	rhOP-1 Treated Group
7	573.2 μm ± 119.8 μm	572.0 μm ± 72.5 μm
14	538.5 μm ± 71.5 μm	674.4 μm ± 144.9 μm ***
56	744.5 μm ± 303.8 μm **	875.2 μm ± 188.7 μm *

Results are expressed as mean ± standard deviation of n=3 animals per group except day 7 rhOP-1 treated group (n=2 animals).

* rhOP-1 treated group at day 56 shows a significant increase in average height compared to the rhOP-1 treated group at day 7 (one way ANOVA, $p < 0.01$)

** Untreated group at day 56 shows a significant increase in average height compared to the untreated group at day 14 (one way ANOVA, $p < 0.05$)

*** rhOP-1 treated group at day 14 shows a significant increase in average height compared to the untreated group at day 14 (one way ANOVA, $p < 0.01$)

303.8 μm $p < 0.05$). The increase in growth plate height observed at day 56 in the untreated animals was due to one animal that exhibited variable growth plate heights adjacent to the defect (animal ID #107). Nine different sections from this animal were measured with growth plate measurements ranging from 395.7 μm to 1422.6 μm (Table 3.3). No other animal in either the rhOP-1 treated or untreated group displayed such variation in growth plate height.

A comparison of growth plate height between the rhOP-1 treated group and the untreated group revealed that there was no statistically significant difference in growth plate height at day 7 or day 56. However, at day 14 the growth plate height in the rhOP-1 treated group (674.4 μm ± 144.9 μm) was significantly greater than that of the untreated group (538.5 μm ± 71.5 μm; $p < 0.01$; Table 3.2).

The increase in growth plate height was localised to the area immediately adjacent to the defect, and growth plate height at the distant aspect of the growth plate showed no significant difference between the rhOP-1 treated and untreated groups at the time points assayed (Table 3.4).

Although not a significant difference, the height of growth plates can vary between animals; therefore to eliminate the inter-animal growth plate height variation, the difference in growth plate height was calculated by subtracting the distal growth plate height from the growth plate height immediately adjacent to the defect. The values for individual animals are shown in Figure 3.3 and support the results shown in Table 3.2. At each time point, the rhOP-1 treatment group displayed a greater average increase in growth plate height than the untreated group, including a significant increase at day 14 (232.1 μm

Table 3.3: Growth plate measurements of sheep #107

Distant growth plate	Growth plate adjacent to defect	Difference in height
364.6 μm	548.6 μm	184.0 μm
405.6 μm	797.0 μm	391.4 μm
340.0 μm	1069.6 μm	729.6 μm
521.1 μm	1422.6 μm	901.5 μm
539.1 μm	1121.2 μm	582.1 μm
382.3 μm	790.4 μm	408.1 μm
504.1 μm	745.2 μm	241.1 μm
396.6 μm	395.7 μm	-0.9 μm
397.6 μm	678.4 μm	280.8 μm
427.9 μm ± 73.4 μm	**841.0 μm ± 314.8 μm**	**413.1 μm ± 282.6 μm**

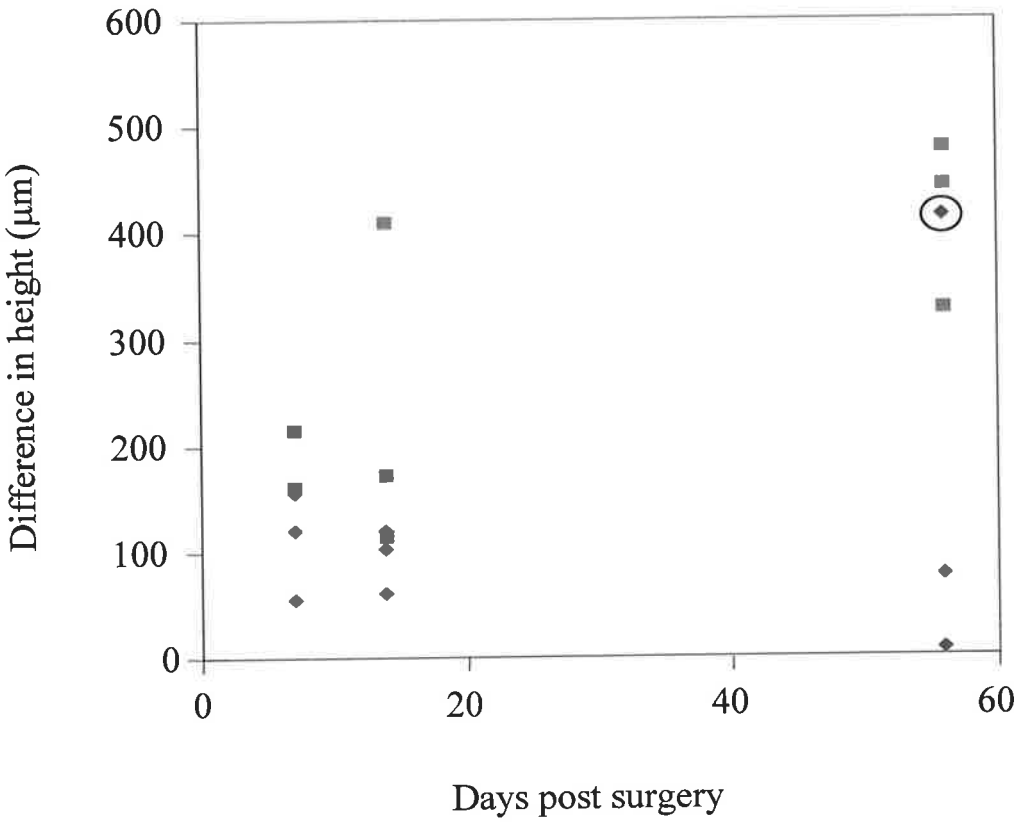
Nine different sections were measured using the Image-Pro® PLUS software with 150 measurements generated for each of the sections. The average of these measurements is shown above. The values shown in bold represent the mean ± standard deviation of the sections listed.

Table 3.4: Growth plate height at the most distant aspect from the defect site in rhOP-1 treated and untreated groups

Days Post Surgery	Untreated Group	rhOP-1 Treated Group
7	461.0 μm ± 102.8 μm	374.9 μm ± 47.4 μm
14	452.5 μm ± 75.2 μm	442.3 μm ± 40.6 μm
56	461.9 μm ± 128.2 μm	442.6 μm ± 69.3 μm

Results are expressed as mean ± standard deviation of n=3 animals per group except day 7 rhOP-1 treated group (n=2 animals). No significant difference was observed between the rhOP-1 treated and untreated groups.

Figure 3.3: Comparison of the average difference in growth plate height adjacent to the defect 7, 14 and 56 days post surgery for rhOP-1 treated (■) and untreated (◆) sheep. Methylmethacrylate-embedded sections were stained with von Kossa/H&E and the growth plate height was measured adjacent to the defect using the Image-Pro® PLUS software. Sections (ranging from 2 to 9 per animal) were measured and the average for 150 measurements per section was generated. There are 3 animals per group, except in the rhOP-1 treated group at day 7 (n=2). The circled value represents animal ID #107, which displayed greater variation in growth plate height adjacent to the defect.


600
500
400
300
200
100
0
Difference in height (µm)
0
20
40
60
Days post surgery

± 160.5 μm compared to 103.7 μm ± 115.7 μm; $p < 0.01$). Again, animal ID #107 (day 56, no treatment) shows an increase in growth plate height that is clearly different from the others in the group and is more similar to that of the rhOP-1 treatment group (circled point, Fig. 3.3). If this animal (ID #107) was removed from the analysis, there would be no increase in growth plate height from day 7 to day 56 in the untreated animals and there would be a statistically significant difference in growth plate height between the rhOP-1 treated and untreated groups at day 56 ($p < 0.0005$). However, as the groups were limited to only three animals per group, this animal was left in the study to allow statistical comparisons to be made.

A rapid increase in growth plate height was observed in the rhOP-1 treated animals in the first seven days of treatment (46% of the total increase at 28.2 μm/day). The untreated animals also showed an increase in growth plate height over the first seven days at a rate of 16.0 μm/day, but the total height increase was only approximately half of the height increase seen in the rhOP-1 treated animals. A further increase in growth plate height of 5 μm/day was seen over the next seven days in the rhOP-1 treated group, compared to no height increase in the untreated animals. Throughout the remaining 42 days of treatment, the growth plate height of the rhOP-1 treated group increased at a rate of 10.3 μm/day, more than twice the rate of the untreated group, which proceeded at 4.3 μm/day, due to the large increase in growth plate height seen in animal ID #107.

3.2.2.2 Proportions of the Resting, Proliferative and Hypertrophic Zones

To determine whether the increase in growth plate height occurred in a specific region, the heights of the resting, proliferative and hypertrophic zones were measured. The different regions were defined according to the following criteria. The resting zone was measured

from the top of the growth plate until the formation of the chondrocyte columns, and the proliferative zone was measured from the commencement of the chondrocyte columns to the last chondrocytes within the columns prior to them increasing in size. The hypertrophic zone was measured from the first chondrocytes increasing in size until the last visible intact chondrocyte on the metaphyseal side of the growth plate. Image-Pro® PLUS software was used, and measurements were taken in the growth plate adjacent to the defect and in the growth plate most distal to the defect, as described previously for total growth plate height (section 3.2.2). The proportion of each zone was calculated as a percentage of the total growth plate height.

The resting, proliferative and hypertrophic zones in the distal area of the growth plate were of similar proportions in both the rhOP-1 treated and untreated groups at all time points. The average percentages were 23.3 ± 5.0% for the resting zone, 51.0 ± 5.6% for the proliferative zone and 25.7 ± 4.4% for the hypertrophic zone.

Seven days after surgery, there were no significant changes in the proportion of the growth plate zones adjacent to the defect in either the rhOP-1 treated or untreated animals (Table 3.5). By day 14, the proportion of the proliferative zone significantly decreased in the rhOP-1 treated animals from 47.1 ± 2.5% in the distal growth plate to 40.7 ± 1.5% in the growth plate adjacent to the defect ($p < 0.05$; Table 3.5). There were no changes observed in the untreated animals. At day 56, there was a significant increase in the proportion of the resting zone in the rhOP-1 treated animals (18.1 ± 4.0% in the distal growth plate to 40.1 ± 3.1% in the growth plate adjacent to the defect, $p < 0.005$) and associated significant decreases in the proliferative (55.0 ± 4.9% in the distal growth plate to 42.2 ± 1.6% in the growth plate adjacent to the defect, $p < 0.05$) and hypertrophic (26.9 ± 3.9% in the distal

Table 3.5: Growth Plate Zone Proportions Adjacent to the Defect

		Resting	Proliferative	Hypertrophic
Day 7	**No rhOP-1**	22.5 ± 8.3	52.7 ± 11.1	24.8 ± 3.1
	rhOP-1	18.2 ± 8.5	59.8 ± 11.1	22.0 ± 2.6
Day 14	**No rhOP-1**	30.8 ± 11.1	53.8 ± 8.1	15.4 ± 4.0
	rhOP-1	33.0 ± 6.5	40.7 ± 1.5 *	26.3 ± 5.9
Day 56	**No rhOP-1**	34.1 ± 13.4	49.7 ± 14.0	16.2 ± 4.3 *
	rhOP-1	40.1 ± 3.1 *	42.2 ± 1.6 *	17.7 ± 2.2 *

The height of each zone was calculated as a percentage of the total growth plate height for each animal, and the average and standard deviation calculated for each group. Results are expressed as mean ± standard deviation of n = 3 animals per group except day 7 rhOP-1 treated group (n=2 animals).

* The proportion of the growth plate zones adjacent to the defect show a significant difference in comparison with that of the normal growth plate zones (one way ANOVA, all $p < 0.05$, except day 56 resting zone when $p < 0.005$).

growth plate to 17.7 ± 2.2% in the growth plate adjacent to the defect, $p < 0.05$) zones (Table 3.5). There was also a significant decrease in the proportion of the hypertrophic zone in the untreated group (26.2 ± 4.0% in the distal growth plate to 16.2 ± 4.3% in the growth plate adjacent to the defect, $p < 0.05$, Table 3.5).

When the growth plate adjacent to the defect of the rhOP-1 treated and untreated animals was compared, there were no significant differences in the proportions of the growth plate zones at any time point. This suggests that the growth plates increased in height in a similar manner, and that rhOP-1 did not affect a specific zone of the growth plate.

3.2.2.3 Changes in Chondrocyte Number Within Each Zone of the Growth Plate

To determine whether the changes in growth plate height were a result of increased cellular proliferation, an area of growth plate adjacent to the defect and within the distal region was selected and the chondrocytes of each zone were counted and converted into the number of cells per square millimetre.

The average number of chondrocytes within each zone of the distal growth plate area was similar between the rhOP-1 treated and untreated animals. The average number of cells was 1271.0 ± 432.8 in the resting zone, 2041.6 ± 377.2 in the proliferative zone and 1518.2 ± 360.2 in the hypertrophic zone.

The number of chondrocytes present in the resting, proliferative and hypertrophic zones did not change significantly in the area adjacent to the defect when compared to the distal area of the growth plate. This number of cells remained constant within each zone throughout the time course in both the rhOP-1 treated and untreated groups (Table 3.6).

Table 3.6: Chondrocyte Numbers in the Growth Plate Zones

		Resting	Proliferative	Hypertrophic
Day 7	**No rhOP-1**	742.0 ± 211.0	2247.2 ± 805.3	1118.7 ± 204.2
	rhOP-1	1164.4 ± 477.5	1662.9 ± 159.9	1070.3 ± 131.9
Day 14	**No rhOP-1**	916.6 ± 240.1	1852.9 ± 103.6	1298.4 ± 279.2
	rhOP-1	643.1 ± 345.1	2169.7 ± 524.0	1056.5 ± 317.5
Day 56	**No rhOP-1**	760.0 ± 308.3	2265.7 ± 776.8	2143.9 ± 539.7
	rhOP-1	990.6 ± 399.1	2523.4 ± 324.7	1234.1 ± 344.2

The number of cells within each zone of a defined area of growth plate were counted and calculated as the number of cells per square millimetre. Results are expressed as mean ± standard deviation of n = 3 animals per group except day 7 rhOP-1 treated group (n=2 animals). The value is the number of chondrocytes per square millimetre in the growth plate adjacent to the defect.

There was no significant difference in the average number of cells per square millimetre between the rhOP-1 treated and untreated animals in any zones at any time point.

This suggests that there is no increase in the rate of cellular proliferation within the growth plate adjacent to the defect.

3.2.3 Changes in Host Growth Plate Architecture

Following ablation, changes to the remaining growth plate were assessed by examining medial spur formation and the transformation of normal growth plate cartilage into fibro-cartilage. A medial spur (also known as tethering) occurs when the growth plate that remains after ablation elongates towards the metaphysis with the future growth of the bone. The medial spur was classified as absent if the growth plate adjacent to the defect remained in the same plane as the unaffected growth plate (0; Fig. 3.4A), present if the growth plate adjacent to the defect had slightly deviated from its normal orientation towards the metaphysis (+; Fig. 3.4B) or markedly elongated when the growth plate adjacent to the defect had deviated a great distance from its original position (++; Fig. 3.4C). The transformation adjacent to the defect from normal growth plate cartilage (0) to fibro-cartilage (+) was described as evident if there was a loss of normal growth plate architecture (Fig. 3.5).

The occurrence of a medial spur did not become apparent until day 56, except for a single untreated animal at day 7 that exhibited a slight tethering (Fig. 3.4B, Table 3.7). Three animals at day 56 (two rhOP-1 treated and one untreated) displayed a markedly elongated medial spur (Fig. 3.4C), and a second untreated animal showed a slight medial spur (Table 3.7).

Eleven of the animals at days 7 and 14 retained normal growth plate architecture adjacent to the defect, including those that displayed expansion of the growth plate (Fig. 3.5A,B).

Figure 3.4: Medial spur formation of the growth plate adjacent to the defect. (A) A day 7 rhOP-1 treated animal without a medial spur (0), (B) a day 7 untreated animal showing the presence of a medial spur (+), and (C) a day 56 rhOP-1 treated animal with a markedly elongated medial spur (++). The defect is represented by (*) and the bar represents 400 μm. Methyl methacrylate sections stained with von Kossa/haematoxylin and eosin.

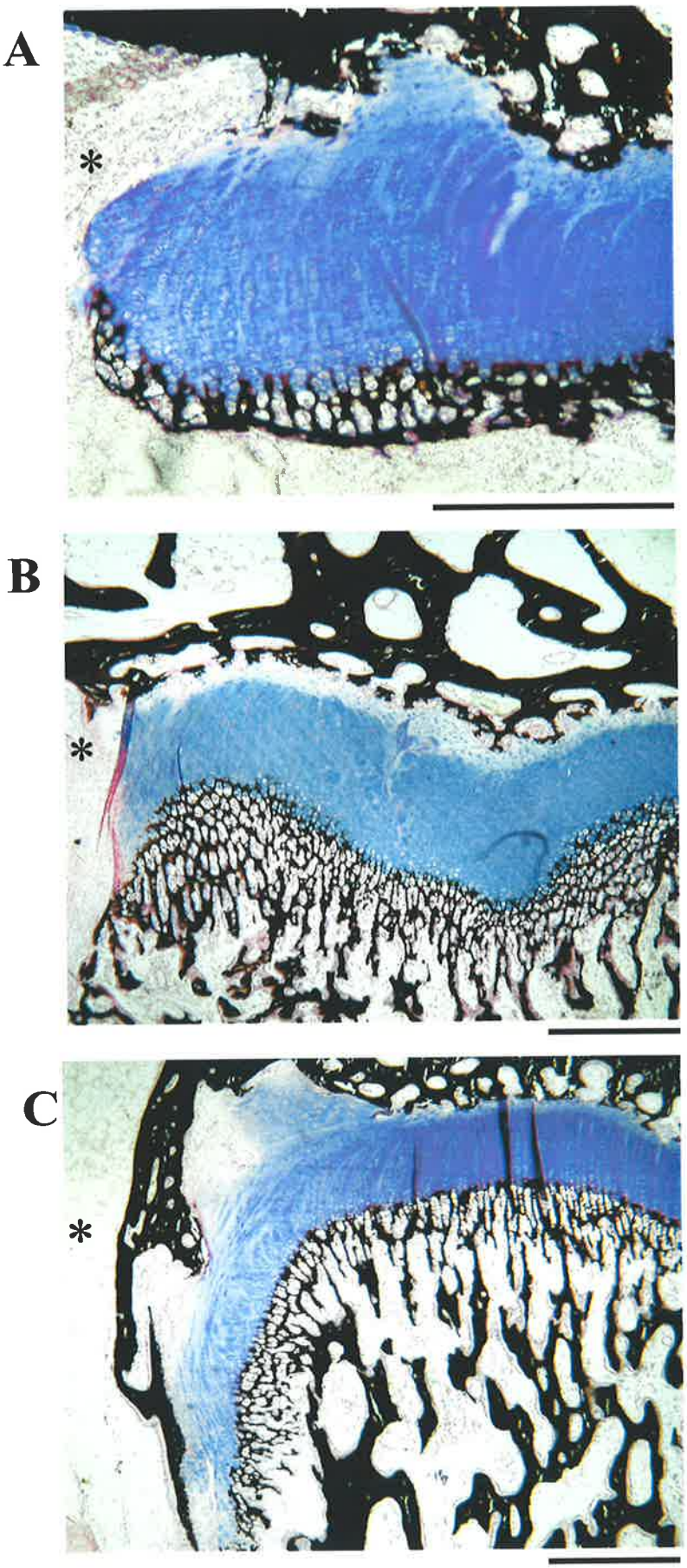

A
*
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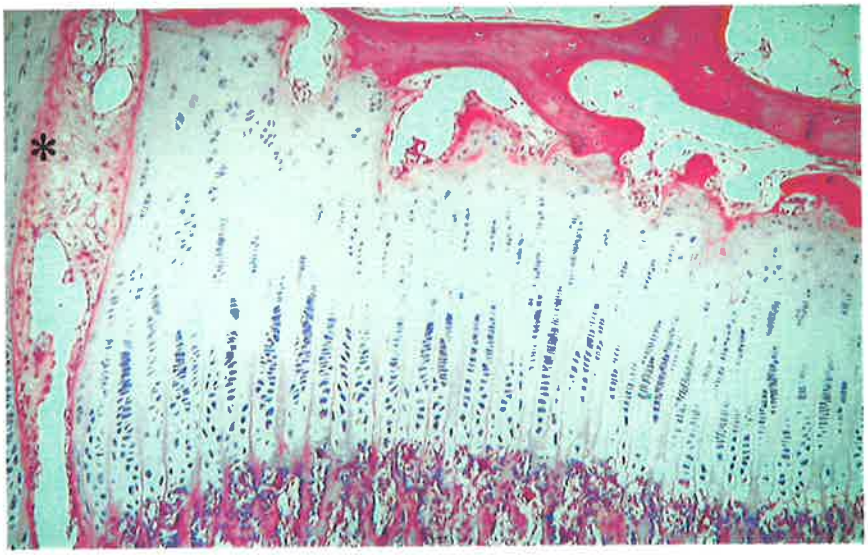
Figure 3.5: Expansion of the growth plate and the de-differentiation of normal growth plate cartilage to fibro-cartilage adjacent to the defect. (A) A day 56 untreated animal with a small increase in growth plate height adjacent to the defect and normal growth plate architecture. (B) A day 56 untreated animal shows a large increase in growth plate height accompanied by a loss of normal growth plate architecture. The defect is represented by (*) and the bar represents 100 μm. Paraffin embedded sections stained with haematoxylin and eosin.

A



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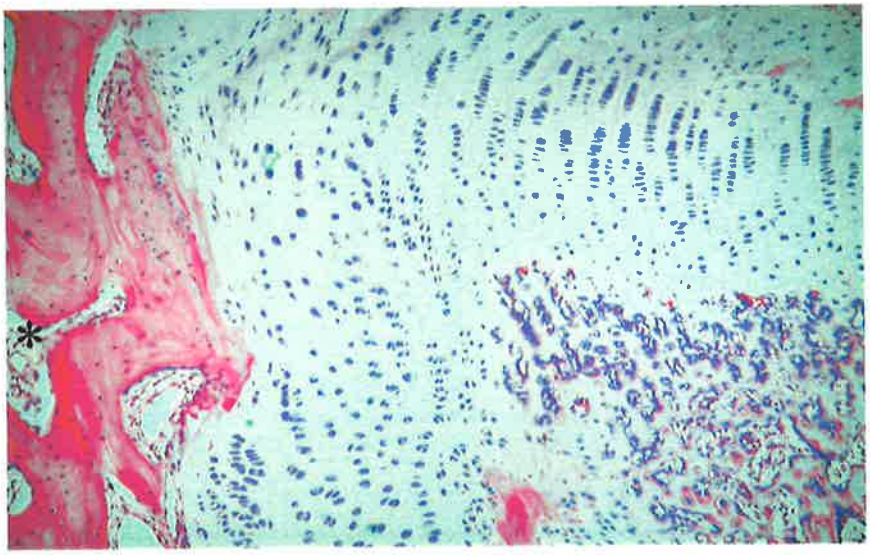

Table 3.7: Response of the Host Growth Plate

Day 7

	#103	#123	#124	#102	#122	#139
rhOP-1 Treatment	-	-	-	+	+	+
Medial Spur	+	0	0	0	0	0
Fibro-cartilage	0	0	0	0	0	0

Day 14

	#105	#118	#121	#104	#119	#120
rhOP-1 Treatment	-	-	-	+	+	+
Medial Spur	0	0	0	0	0	0
Fibro-cartilage	0	0	0	0	0-+	0

Day 56

	#107	#113	#116	#108	#114	#117
rhOP-1 Treatment	-	-	-	+	+	+
Medial Spur	+	++	0	++	++	0
Fibro-cartilage	+	0	0	0-+	+	0-+

Animals were rhOP-1 treated (+) or untreated (-). The formation of a medial spur was scored as (0) no medial spur present, (+) the presence of a medial spur and (++) a marked elongation of the medial spur. Fibro-cartilage was a description of growth plate architecture adjacent to the defect with (0) regular cartilage and (+) a loss of normal growth plate architecture.

There was a single rhOP-1 treated animal at day 14 that showed disorganisation within the lower proliferative and hypertrophic zones of the growth plate adjacent to the defect. Of the day 56 animals, all rhOP-1 treated animals showed a loss of normal growth plate architecture, as did one untreated animal (Fig. 3.5C, Table 3.7). The growth plate of these animals was disorganised, and lacked the ordered proliferative columns and enlarged hypertrophic chondrocytes seen within a normal growth plate. Instead, the chondrocytes were randomly distributed throughout the matrix, often present as single or paired cells rather than a nest of chondrocytes that is normally seen within the proliferative zone.

3.2.4 Response of the Defect Site

The generation of an organised fibrous capsule surrounding the fat implant provides a stable cavity and helps ensure that the fat remains viable and in the correct position (Foster, 1989). The degree of fibrosis around the autologous fat implant was examined and graded as follows: a small rim of fibrous tissue surrounding the fat (0; Fig. 3.6A), the presence of collagen fibres and fibroblasts (+; Fig. 3.6B) or an organised fibrous capsule (++; Fig. 3.6C).

The presence of collagen fibres, fibroblasts and organised capsules were seen as early as day 7. At this time point all of the untreated animals showed the presence of collagen fibres and fibroblasts, and were close to having formed an organised capsule. Only one of the rhOP-1 treated animals showed collagen fibres and fibroblasts around the fat implant at day 7. At day 14 all animals demonstrated some form of fibrosis except one rhOP-1 treated animal that showed no evidence of fibrous tissue surrounding the fat. The untreated day 56 animals all showed fibrosis around the fat, however of the rhOP-1 treated animals only one displayed fibrosis (Table 3.8).

Figure 3.6: Fibrous capsule formation around the fat implant in the defect. (A) A day 56 rhOP-1 treated animal with a small rim of fibrous tissue surrounding the fat (0), (B) a day 7 untreated treated animal showing the presence of collagen fibres and fibroblasts (+) and (C) a day 7 rhOP-1 treated animal with an organised fibrous capsule (++). The arrow indicates the boundary of the fat implant and the fibrous capsule. The bar represents 300 μm. Methyl methacrylate sections stained with von Kossa/ haematoxylin and eosin.

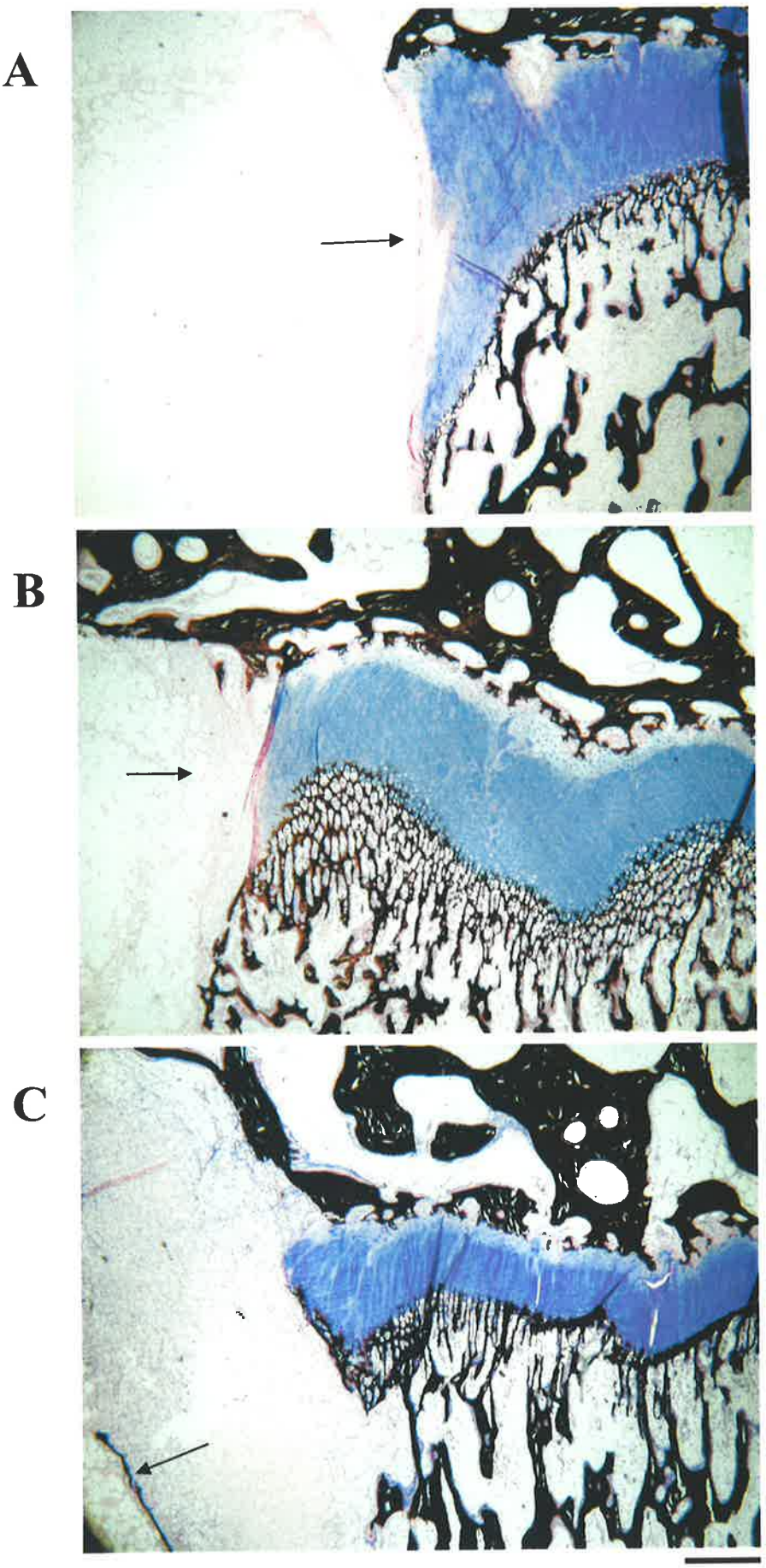
A
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Table 3.8: Response of the Defect Site

Day 7

	#103	#123	#124	#102	#122	#139
rhOP-1 Treatment	-	-	-	+	+	+
Fibrous Capsule	+	+	+-++	0	+-++	0

Day 14

	#105	#118	#121	#104	#119	#120
rhOP-1 Treatment	-	-	-	+	+	+
Fibrous Capsule	+	++	+	0-+	+	0

Day 56

	#107	#113	#116	#108	#114	#117
rhOP-1 Treatment	-	-	-	+	+	+
Fibrous Capsule	+-++	+	+	+-++	0	0

Animals were rhOP-1 treated (+) or untreated (-). The degree of fibrosis around the fat implant was described as absent (0) if only a small rim surrounded the fat, present (+) if collagen fibres and fibroblasts were present, or as an organised capsule (++).

The fat implant in all animals, both rhOP-1 treated and untreated, remained viable throughout the experimental time course. A single rhOP-1 treated animal at day 7 had predominantly fibrous tissue present within the defect, with only a few small areas of fat cells.

It appears that treatment with rhOP-1 may impede the formation of an organised fibrous capsule, as 56% of the rhOP-1 treated animals did not display any form of fibrosis. In comparison, all of the untreated animals demonstrated some degree of fibrosis around the fat implant.

3.2.5 Response of the Surrounding Bone

The effect of the Langenskiöld procedure and treatment with rhOP-1 on the epiphyseal and metaphyseal bone was determined through calculation of bone volume and bone surface area (section 2.7.2). Due to sectioning problems and limited tissue availability, only 13 of the 18 animals could be analysed using this method, restricting the conclusions that could be drawn from these results.

Within the epiphysis there was significantly greater bone volume above the defect than above the remaining growth plate in the untreated animals at day 14 ($p < 0.01$). However, this trend did not continue and no differences in bone volume were observed at any other time points in either of the treatment groups. There was also no significant difference in bone volume above the defect between the rhOP-1 treated and untreated animals at any time point.

Analysis of the metaphyseal bone volume demonstrated that the only difference seen was in the rhOP-1 treated animals at day 7, which had a significantly greater amount of bone under the defect than under the growth plate ($p < 0.05$). This trend did not continue, as there were no differences in bone volume observed at day 14 or 56. The untreated animals showed similar bone volumes under the defect and under the growth plate at all time points.

Calculations of epiphyseal and metaphyseal bone surface area in both the rhOP-1 treated and untreated animals showed no significant difference surrounding either the defect or the growth plate. There was also no difference surrounding the defect between the rhOP-1 treated or untreated animals.

As the changes in the epiphyseal and metaphyseal bone volume and bone surface area are similar in the areas surrounding the defect and the growth plate in individual animals, and similar surrounding the defect between the rhOP-1 treated and untreated animals, there does not appear to be a response of the bone to the surgical procedure or treatment with rhOP-1.

3.2.6 Subperiosteal Bone Formation

Animal ID #117 was treated with rhOP-1 and sacrificed 56 days after surgery. At the time of dissection of the tibia, it was noted that a large outgrowth protruded from the bone. Upon further examination, it was determined that the position of the top of the outgrowth corresponded to the area adjacent to that of the initial defect site. Histological analysis of the protrusion indicated that it was composed of bone and there was no evidence of cartilage or chondrocytes within the mass, indicating that it may have formed via

intramembranous ossification. The most likely explanation for this protrusion is the formation of a subperiosteal bone spur, a peripheral bone bridge that forms due to the disruption of the periosteum. This phenomenon has been seen clinically and can be alleviated through the excision of the periosteum at the time of surgery (Foster, B.K., personal communication). However, although unlikely, the possibility that the protrusion formed as a result of treatment with rhOP-1 cannot be ruled out.

3.3 Detection of the Injected rhOP-1

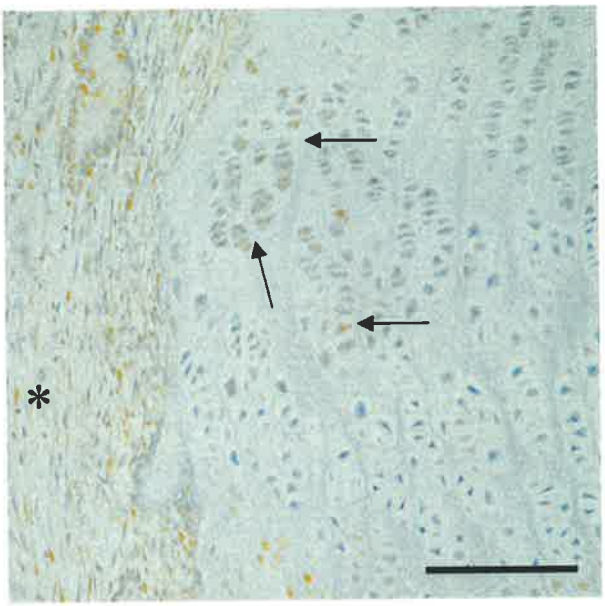
Immunolocalisation with a monoclonal anti-rhOP-1 antibody was performed to establish the period of time the injected rhOP-1 remained at the growth plate/defect interface (section 2.4.1). Four animals (one rhOP-1 treated at day 7, one untreated at day 14, and two untreated at day 56) displayed staining within several chondrocytes in the growth plate immediately adjacent to the defect (Fig. 3.7A). Throughout the time course, both the rhOP-1 treated and untreated animals displayed staining for OP-1 in the fibrous capsule surrounding the fat implant (Fig. 3.7A), the cells surrounding the trabeculae of the primary spongiosa (Fig. 3.7B) and in the osteocytes embedded within these trabeculae (data not shown). There was no staining within the normal growth plate area of the rhOP-1 treated or untreated animals (Fig. 3.7B), or the control animals (Fig. 3.7C).

Discussion

If left untreated, injuries to the growth plate of children's long bones can result in limb length discrepancies and angular deformities. The Langenskiöld procedure is regularly used to prevent or reverse these abnormalities through the resection of the bone bridge that is impeding growth and insertion of an interpositional material, generally fat, to prevent

Figure 3.7: Immunohistochemical localisation of rhOP-1 in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a monoclonal anti-OP-1 antibody and the antigen was detected using the DAB substrate. (A) A day 7 rhOP-1 treated animal showing positive staining within the chondrocytes of the growth plate immediately adjacent to the defect (arrows indicate nests of positive cells). There is positive staining for OP-1 in the fibrous capsule of the defect (*). (B) A day 56 untreated animal showing no staining within the distal growth plate. (C) A control animal showing no staining within the growth plate. There is positive staining around the trabeculae of the metaphysis in all animals. The bar represents 100 μm.

A



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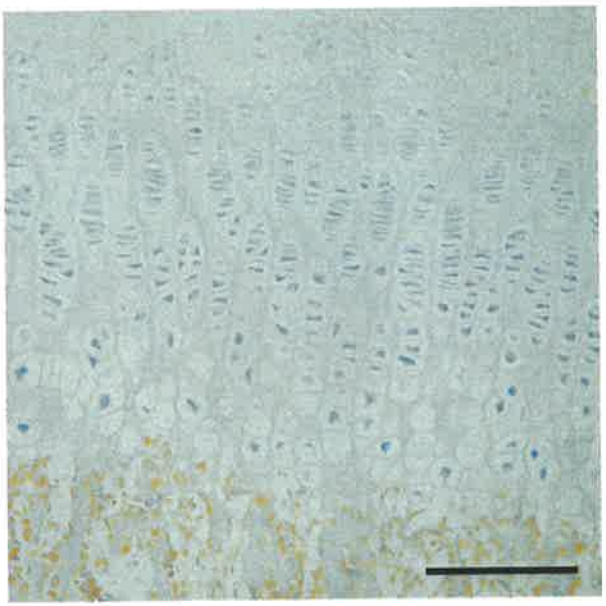


C

reformation of the bone bridge. However, this surgical procedure does not promote regeneration of the growth plate and thus, normal function of the growth plate is not restored. The aim of this study in sheep was to use the Langenskiöld procedure in conjunction with rhOP-1, a growth factor known to induce chondrogenesis, to promote regeneration of the growth plate. Histological analysis of the growth plate, defect site and surrounding bone was performed to assess the response of the growth plate to the Langenskiöld procedure and the effects of rhOP-1 administration.

Following the Langenskiöld procedure, the limbs of both the rhOP-1 treated and untreated animals continued to grow at an equivalent rate to that of the control (no defect) animals. This indicates that the use of fat as the interpositional material for the Langenskiöld procedure did not inhibit long bone growth in this experimental model and treatment with rhOP-1 did not increase or decrease the overall rate of limb growth. This is in contrast to the study by Johnstone *et al.* (Johnstone *et al.*, 2002) that used type I collagen as the interpositional material. In that study, all animals that underwent the Langenskiöld procedure experienced growth arrest by 14 days post surgery. Therefore, the use of fat as the interpositional material in this study was more successful than type I collagen in preventing significant bone formation that results in premature growth arrest.

The administration of rhOP-1 to the growth plate/defect interface did not appear to enhance bone formation within the defect. The thin spicules of bone observed were similar to those reported in previous studies, which described the presence of thin bone bridges adjacent to the defect that did not significantly affect the longitudinal growth of the bone (Foster, 1989; Wirth *et al.*, 1994). Complete bone bridge formation spanning the defect was seen in one of the nine animals treated with rhOP-1, compared to no complete bone

bridges in the untreated animals, suggesting that rhOP-1 does not accelerate bone formation at this dosage. The complete bone bridge formation seen in the rhOP-1 treated animal is likely a result of the fat moving away from the defect site allowing hematoma formation, or the fat undergoing necrosis, providing insufficient interposition to prevent the formation of bone (Hasler and Foster, 2002). Hematoma formation is prevented in the clinical setting through the addition of bone wax to the interface of the growth plate and defect to prevent bleeding into the cavity (Hasler and Foster, 2002). This technique should be incorporated into the surgical procedure in future experiments to assist in the prevention of bone bridge formation.

Throughout the 56-day experimental period, animals treated with rhOP-1 showed an increase in growth plate height adjacent to the defect of 432 μm, compared with a 282 μm increase seen in the untreated animals. Forty six percent of the total increase seen in the rhOP-1 treated animals was by day 7 (197.1 μm ± 42.7 μm), indicating that rhOP-1 has an immediate effect on the growth plate, as seen in the *in vitro* studies (Klein-Nulend *et al.*, 1998a; Klein-Nulend *et al.*, 1998b). The increase observed in the untreated group at day 7 may be a result of surgical trauma, as there is no further increase in growth plate height seen at day 14. In articular cartilage, the high content of proteoglycans gives the tissue a large osmotic swelling pressure (Grodzinsky, 1983). In a canine model investigating the healing of lacerated articular cartilage, it was reported that there was an increase in the water content of the cartilage (Palmoski and Brandt, 1982). Therefore, the initial increase in growth plate height seen in both the rhOP-1 treated and untreated groups at day 7 may be a result of a similar phenomenon in growth plate cartilage. The increase in growth plate height seen at day 56 in the untreated group is a result of a single animal (ID #107) that displayed growth plate height increases ranging from 0 to 900 μm. If this animal is

excluded from the analysis, the untreated animals show no further increase in growth plate height following the initial increase in the first seven days.

The rate of growth plate height increase in the rhOP-1 treated animals was greatest during the first seven days of treatment and slowed down thereafter. This may be related to the amount of rhOP-1 present at the interface of the growth plate and the defect. To achieve prolonged growth plate expansion, a continuous supply of rhOP-1 at the defect site may be necessary. This could be achieved through the use of a slow release device like a mini osmotic pump (Jelic *et al.*, 2001).

The majority of the growth plate height expansion in the rhOP-1 treated animals occurred within the resting zone. The number of chondrocytes per square millimetre within the resting zone in the normal area of growth plate and in the growth plate adjacent to the defect did not change significantly. This suggests that the increase in height may be a result of an increase in matrix synthesis rather than an increase in cellular proliferation. A study by Xian *et al.* (Xian *et al.*, 2003) reported that there was no up-regulation of cell proliferation in the growth plate adjacent to the defect site in a rat drill-hole model of growth plate injury, further supporting an increase in matrix synthesis as the cause of growth plate height expansion.

The increase in matrix volume in the growth plate adjacent to the defect in the rhOP-1 treated animals is supported by reports which show that rhOP-1 increases the production of proteoglycans and type II collagen by articular chondrocytes in culture (Chen *et al.*, 1993; Flechtenmacher *et al.*, 1996; Lietman *et al.*, 1997). In addition, an *in vivo* study using rhOP-1 for articular cartilage repair showed an increase in aggrecan, thus demonstrating

that rhOP-1 promotes the production of a cartilage-like matrix (Louwerse *et al.*, 2000). *In vitro* studies by Asahina *et al.* (Asahina *et al.*, 1996) and Haaijman *et al.* (Haaijman *et al.*, 1997) have reported an increase in cellular proliferation in ATDC5 cells, a cell line with the potential to differentiate into chondrocytes, and in an embryonic mouse long bone culture system, respectively. However, the effects of rhOP-1 on cellular proliferation *in vivo* have not been reported. Future *in vivo* experiments could incorporate methods to measure both matrix synthesis ($[^{35}S]$-SO_4 incorporation into proteoglycans) and cell proliferation (BrdU labelling).

The changes in the histology of the growth plate adjacent to the defect were similar between the rhOP-1 treated and untreated animals. Marked medial spur formation (or tethering of the growth plate) was not observed until day 56 in both treatment groups. In most cases, the medial spur elongation was accompanied by the de-differentiation of the growth plate chondrocytes into fibro-cartilage. Other studies have described changes in the organisation of the growth plate adjacent to injury sites, which have ultimately been associated with the formation of a bone bridge (Wirth *et al.*, 1994; Lee *et al.*, 2000; Gruber *et al.*, 2002; Johnstone *et al.*, 2002; Xian *et al.*, 2003). In all of these cases, bone formation occurred when the injury to the growth plate stretched from the epiphysis to the metaphysis, allowing communication between the two areas. When the injury was contained within the growth plate, normal chondrocyte morphology persisted and the growth plate repaired with little bone formation (Phieffer *et al.*, 2000; Gruber *et al.*, 2002).

Analysis of the development of a fibrous capsule surrounding the fat implant revealed that the animals treated with rhOP-1 had markedly inhibited formation of the fibrous capsule compared to the untreated animals (44% versus 100% respectively). Of the 8 animals that

showed evidence of bone formation within the defect, 7 of these had some degree of fibrous capsule formation. It has been reported that the fibrous capsule around the fat provides a stable environment for continued viability and serves to secure the fat in the correct position (Foster, 1989). However, the results of this study indicate that the collagen fibres and fibroblasts present within the fibrous capsule may be the template on which ossification can occur. Therefore, the inhibition of fibrous capsule formation by treatment with rhOP-1 may be beneficial in preventing significant bone formation within the defect.

Immunolocation was performed to establish the period of time rhOP-1 remained at the interface of the growth plate and the defect. The staining pattern observed was the same in the rhOP-1 treated and untreated animals, indicating that the antibody obtained was not specific for the injected rhOP-1 as expected, and was cross-reactive with the endogenous OP-1 or other bone morphogenetic proteins of the sheep. Future experiments could address this problem through the generation of a tagged protein (for example, green fluorescent protein or a His_6 tag) that would enable specific detection of the injected protein, rather than identification of endogenous molecules.

The results within this chapter demonstrate the advantages of using a large sheep model for this study. The animals underwent sufficient growth to allow comparison of the overall limb growth of the control animals with the animals that underwent the Langenskiöld procedure. The large increase in limb length was also sufficient to visualise the appearance of medial spur formation in the animals in the day 56 groups. This model would therefore also be suitable for the future testing of other growth factors and molecules of interest on the growth plate repair process.

It is proposed that following the surgical procedure and fat insertion, the animals underwent a traumatic response that resulted in the expansion of the growth plate adjacent to the defect. A fibrous capsule was formed around the implanted fat in the defect, the collagen fibres of which may have been a template for bone formation. The remaining growth plate tethered into a medial spur 56 days post surgery, possibly due to the fibrous capsule attaching to the growth plate, anchoring it as the limb underwent further growth. In some animals, the presence of the medial spur was accompanied by the de-differentiation of the growth plate chondrocytes into a more primitive form.

The administration of rhOP-1 following the Langenskiöld procedure resulted in an increase in height in the growth plate adjacent to the defect that was greater than that seen in the untreated animals. Although expansion was seen in the untreated animals at day 7, the continued expansion of the growth plate in the rhOP-1 treated animals throughout the time course indicates that there is a specific response to the rhOP-1.

Summary

The use of fat as an interpositional material successfully inhibited bone bridge formation, allowing normal limb growth. The observation of expansion within the growth plate in the rhOP-1 treated animals indicates that the growth plate chondrocytes may be increasing their matrix synthesis in order to regenerate the growth plate. Treatment with rhOP-1 did not affect the growth rate of the limbs or the effectiveness of the Langenskiöld procedure, and may play a role in the inhibition of fibrous capsule formation, the site at which the majority of bone formation occurs. The next chapter evaluates this process at the molecular level.

Chapter 4

Molecular Analysis Following the Langenskiöld Procedure

Introduction

OP-1 is a member of the bone morphogenetic protein (BMP) family, a sub-group of the TGF-β superfamily. Members of the BMP family are found within bone and are known to induce ectopic bone formation through the initiation of endochondral ossification (Reddi, 1981). The osteogenic effects of the BMPs were first described in 1965 (Urist, 1965) when demineralised bone matrix was implanted into non-bony sites and induced new bone and bone marrow formation. OP-1 has been shown to be important in stimulating the proliferation (Knusten *et al.*, 1993) and differentiation (Cheifetz *et al.*, 1996) of cells of the osteoblastic lineage and may also contribute to bone resorption by affecting the recruitment of osteoclasts (Hentunen *et al.*, 1995). The strong osteogenic properties of OP-1 have been utilised to heal segmental or non-union bone defects that would normally be unable to repair themselves in both animal models (Cook *et al.*, 1994a; Cook *et al.*, 1994b; Cook *et al.*, 1995) and in clinical trials (Geesink *et al.*, 1999; Friedlaender *et al.*, 2001). Results from clinical trials indicated that the addition of OP-1 to critical size fibular and tibial defects was a safe and effective substitute for bone autografts, providing clinical and radiographic results comparable to those achieved with bone autograft, without donor site morbidity (Friedlaender *et al.*, 2001).

In addition to its presence within the periosteum and osteoblasts (Vukicevic *et al.*, 1994; Helder *et al.*, 1995; Anderson *et al.*, 2000), OP-1 has been localised to the superficial and middle layers of articular cartilage (Chubinskaya *et al.*, 2000; Muehleman *et al.*, 2002) and to the hypertrophic chondrocytes of the growth plate (Houston *et al.*, 1994; Vukicevic *et al.*, 1994). Its presence within cartilage suggests several roles for this protein beyond that directly involved with bone formation. Firstly, as both OP-1 and its receptors are found

within articular cartilage it may have a possible role in maintaining cartilage homeostasis (Chubinskaya *et al.*, 2000; Muehleman *et al.*, 2002), and secondly, it may have an organisational role in long bone development (Helder *et al.*, 1995). As OP-1 has been shown to stimulate the expression of the osteoblastic phenotype *in vitro*, it may activate the osteoblastic precursors migrating into the metaphyseal edge of the growth plate, possibly after release from the growth plate matrix through the action of chondroclasts (Houston *et al.*, 1994).

As well as the reported osteogenic properties of OP-1, there are many examples describing the chondrogenic potential of OP-1. Studies using recombinant human OP-1 (rhOP-1) have shown that it promotes differentiation of rat calvarial cells and ATDC5 cells *in vitro* and perichondrium *in vivo* into cells with chondrogenic potential (Asahina *et al.*, 1993; Dieudonne *et al.*, 1994; Asahina *et al.*, 1996; Klein-Nulend *et al.*, 1998a; Klein-Nulend *et al.*, 1998b). It also has the ability to stimulate mature articular chondrocytes to increase matrix production through an up-regulation of proteoglycan and type II collagen synthesis (Chen *et al.*, 1993; Flechtenmacher *et al.*, 1996; Hidaka *et al.*, 2001). The proteoglycan monomers and the composition of the glycosaminoglycans produced in response to rhOP-1 treatment are the same as those produced by normal untreated chondrocytes (Lietman *et al.*, 1997). When added to chick sternal chondrocyte cultures, rhOP-1 supported hypertrophy as shown by the up-regulation of type X collagen and down-regulation of type I collagen (Chen *et al.*, 1995).

Several *in vivo* studies have been conducted in rabbits, goats, sheep and dogs using recombinant OP-1 to accelerate the repair of articular cartilage defects by an ingrowth of mesenchymal cells to the injury site that transform into articular cartilage-like tissue (Grgic

et al., 1997; Louwerse *et al.*, 2000; Jelic *et al.*, 2001; Cook *et al.*, 2003). Recombinant OP-1 has also been used in dogs to regenerate thyroid cartilage defects (Katic *et al.*, 2000). In this model the addition of recombinant OP-1 induced bone, cartilage and ligament-like tissue. In both articular cartilage and thyroid cartilage repair the newly formed cartilage integrated well with the host cartilage, indicating that recombinant OP-1 can induce remodelling of the cartilage matrix (Katic *et al.*, 2000; Jelic *et al.*, 2001). However, conflicting results have been reported for subchondral defects (Grgic *et al.*, 1997; Louwerse *et al.*, 2000; Cook *et al.*, 2003).

The majority of reported studies have focussed on the use of rhOP-1 in the repair of articular cartilage defects. Previous work in our laboratory established that rhOP-1 could promote the outgrowth of the growth plate cartilage adjacent to a region where the growth plate had been ablated (Johnstone *et al.*, 2002). However, the expansion was limited by the formation of mineral within the defect site, which may have been facilitated by the presence of the type I collagen carrier used to plug the defect site.

The aim of the present study was to investigate the effects of rhOP-1 on growth plate repair when used in conjunction with the Langenskiöld procedure, a surgical practice designed to prevent bone bridge formation (Langenskiöld, 1967).

This chapter reports the effects of rhOP-1 on the molecular modifications of the growth plate following injury. Bone- and cartilage-specific markers within the growth plate adjacent to the defect were evaluated using immunohistochemistry to determine any change in molecular expression. The collagen structure and composition of the growth

plate adjacent to the defect were evaluated using polarised light microscopy and *in situ* hybridisation.

Results

4.1 Immunohistochemical Analysis

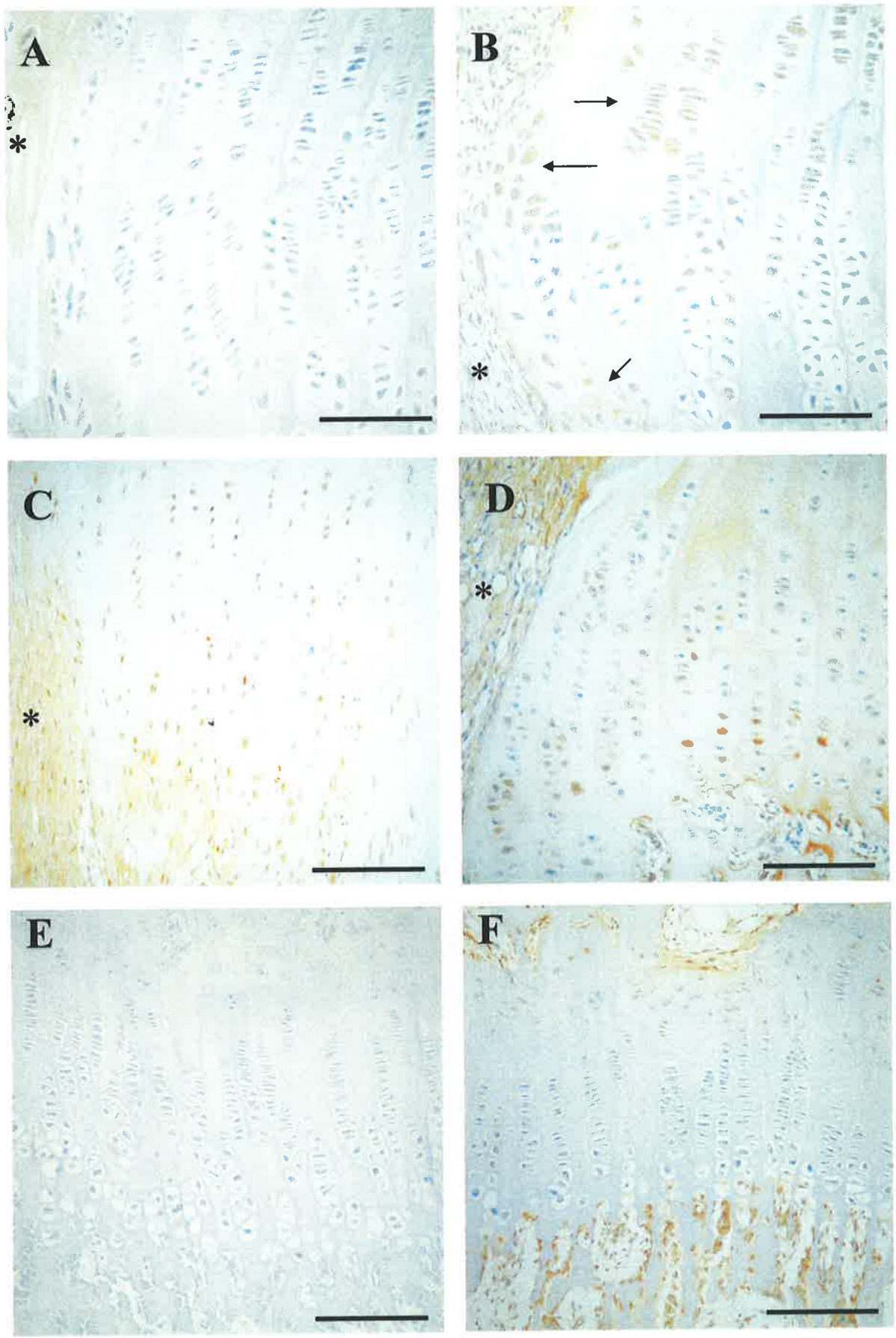
To investigate the molecular changes in the growth plate adjacent to the defect, immunohistochemical analysis was performed. Paraffin sections (5 μm) were incubated overnight with antibodies against several types of collagen, bone matrix proteins and glycosaminoglycans (for method see section 2.4.1 and for antibody concentrations see Table 2.3). Sections were incubated with biotinylated secondary antibodies, amplified with streptavidin and the signal detected using the DAB colour substrate. The rhOP-1 treated animals, untreated animals and normal animals without defects were compared. The area adjacent to the defect in rhOP-1 treated and untreated animals was compared with the observed molecular expression of the normal animals.

4.1.1 Collagen Immunolocalisation

4.1.1.1 Type I Collagen

Type I collagen was not observed within the growth plate in any of the untreated animals at day 7 (Fig. 4.1A). In contrast, type I collagen was found intracellularly within the resting, proliferative and hypertrophic chondrocytes of the growth plate immediately adjacent to the defect in all 3 of the rhOP-1 treated animals (Fig 4.1B). There was no type I collagen detected within the growth plate located at the distal aspect from the defect (data not shown).

Figure 4.1: Immunohistochemical localisation of type I collagen in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a polyclonal anti-type I collagen antibody and the antigen detected using the DAB substrate. An untreated animal (A) and a rhOP-1 treated animal (B) at day 7, an untreated animal (C) and a rhOP-1 treated animal (D) at day 56, a rhOP-1 treated animal at day 7 incubated overnight without primary antibody, as a negative control (E) and a normal animal (F) are shown. The defect is indicated by (*). Arrows in (B) indicate stained nests of chondrocytes. All chondrocytes within the growth plate of (C) and (D) are positively stained. The bar represents 100 μm.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By day 14, 2 of the 3 untreated sheep displayed type I collagen within the resting, proliferative and hypertrophic chondrocytes of the growth plate flanking the defect (data not shown). All of the rhOP-1 treated animals at day 14 displayed type I collagen in the chondrocytes of the growth plate. By 56 days post surgery, type I collagen localisation was observed within all chondrocytes of the growth plate adjacent to the defect in both the rhOP-1 treated and untreated animals (Fig. 4.1C-D). The collagen fibres of the fibrous capsule around the fat implant in the defect stained positively for type I collagen, as expected (Fig. 4.1B-D). There was no staining observed in the fibroblasts of the fibrous capsule or the fat cells of the implant in any animals.

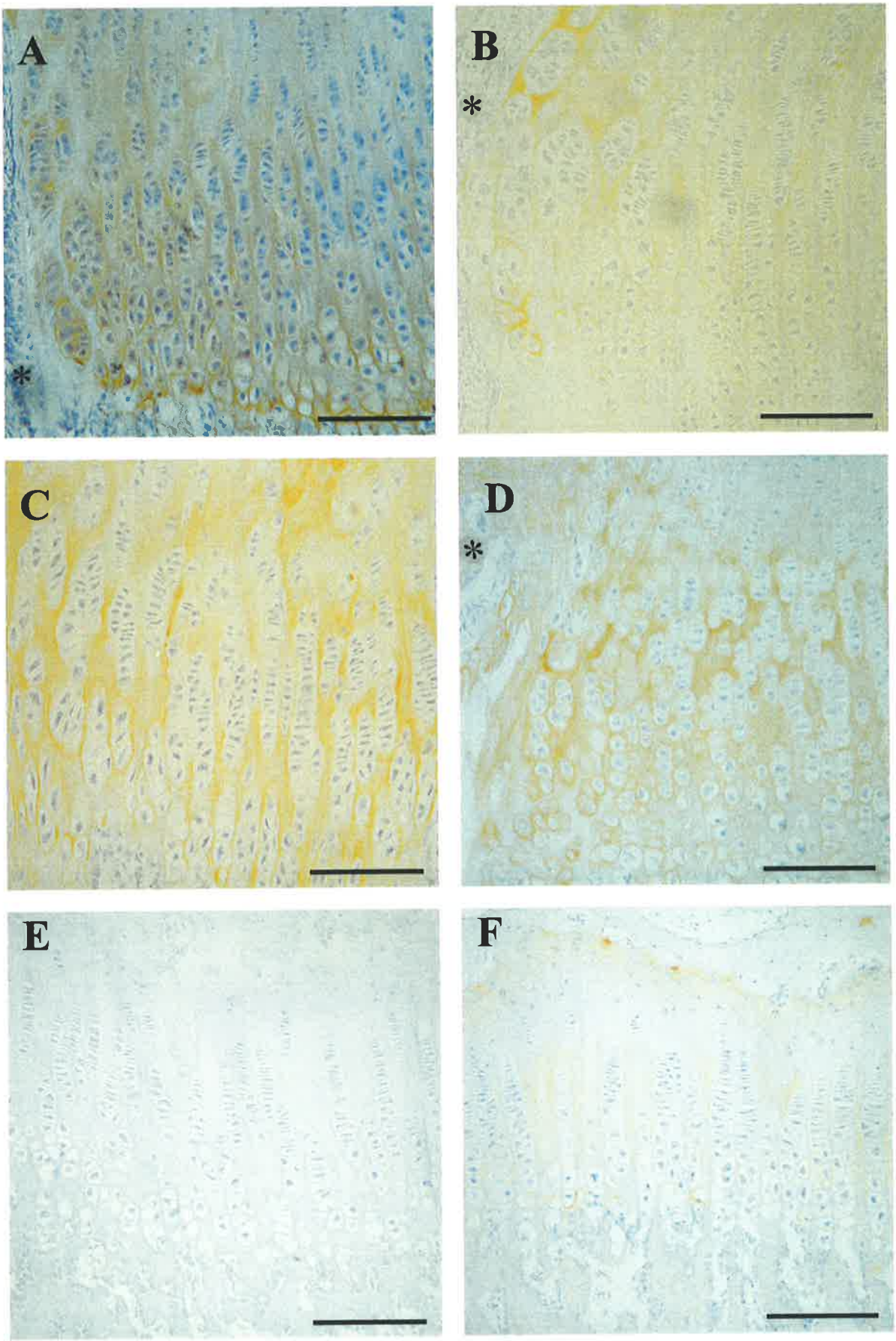
Type I collagen was not detected in control sections that were incubated without the primary antibody (Fig. 4.1E). Type I collagen is not normally observed in growth plate cartilage, but localises to the osteoblasts and osteocytes around and within the trabeculae of the epiphysis and metaphysis (Fig. 4.1F).

4.1.1.2 Type II Collagen

Type II collagen was observed throughout the matrix of the resting, proliferative and hypertrophic zones, and within the trabeculae of the primary spongiosa. Throughout the 8-week experimental period no difference in type II collagen was observed between the rhOP-1 treated and untreated animals at day 7 (Fig. 4.2A-B), day 14 or day 56 (Fig. 4.2C-D). There was no type II collagen detected within the defect or the fibrous capsule surrounding the defect (data not shown).

Type II collagen was not detected in the control sections that had been incubated without the anti-type II collagen antibody (Fig. 4.2E). In normal animals, type II collagen was

Figure 4.2: Immunohistochemical localisation of type II collagen in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a monoclonal anti-type II collagen antibody and the antigen detected using the DAB substrate. An untreated animal (A) and a rhOP-1 treated animal (B) at day 7, an untreated animal (C) and a rhOP-1 treated animal (D) at day 56, a rhOP-1 treated animal at day 7 incubated overnight without primary antibody, as a negative control (E) and a normal animal (F) are shown. The defect is indicated by (*). The bar represents 100 μm.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present throughout the growth plate (Fig. 4.2F). It was also present in the centre of the trabeculae within the primary spongiosa. The type II collagen persisted until remodelling had progressed in the secondary spongiosa and replaced the predominant cartilage collagen with type I collagen (data not shown).

4.1.1.3 Type X Collagen

Type X collagen was localised to the hypertrophic chondrocytes of the growth plate and between the columns of proliferative chondrocytes. No difference was observed for type X collagen in rhOP-1 treated and untreated animals at day 7 (Fig. 4.3A-B), day 14 or day 56 (Fig. 4.3C-D). Type X collagen was not detected in the defect of any of the rhOP-1 treated or untreated animals at any time point, in either the fat implant or the surrounding fibrous capsule.

No type X collagen was detected in the sections incubated without the anti-type X collagen antibody (Fig. 4.3E). The normal animals displayed type X collagen within the hypertrophic chondrocytes and between the columns of the proliferative chondrocytes (Fig. 4.3F).

4.1.2 Osteopontin Immunolocalisation

Osteopontin was not observed within the growth plate of untreated animals at day 7 (Fig. 4.4A). However, rhOP-1 treated animals at day 7 demonstrated osteopontin localised to the proliferative chondrocytes of the growth plate in the area immediately adjacent to the defect (Fig. 4.4B). Osteopontin was also observed within the fibrous tissue of the defect in rhOP-1 treated animals at day 7 (Fig.4.4B).

Figure 4.3: Immunohistochemical localisation of type X collagen in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a polyclonal anti-type X collagen antibody and the antigen detected using the DAB substrate. An untreated animal (A) and a rhOP-1 treated animal (B) at day 7, an untreated animal (C) and a rhOP-1 treated animal (D) at day 56, a rhOP-1 treated animal at day 7 incubated overnight without primary antibody, as a negative control (E) and a normal animal (F) are shown. The defect is not shown. The bar represents 100 μm.

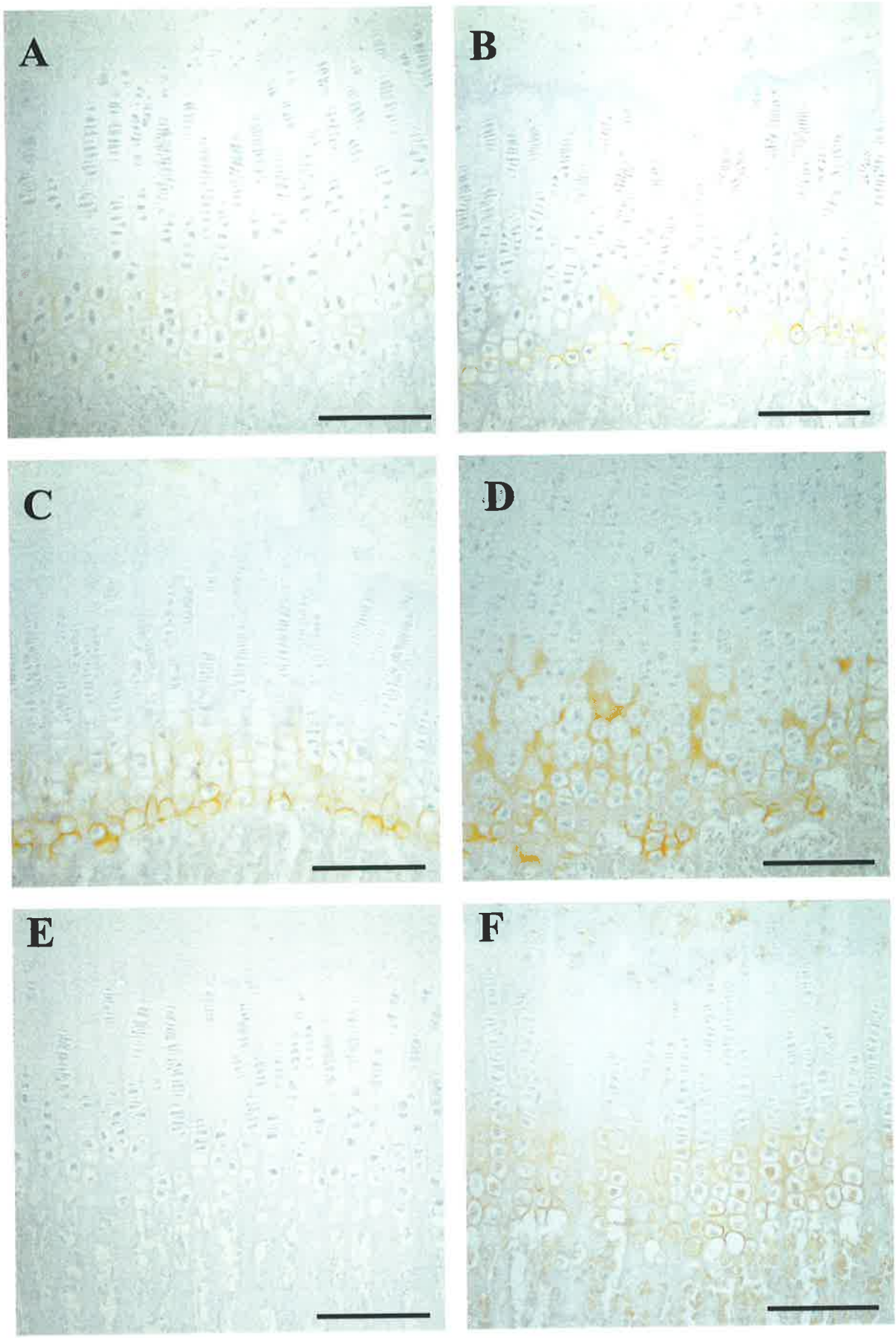
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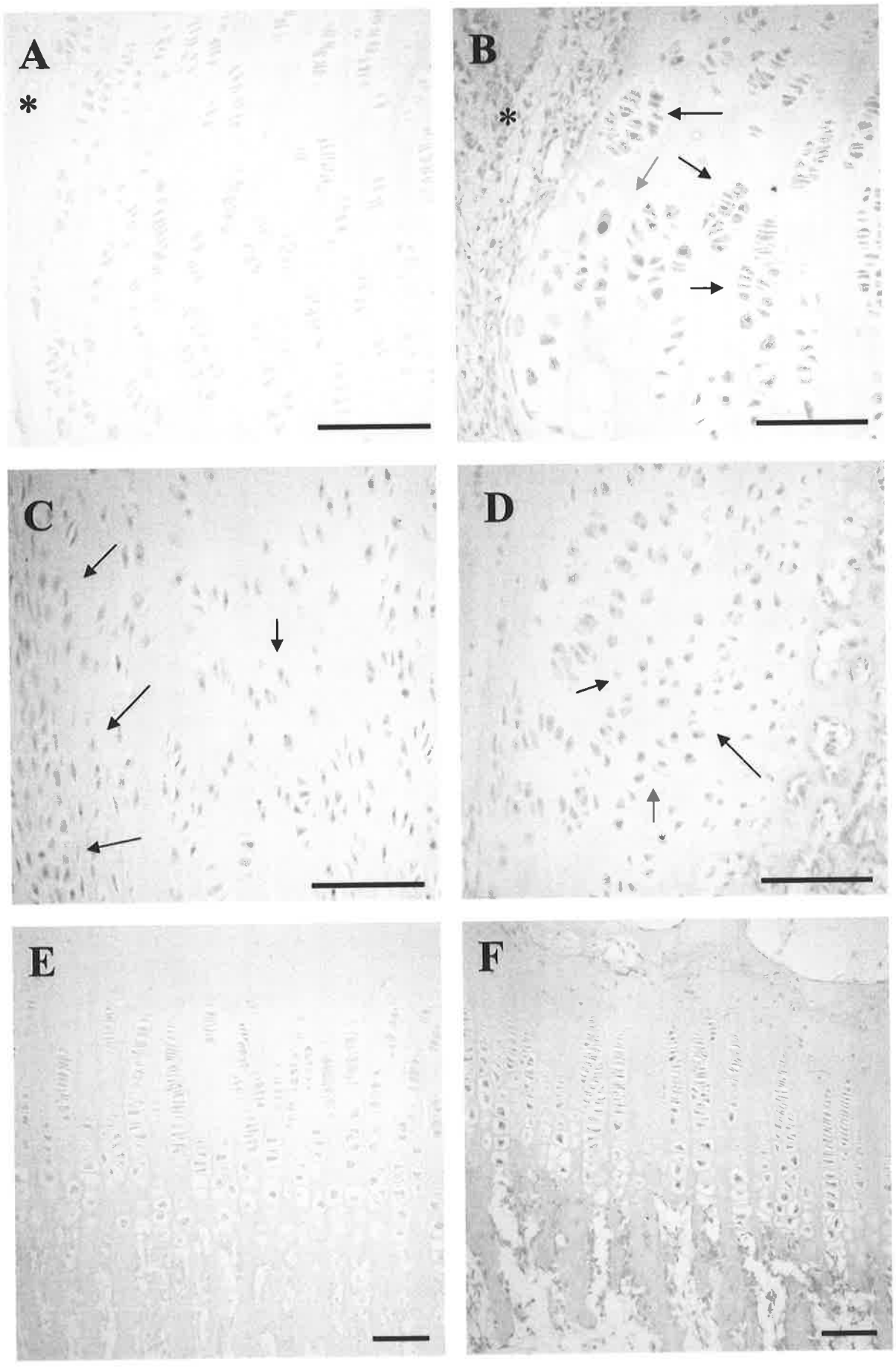
Figure 4.4: Immunohistochemical localisation of osteopontin in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a polyclonal anti-osteopontin antibody and the antigen detected using the DAB substrate. An untreated animal (A) and a rhOP-1 treated animal (B) at day 7, an untreated animal (C) and a rhOP-1 treated animal (D) at day 56, a rhOP-1 treated animal at day 7 incubated overnight without primary antibody, as a negative control (E) and a normal animal (F) are shown. The defect is indicated by (*). Arrows in (B-D) indicate stained areas of chondrocytes. The bar represents 100 μm.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At day 14, all the rhOP-1 treated animals and one of the untreated animals exhibited osteopontin within the chondrocytes of the growth plate (data not shown). By day 56 post surgery, all rhOP-1 treated and untreated animals displayed osteopontin within the chondrocytes of the growth plate adjacent to the defect and also in the fibrous tissue of the defect (Fig. 4.4C-D).

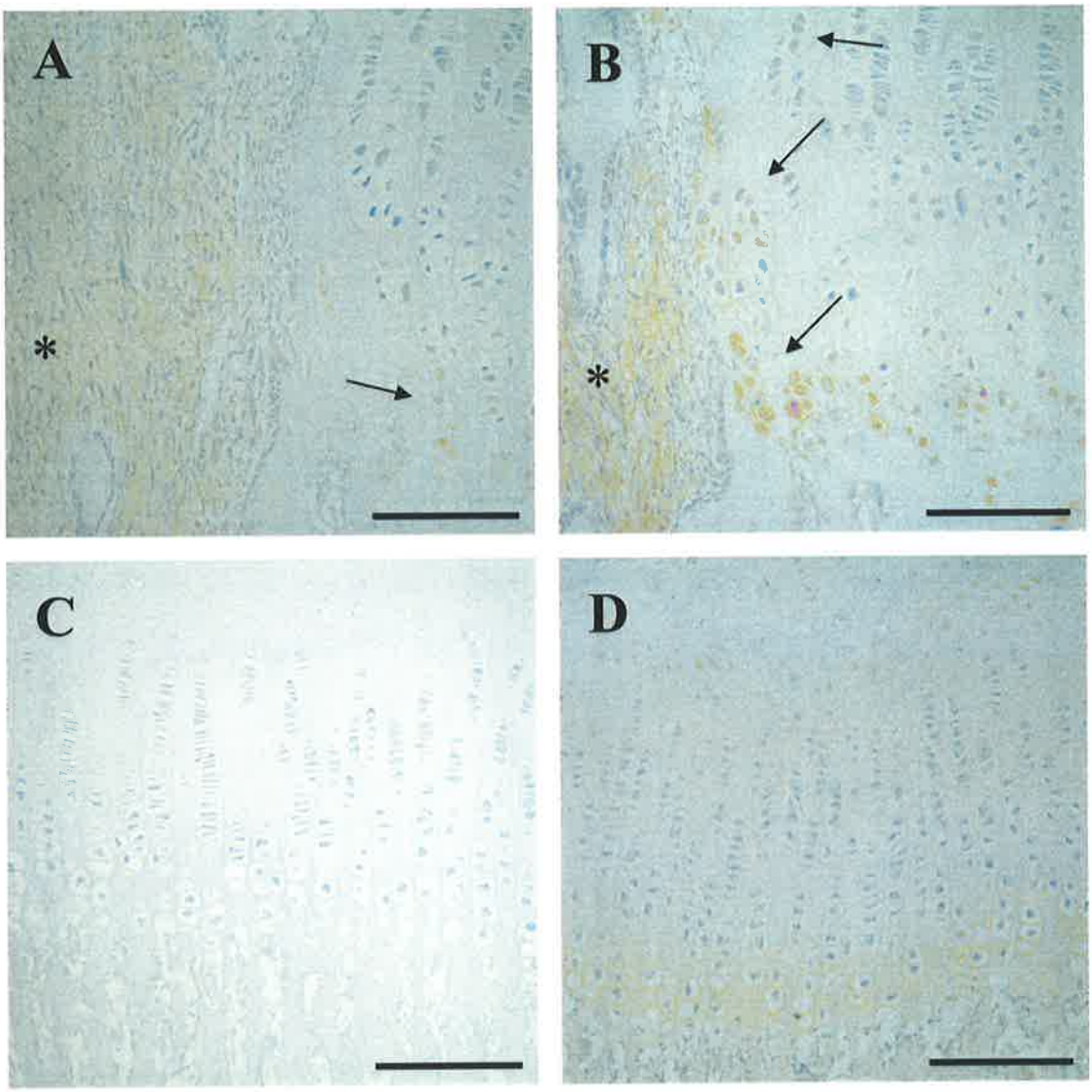
Osteopontin was not detected in control sections that were incubated without the osteopontin antibody (Fig. 4.4E). In normal animals, osteopontin was observed in the osteoblasts surrounding the trabeculae in the metaphysis, but not throughout the growth plate (Fig. 4.4F). A few proliferative chondrocytes were positive for osteopontin. However, these were sporadic and the staining was not uniform across the entire section.

4.1.3 Osteonectin Immunolocalisation

In both rhOP-1 treated and untreated groups, osteonectin was present in the fibrous capsule of the defect and within the hypertrophic chondrocytes of the growth plate (Fig. 4.5A). In the growth plate immediately adjacent to the defect, both treatment groups demonstrated intracellular osteonectin within the resting, proliferative and hypertrophic chondrocytes (Fig. 4.5B). This localisation was present at all time points.

There was no osteonectin detected in sections incubated without the osteonectin antibody (Fig. 4.5C). Osteonectin was detected within the hypertrophic chondrocytes of the growth plate in normal animals (Fig. 4.5D). There was a light staining of the matrix throughout the growth plate and osteonectin was observed within the marrow of the metaphysis (Fig. 4.5D).

Figure 4.5: Immunohistochemical localisation of osteonectin in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a polyclonal anti-osteonectin antibody and the antigen detected using the DAB substrate. A day 7 rhOP-1 treated animal showing positive staining within the fibrous capsule of the defect (A) and a day 7 rhOP-1 treated animal showing positive staining in chondrocytes in the growth plate adjacent to the defect (B). A day 7 rhOP-1 treated animal incubated overnight without primary antibody, as a negative control (C) and a normal animal (D). The defect is indicated by (*). Arrows in (A and B) indicate stained areas of chondrocytes. The bar represents 100 μm.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4.1.4 Glycosaminoglycan and Proteoglycan Immunolocalisation

To observe changes in cartilage structure or composition, antibodies were used to detect various glycosaminoglycans present within the growth plate. These included 2B6, an antibody against digested chondroitin-4-sulfate neo epitope, 5D4, an antibody that detects keratan sulfate, and 7D4, an antibody against native chondroitin-4-sulfate that co-localises with type X collagen. Antibodies against decorin and biglycan core proteins, to which the glycosaminoglycans attach, were also used.

4.1.4.1 Chondroitin-4-Sulphate Neo-epitope

Detection of chondroitin-4-sulphate neo-epitope using the 2B6 antibody was seen throughout the growth plate in all rhOP-1 treated and untreated animals at all time points (Fig. 4.6A-D). The presence of 2B6 was also throughout the trabeculae of the metaphysis and within the fibrous tissue and bone in the defect.

No chondroitin-4-sulphate neo-epitope was detected when the sections were incubated without the primary antibody (Fig. 4.6E). Normal animals exhibited the 2B6 neo-epitope throughout the growth plate and within the trabeculae of the metaphysis (Fig. 4.6F).

4.1.4.2 Keratan Sulphate

Keratan sulphate, as detected by the 5D4 antibody was observed throughout all zones of the growth plate, as well as in the trabeculae of the metaphysis (Fig. 4.7A-D). There was no difference observed between the rhOP-1 treated and untreated groups at any of the time points. There was no evidence of keratan sulphate in the fibrous capsule of the defect.

Insert the following table after page 97

Table 4.1 Glycosaminoglycan Epitope Antibody Specificity

Antibody	Specificity	References
2-B-6	Recognises an unsaturated, disulphated disaccharide which is produced by chondroitinase digestion of native chondroitin sulphate and dermatan sulphate chains.	Caterson *et al.*, 1985; Byers *et al.*, 1992.
5-D-4	Recognises native chains of skeletal keratan sulphate.	Caterson *et al.*, 1983; Byers *et al.*, 1992.
7-D-4	Recognises an epitope within native chondroitin sulphate chains that is thought to be oversulphated and is usually destroyed by chondroitinase digestion.	Caterson *et al.*, 1990; Byers *et al.*, 1992.

Byers, S., Caterson, B., Hopwood, J.J. and Foster, B.K. (1992). Immunolocation Analysis of Glycosaminoglycans in the Human Growth Plate. J Histochem Cytochem *40(2)* 275-282.

Caterson, B., Christner, J.E. and Baker, J.R. (1983). Identification of a monoclonal antibody that specifically recognizes corneal and skeletal keratan sulphate. J Biol Chem *258* 8848-8854.

Caterson, B., Christner, J.E. and Couchman, J.R. (1985). Production and characterisation of monoclonal antibodies directed against connective tissue proteoglycans. Fed Proc *44* 386-393.

Caterson, B., Griffin, J., Mahmoodian, F. and Sorrell, J.M. (1990). Monoclonal antibodies against chondroitin sulphate isomers: their use as probes for investigating proteoglycans metabolism. Biochem Soc Trans *18* 820-823.

Figure 4.6: Immunohistochemical localisation of chondroitin-4-sulphate in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a polyclonal anti-2B6 antibody and the antigen detected using the DAB substrate. An untreated animal (A) and a rhOP-1 treated animal (B) at day 7, an untreated animal (C) and a rhOP-1 treated animal (D) at day 56, a rhOP-1 treated animal at day 7 incubated overnight without primary antibody, as a negative control (E) and a normal animal (F) are shown. The defect is indicated by (*). The bar represents 100 μm.

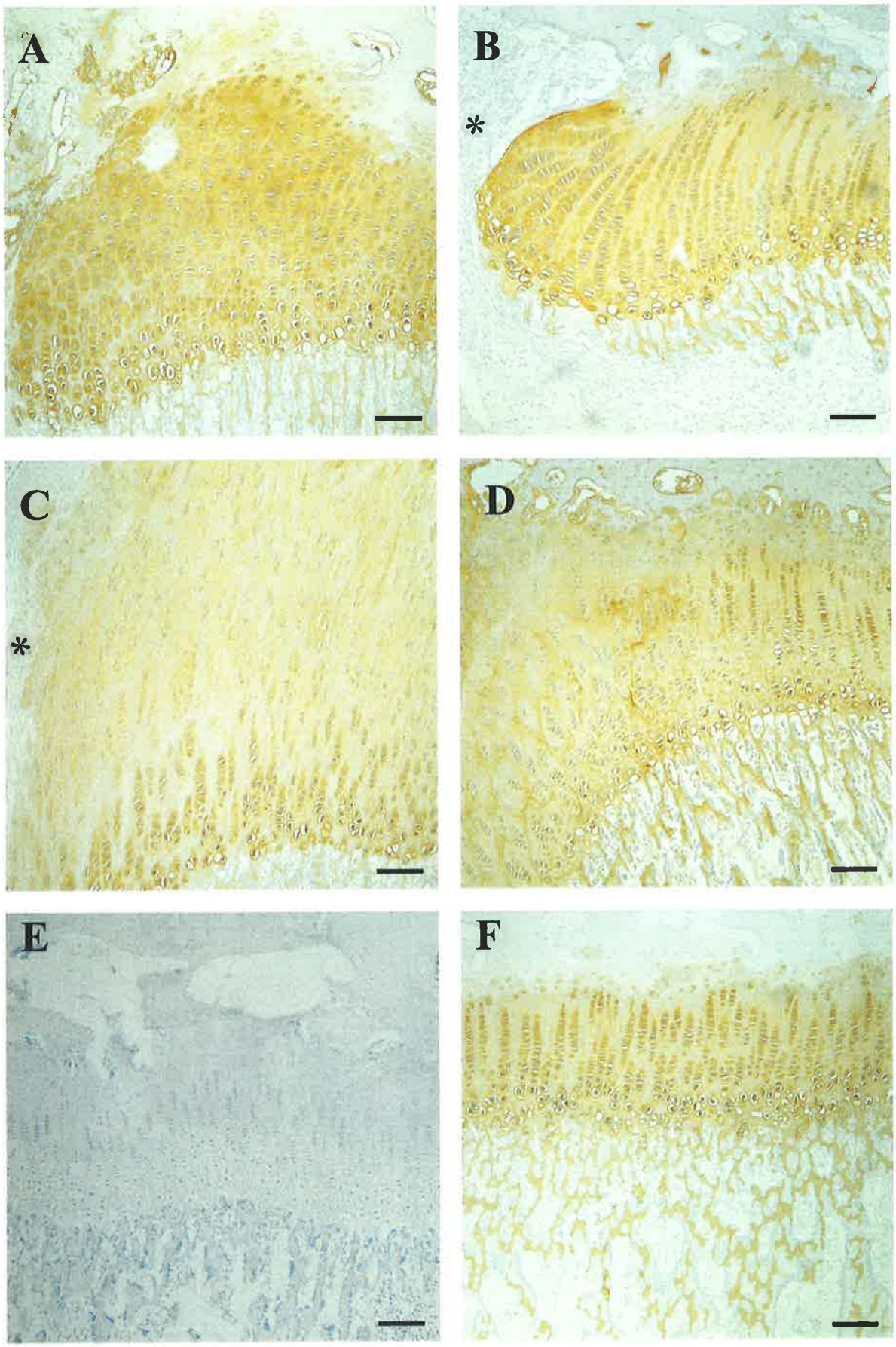
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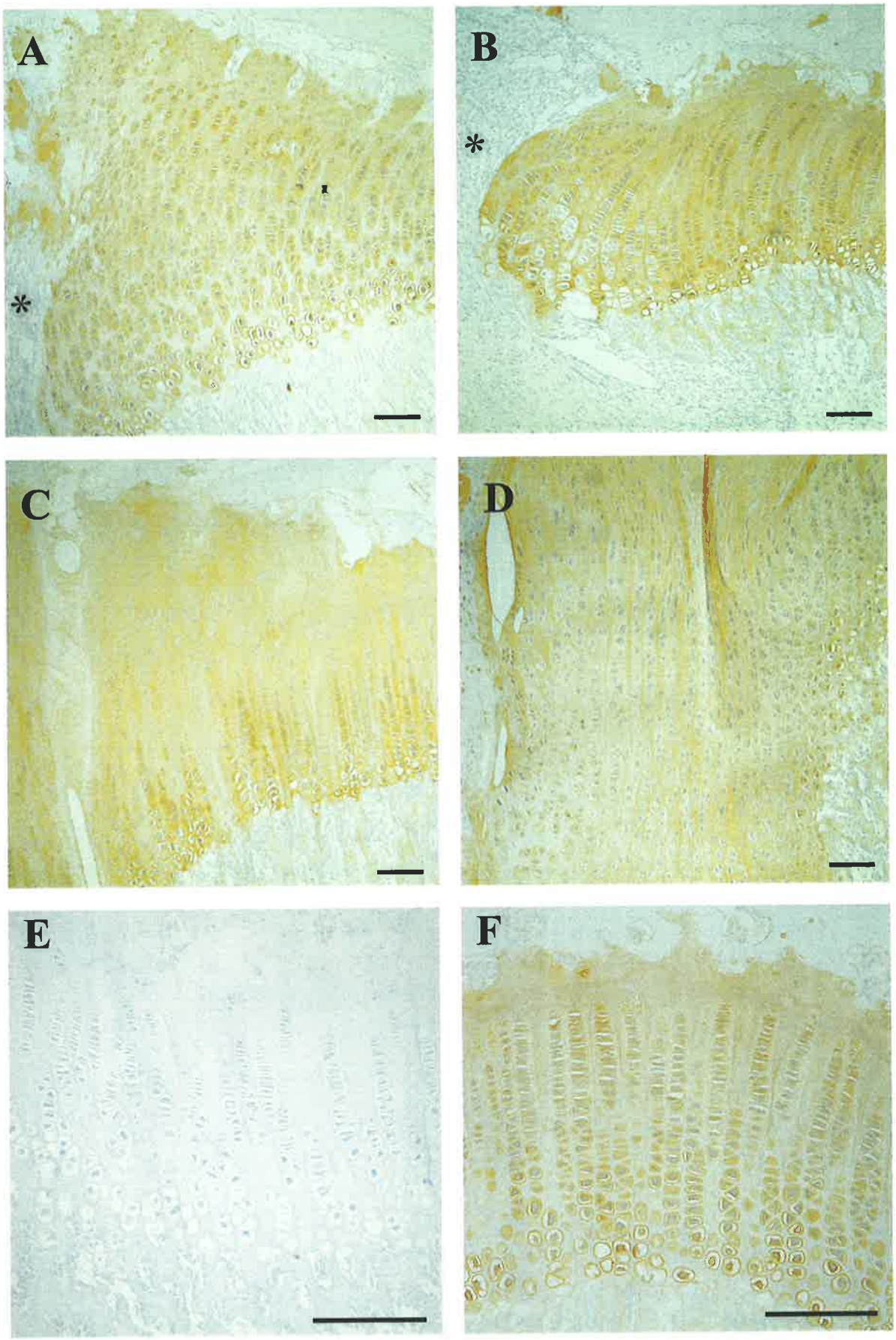
Figure 4.7: Immunohistochemical localisation of keratan sulphate in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a polyclonal anti-5D4 antibody and the antigen detected using the DAB substrate. An untreated animal (A) and a rhOP-1 treated animal (B) at day 7, an untreated animal (C) and a rhOP-1 treated animal (D) at day 56, a rhOP-1 treated animal at day 7 incubated overnight without primary antibody, as a negative control (E) and a normal animal (F) are shown. The defect is indicated by (*). The bar represents 100 μm.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No keratan sulphate was observed in sections incubated without the 5D4 antibody (Fig. 4.7E). Detection of keratan sulphate was found throughout all zones of the growth plate in the normal animals (Fig. 4.7F).

4.1.4.3 Native Chondroitin Sulphate

Native chondroitin sulphate, detected using the 7D4 antibody, was observed in the hypertrophic zone, between the chondrocyte columns of the proliferative zone, and in the trabeculae of the metaphysis in both the rhOP-1 treated and untreated groups at all time points (Fig. 4.8A-D). The fibrous tissue and bone within the defect was also positive for the 7D4 epitope.

No staining was observed when sections were incubated without the 7D4 antibody (Fig. 4.8E). The distribution of 7D4 in the normal growth plates was found within the hypertrophic chondrocytes, and lighter staining throughout the remaining growth plate (Fig. 4.8F).

4.1.4.4 Decorin

Decorin was observed within only 2 or 3 chondrocytes of the growth plate of the untreated animals at day 7 (Fig. 4.9A). The rhOP-1 treated animals at day 7 displayed decorin in approximately 50 percent of the chondrocytes in the growth plate immediately adjacent to the defect (Fig. 4.9B). Decorin was mainly observed within the osteoblasts surrounding the trabeculae in the primary spongiosa, and within the trabeculae of the secondary spongiosa.

By day 14, decorin was detected in the resting chondrocytes and some of the proliferative chondrocytes in both the rhOP-1 treated and untreated groups. Decorin was observed

Figure 4.8: Immunohistochemical localisation of native chondroitin sulphate in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a polyclonal anti-7D4 antibody and the antigen detected using the DAB substrate. An untreated animal (A) and a rhOP-1 treated animal (B) at day 7, an untreated animal (C) and a rhOP-1 treated animal (D) at day 56, a rhOP-1 treated animal at day 7 incubated overnight without primary antibody, as a negative control (E) and a normal animal (F) are shown. The defect is indicated by (*). The bar represents 100 μm.

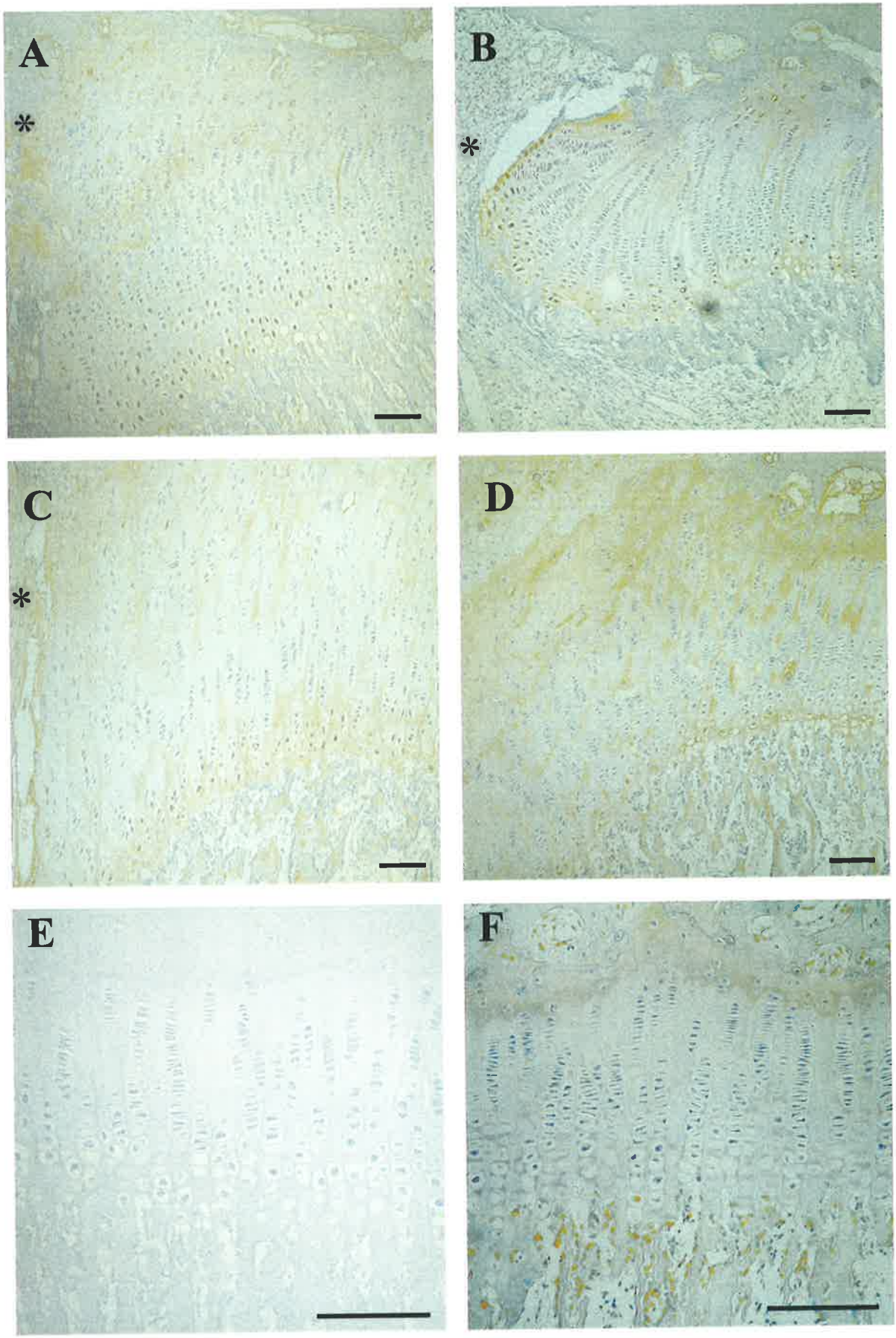
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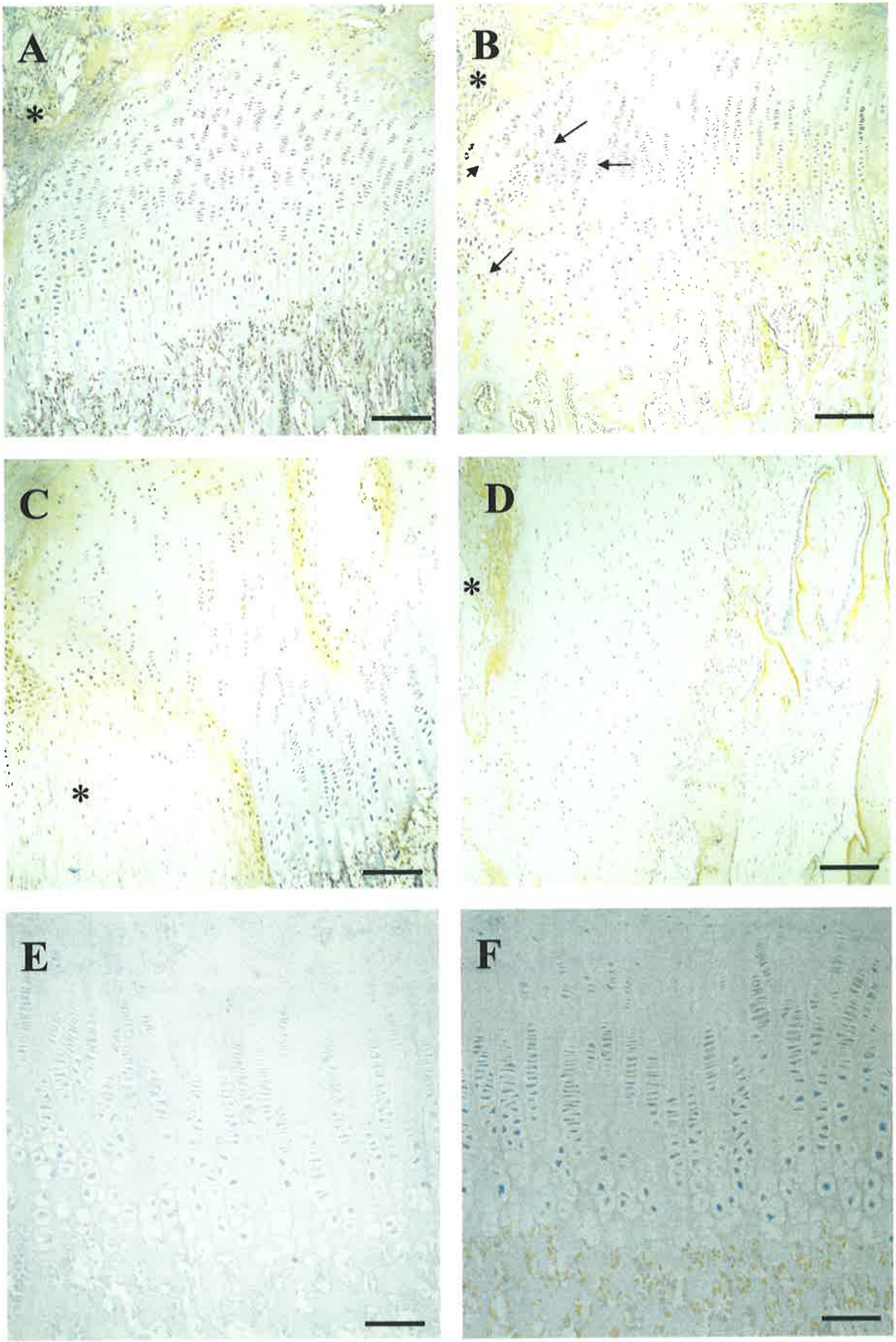
Figure 4.9: Immunohistochemical localisation of decorin in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a polyclonal anti-decorin antibody and the antigen detected using the DAB substrate. An untreated animal (A) and a rhOP-1 treated animal (B) at day 7, an untreated animal (C) and a rhOP-1 treated animal (D) at day 56, a rhOP-1 treated animal at day 7 incubated overnight without primary antibody, as a negative control (E) and a normal animal (F) are shown. The defect is indicated by (*). The bar represents 100 μm.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within the osteoblasts and osteocytes of the metaphysis, as described for day 7 sections (data not shown). Treated and untreated animals at day 56 displayed decorin within the growth plate adjacent to the defect (Fig 4.9C-D) in addition to the decorin within the metaphysis.

No decorin was seen in sections that were incubated without primary antibody (Fig 4.9E). Normal animals displayed decorin within and surrounding the trabeculae in the metaphysis of the bone. There was no decorin observed within the growth plate in any of the animals (Fig 4.9F).

4.1.4.5 Biglycan

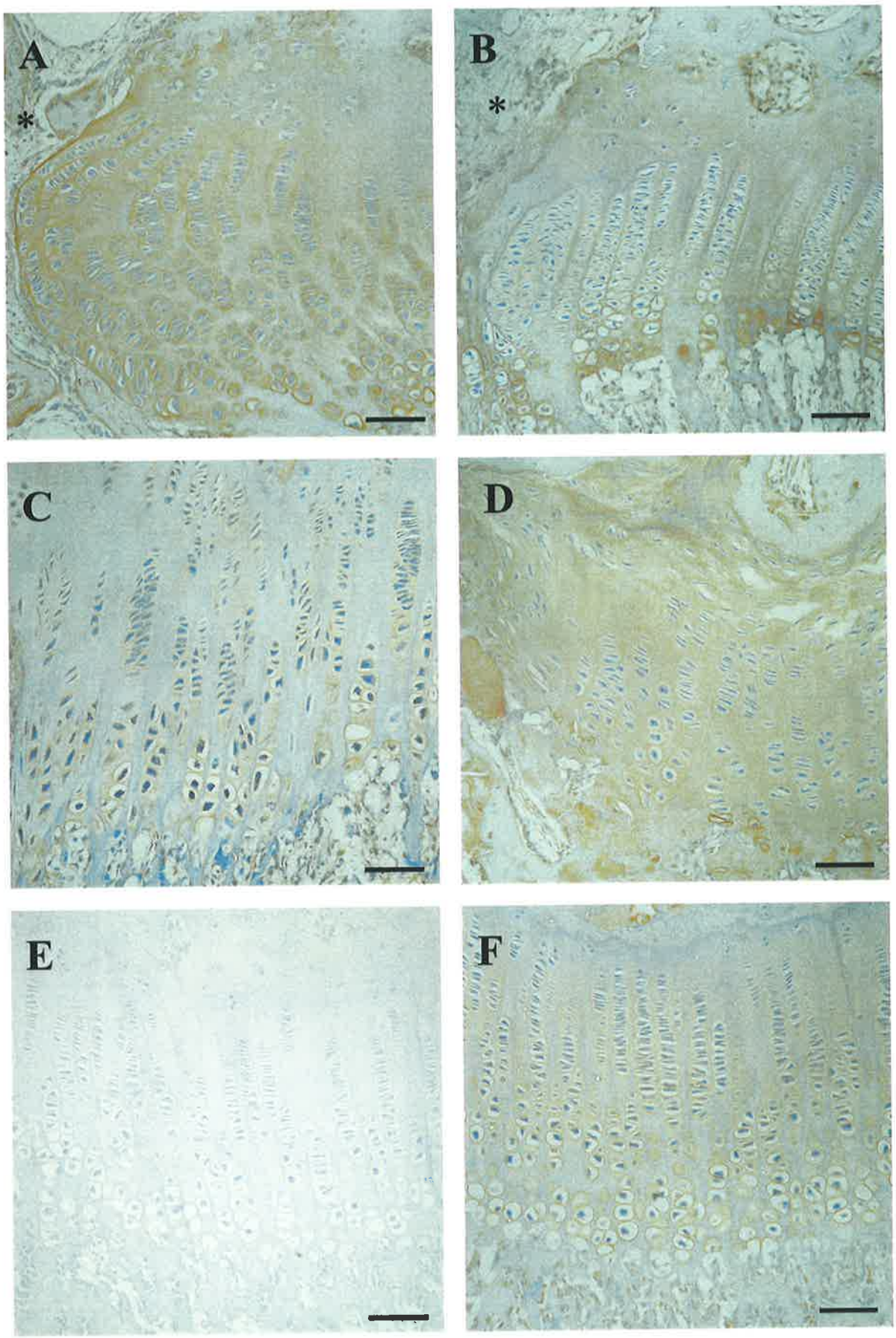
The detection of biglycan was limited to the growth plate in both rhOP-1 treated and untreated animals at all time points (Fig. 4.10A-D). Biglycan was not observed within the trabeculae of the epiphysis or metaphysis or within the defect in any of the animals. There was no difference in biglycan distribution between the rhOP-1 treated and untreated animals at any of the time points.

Incubation without the biglycan antibody resulted in no detection of biglycan (Fig. 4.10E). In normal animals, biglycan was present throughout all zones of the growth plate and not within the epiphyseal or metaphyseal bone (Fig. 4.10F).

4.2 Fibrocartilage and polarised light

Polarised light was used in conjunction with Sirius Red staining of growth plate sections to further examine the collagen changes that occurred in the growth plate immediately adjacent to the defect. Sirius Red is a strong, anionic dye that stains collagen via its

Figure 4.10: Immunohistochemical localisation of biglycan in growth plate sections. Paraffin sections (5 μm) were incubated overnight with a polyclonal anti-biglycan antibody and the antigen detected using the DAB substrate. An untreated animal (A) and a rhOP-1 treated animal (B) at day 7, an untreated animal (C) and a rhOP-1 treated animal (D) at day 56, a rhOP-1 treated animal at day 7 incubated overnight without primary antibody, as a negative control (E) and a normal animal (F) are shown. The defect is indicated by (*). The bar represents 100 μm.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sulphonic acid groups, which react with the basic groups present in collagen molecules (Junqueira *et al.*, 1979) allowing the detection of very thin collagen fibrils that are not visible under normal light microscopy (Junqueira *et al.*, 1979)

In normal animals, the type II collagen present within the growth plate presents blue/green in colour and aligns parallel with the columns of the proliferative zone (Fig. 4.11A). Articular cartilage (Fig. 4.11B) was also blue in colour, typical of type II collagen, however it lacked the organised columns seen in the growth plate. Both the metaphyseal (Fig. 4.11C) and epiphyseal (Fig. 4.11D) bone were bright yellow, indicative of collagen type I. The outer edges of the trabeculae showed traces of red, which also denotes type I collagen.

In the untreated animals, there was a progression from normal growth plate architecture to that of fibrocartilage. In day 7 animals, the presence of type II collagen was prominent, and remained associated with the chondrocyte columns within the proliferative zone (Fig. 4.12A). This pattern continued at day 14 (Fig. 4.12B) but by day 56 there was a change from type II collagen in the growth plate adjacent to the defect to type I collagen (Fig. 4.12C). The medial spur that had formed still displayed type II collagen, but it was not aligned in columns as seen in a normal growth plate. The collagen fibres within the fibrous capsule surrounding the fat implant were yellow, indicating the presence of type I collagen (Fig. 4.12D).

In the rhOP-1 treated animals, the day 7 animals displayed normal growth plate architecture, with type II collagen aligned parallel to the proliferative columns of the growth plate, except at the boundary of the defect. At this point, there was yellow staining

Figure 4.11: Polarised light microscopy of normal growth plate cartilage. The growth plate (A), articular cartilage (B), metaphyseal trabeculae (C) and epiphyseal bone (D) are shown. The type II collagen within the growth plate and articular cartilage is a blue/green colour, whereas the type I collagen found in the epiphyseal and metaphyseal bone is a yellow colour. The type II collagen in the growth plate is aligned parallel to the chondrocyte columns (arrow), and is randomly distributed throughout the articular cartilage. The bar represents 200 μm.

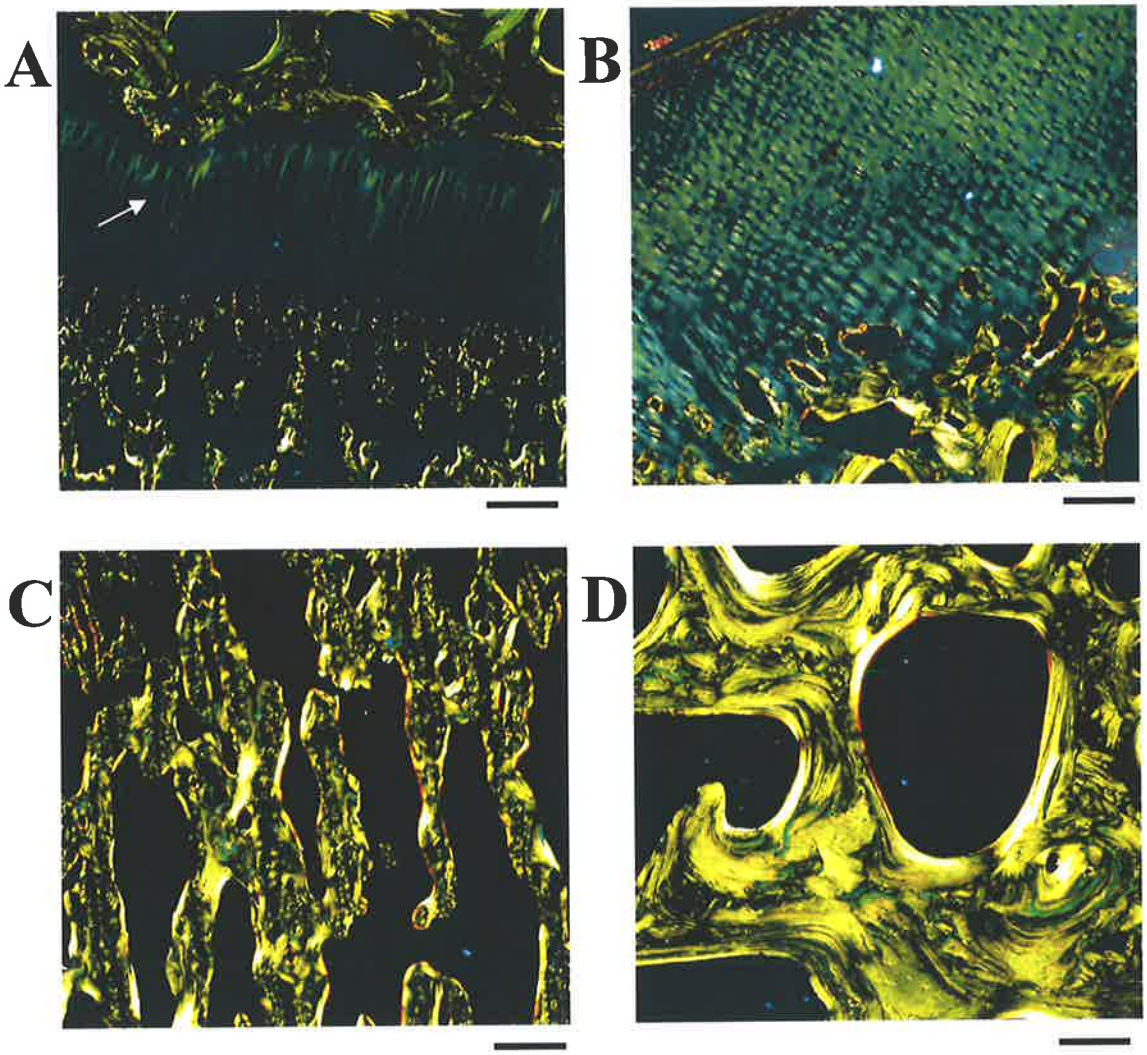
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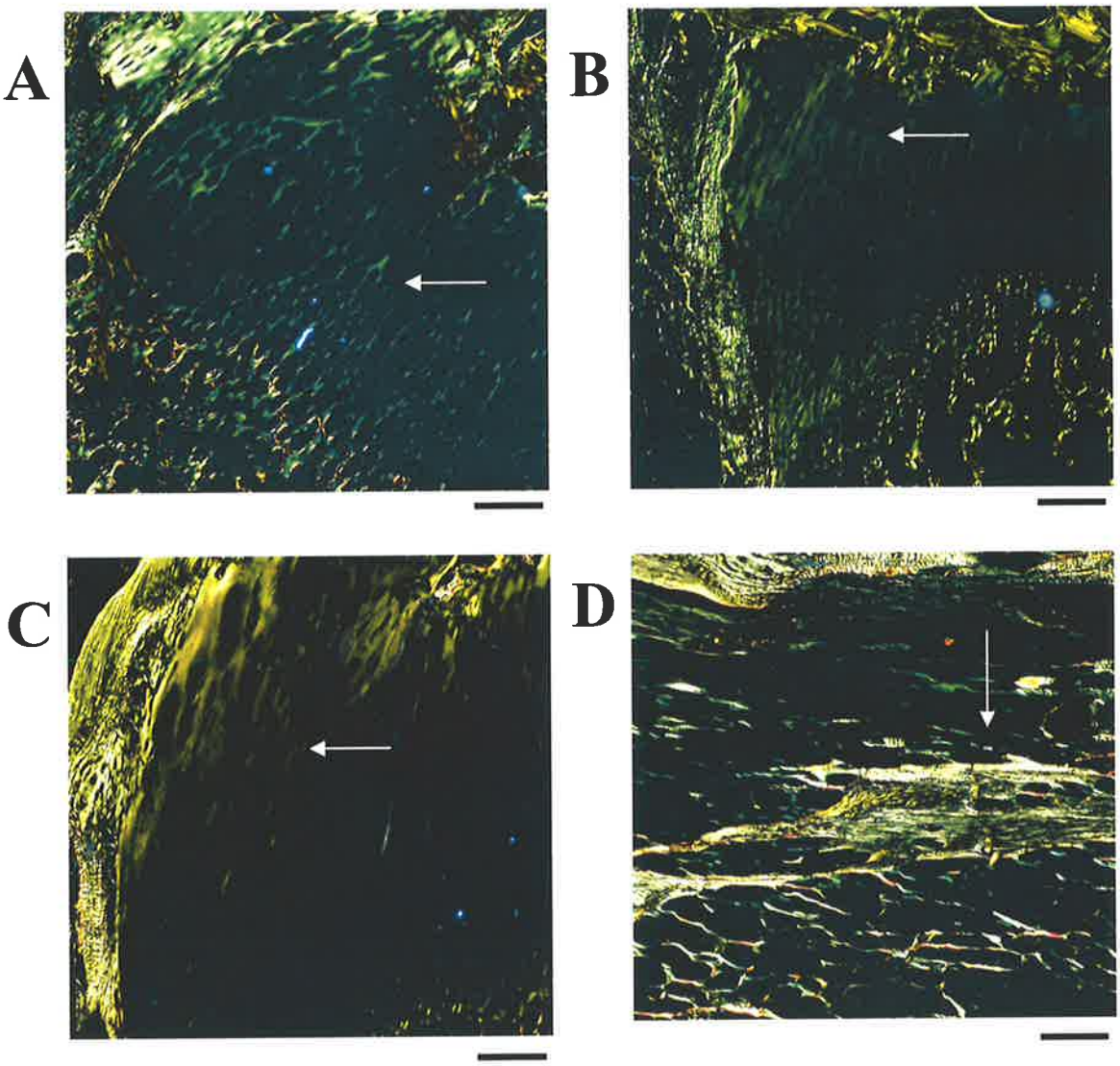
Figure 4.12: Polarised light microscopy of growth plate cartilage from untreated animals. The growth plate at day 7 (A), 14 (B) and 56 (C) are shown. The defect is indicated by (*). Arrows indicate a change in the collagen expression. The fibrous capsule of the defect is shown in (D). The bar represents 100 μm.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rather than blue, indicating expression of type I collagen (Fig. 4.13A, arrow). At day 14 the type II collagen expression had ceased adjacent to the defect and had been replaced with type I collagen (Fig.4.13B). By day 56, the type II collagen within the growth plate adjacent to the defect no longer possessed its columnar arrangement. The type II collagen was throughout the growth plate, but did not extend into the medial spur, which displayed no type I or type II collagen staining (Fig. 4.13C). The four rhOP-1 treated animals that displayed fibrous capsule formation in the defect displayed staining for type I collagen in the capsule. There were small clusters of collagen staining that did not appear the same as the staining of the collagen fibres in the fibrous capsule. This may represent new bone formation (Fig.4.13D).

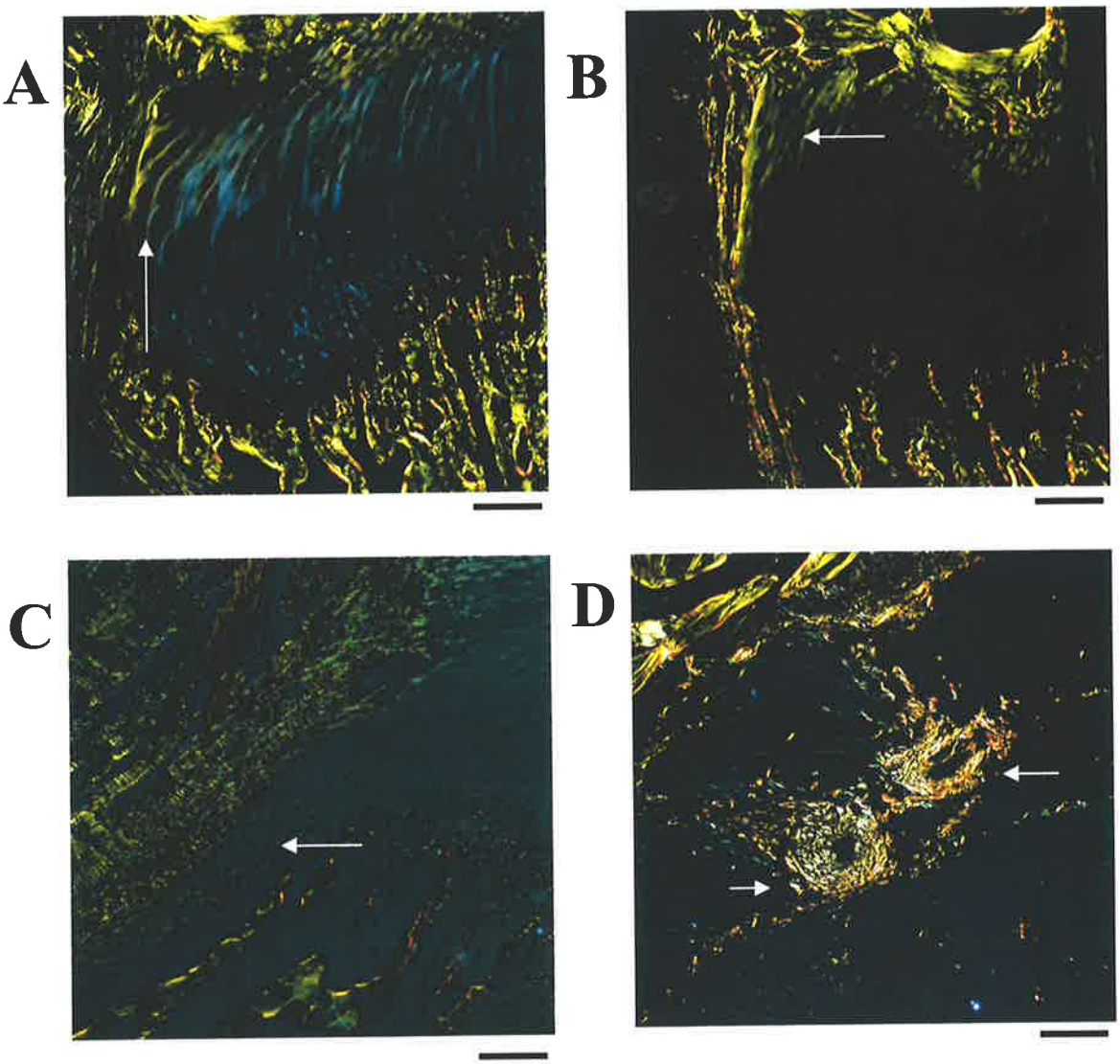
4.3 *In situ* hybridisation

Having established that there were changes in the type of collagen present in the growth plate following injury, *in situ* hybridisation was performed to determine if there was a change in the mRNA expression in the chondrocytes of the growth plate adjacent to the defect. Digoxigenin-labelled riboprobes were synthesised and *in situ* hybridisation performed as described in section 2.5.4.

4.3.1 Type I Collagen

Antisense and sense digoxigenin-labelled type I collagen riboprobes were synthesised for *in situ* hybridisation as described in section 2.5.2. The cross-reactivity of the riboprobes was tested using Northern hybridisation to RNA isolated from the resting and proliferative growth plate zones of sheep (section 2.5.1). Hybridisation with the antisense riboprobe identified a single band of approximately 5 kb in the resting and proliferative zone RNA

Figure 4.13: Polarised light microscopy of growth plate cartilage from rhOP-1 treated animals. The growth plate at day 7 (A), 14 (B) and 56 (C) are shown. The defect is indicated by (*). Arrows indicate a change in the collagen expression. The fibrous capsule of the defect is shown in (D). The bar represents 100 μm.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(Fig. 4.14A). The size of the detected collagen is similar in size to that of other species, which range from 4.6 kb to 6.3 kb (see Appendix A). Hybridisation with the sense riboprobe did not detect any RNA in either the resting or proliferative cells (data not shown).

In situ hybridization was performed on paraffin-embedded sections that were hybridised overnight with a type I collagen antisense digoxigenin-labelled riboprobe (section 2.5.4). Type I collagen mRNA was not observed within the growth plate in any of the rhOP-1 treated or untreated animals at any time point (Fig. 4.14B). However, there was expression in osteoblasts surrounding the trabeculae in the metaphysis (Fig. 4.14C), indicating that the *in situ* hybridization procedure had worked. The fibrous capsule within the defect also stained positively for type I collagen mRNA (Fig. 4.14B). The small clusters of type I collagen identified under polarised light also stained positively for type I collagen mRNA, supporting the possibility of new intramembranous bone formation.

4.3.2 Type II Collagen

Antisense and sense digoxigenin-labelled riboprobes for type II collagen were constructed as described in section 2.5.2. Northern hybridisation with the antisense riboprobe detected a single band at approximately 4.5 kb in the RNA from the resting and proliferative zones of the sheep growth plate, with greater expression within the proliferative chondrocytes than in the resting chondrocytes (Fig. 4.15). The size of the detected type II collagen is similar to that of other species (see Appendix B). Hybridisation with the sense riboprobe did not detect any RNA in either the resting or proliferative RNA (data not shown).

Figure 4.14: Northern analysis and *in situ* hybridisation of type I collagen. (A) Northern hybridisation with an antisense [α^{32}P]-dUTP labelled type I collagen riboprobe. *In situ* hybridisation was performed on paraffin embedded sections using a digoxigenin-labelled riboprobe. The growth plate (B), fibrous capsule (B) and trabeculae within the metaphysis (C) of a rhOP-1 treated animal are shown. The defect is indicated by (*). The arrow indicates cells expressing type I collagen. The bar represents 100 μm.

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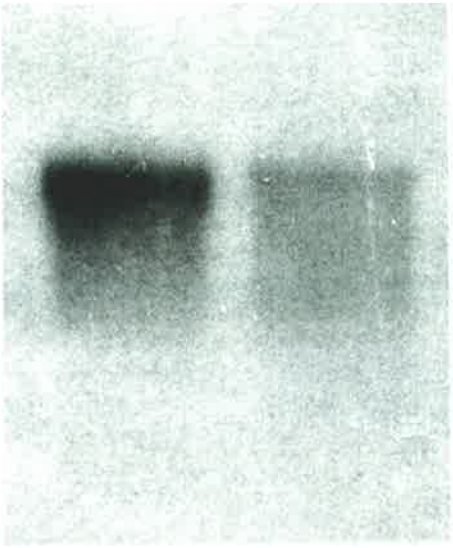

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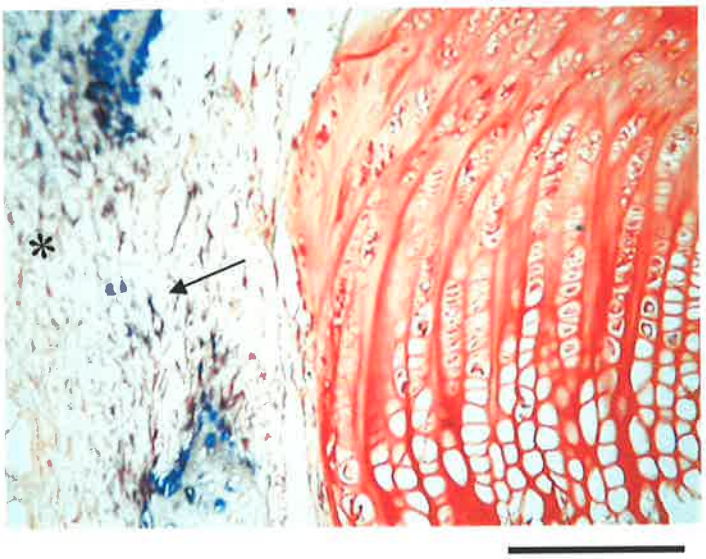


C

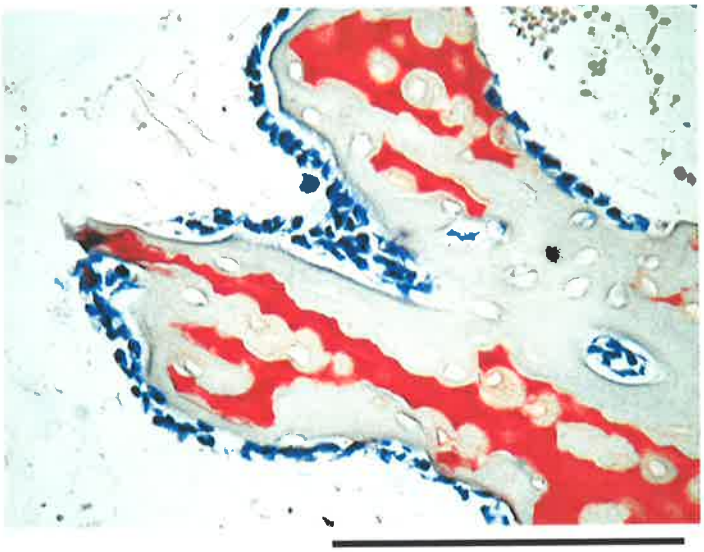
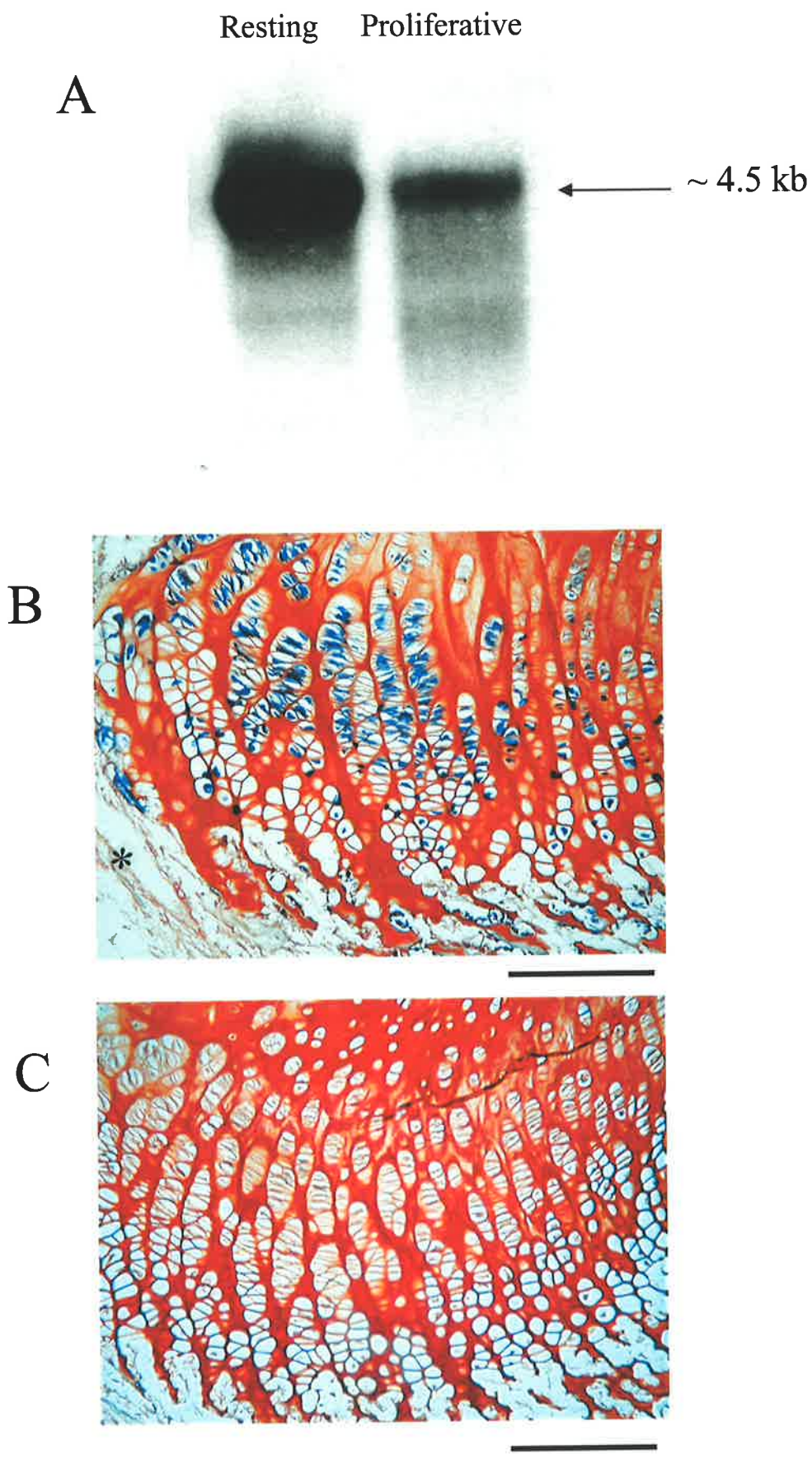


Figure 4.15: Northern analysis and *in situ* hybridisation of type II collagen. (A) Northern hybridisation with an antisense [α^{32}P]-dUTP labelled type II collagen riboprobe. *In situ* hybridisation was performed on paraffin embedded sections using a digoxigenin-labelled riboprobe. The growth plate of a day 7 rhOP-1 treated animal is shown (B,C). There was positive staining in the chondrocytes of some sections (B) but under the same conditions, there was no staining within the chondrocytes in the majority of sections (C). The bar represents 100 μm.


Resting
Proliferative
A
~ 4.5 kb
B
C

Following hybridisation of paraffin-embedded sections with the type II collagen riboprobe, irregular staining results were observed. A day 56 section from both the rhOP-1 treated and untreated group showed type II collagen expression throughout the chondrocytes of the growth plate (Fig. 4.15B,C). A day 7 untreated section showed expression in a few of the chondrocytes of the growth plate, but the staining was not consistent throughout the section. The remaining sections displayed no staining within the chondrocytes of the growth plate. No type II collagen staining was identified within the defect of any animals.

Discussion

Previous studies have shown that the administration of rhOP-1 to cartilage *in vitro* and *in vivo* has both chondrogenic and osteogenic effects (Dieudonne *et al.*, 1994; Haaijman *et al.*, 1997; Johnstone *et al.*, 2002). In this study, it was hypothesised that the chondrogenic potential of rhOP-1 could be utilised to enhance regeneration of the growth plate following injury. The previous chapter reported that administration of rhOP-1 resulted in an increase in height of the growth plate adjacent to the defect. This chapter focused on the molecular changes in and around the growth plate, and examined the collagenous content of the growth plate adjacent to the injury site.

The growth plate adjacent to the defect displayed molecules consistent with the cartilage phenotype, including collagen types II and X, biglycan and glycosaminoglycan epitopes from chondroitin sulphate, chondroitin-4-sulphate and keratan sulphate. There were no changes in the temporal or spatial immunolocalisation of these molecules between the rhOP-1 treated and untreated animals, suggesting that rhOP-1 did not disrupt the chondrogenic phenotype.

In addition to the cartilage-specific molecules within the expanded growth plate, immunolocalisation studies at day 7 demonstrated the presence of type I collagen, osteopontin and decorin in the growth plate adjacent to the defect in the rhOP-1 treated animals. The same molecules were later detected in the untreated animals at day 56. These molecules are usually found in bone and the appearance of these molecules in growth plate chondrocytes suggests an alteration in protein expression. The fact that the appearance of these osteogenic molecules was in both the rhOP-1 treated and untreated animals indicated that it was not a direct effect of the rhOP-1 treatment. Rather, treatment with rhOP-1 accelerated the response, with the molecules appearing at day 7 rather than at day 56.

The appearance of type I collagen at day 7 in the rhOP-1 treated animals is supported by the analysis of the growth plate with polarised light. At day 7 the rhOP-1 treated animals showed a change in the collagen adjacent to the defect, and by day 56 no collagen was visible within the medial spur. The birefringence of collagens is enhanced if they are aligned together, as with the type II collagen fibres which run parallel to the proliferative chondrocyte columns. If the growth plate loses its organised structure, the collagen fibres become randomly arranged and therefore less birefringent. The untreated animals did not exhibit a change in the collagen adjacent to the defect until day 56, again supporting the argument that rhOP-1 accelerated the disorganisation of cartilage structure, rather than initiating it.

In situ hybridisation was used to determine whether the detection of type I collagen in the growth plate was due to a change in mRNA expression at the same site. Neither type I nor type II collagen was detected within the growth plate using digoxigenin-labelled antisense riboprobes. As the riboprobes bound to the sheep mRNA on the Northern membrane, this

indicates that the riboprobes are cross-reactive with the sheep mRNA for the various molecules. The type I collagen riboprobe did show expression in the osteoblasts surrounding the trabeculae in the epiphysis and the metaphysis, indicating that the mRNA in the sections had not degraded. It is unlikely that the riboprobes could not bind to the mRNA within the chondrocytes of the growth plate as a result of an inability to penetrate the matrix of the growth plate, as several enzymes were used to facilitate matrix digestion (for enzymes used see section 2.5.4). A more likely possibility is that the chondrocytes had collapsed within their lacunae during the fixation period, thus preventing access of the riboprobe. In retrospect, a fixative that conserved the structure of the lacunae and the chondrocytes would have been more appropriate.

There are several possibilities to explain the presence of the type I collagen, osteopontin and decorin within the growth plate. They may be present due to a wound healing response, they may be independently expressed in response to the surgical procedure, or they may be the result of the cells switching from a chondrogenic to an osteogenic phenotype.

The wound healing process begins with the marked infiltration of platelets and inflammatory cells to the wound site (Werner and Grose, 2003). This is followed by the proliferative phase in which cells proliferate and begin to synthesise and deposit large amounts of extracellular matrix. Osteopontin has been implicated in the recruitment of macrophages during skin healing (Giachelli *et al.*, 1998; Liaw *et al.*, 1998), and thus could be released by neutrophils present within a blood clot for the attraction of macrophages to the growth plate injury site. The presence of type I collagen and decorin could coincide with the reparative stages of healing, when large amounts of extracellular matrix are

synthesised, as decorin has been associated with type I collagen and the regulation of ordered matrix assembly (Scott and Orford, 1981; Scott *et al.*, 1986; Scott, 1988).

It may be possible that the expression of the bone proteins are not related to each other. Osteopontin is an effective inhibitor of hydroxyapatite crystal formation and growth (Boskey *et al.*, 1993) and may be up regulated in chondrocytes of the growth plate adjacent to the injury site to prevent mineralisation and bone bridge formation. There are several studies reporting the transition of type II collagen to type I collagen within chondrocytes, which may explain the presence of type I collagen within the chondrocytes of the growth plate adjacent to the defect. Type I collagen has been reported to accumulate within the matrix of developing articular chondrocytes (Sasano *et al.*, 1996) and treatment with retinoic acid (Wu *et al.*, 1997) or BrdU (Askew *et al.*, 1991) can result in a shift from type II collagen to type I collagen. *In vitro* experiments have also shown that chondrocytes have the ability to reinitiate type I collagen synthesis in response to changes in the cellular environment (Schenk *et al.*, 1967). The damage to the growth plate may have been enough to initiate this change in collagen. The trauma from the surgical ablation of the growth plate may have initiated the transition from type II collagen to type I collagen, with the presence of osteopontin and decorin secondary to the initial switch from a chondrogenic to an osteogenic phenotype. These molecules are important in the regulation of the types of collagen synthesised and in the turnover of matrix components important in collagen fibril formation (Scott and Orford, 1981; Scott *et al.*, 1986; Scott, 1988; Liaw *et al.*, 1998).

The expression of type I collagen, osteopontin and decorin within the cells of the growth plate may indicate the transition of cells from a chondrogenic to an osteogenic phenotype, as all three molecules are normally expressed within bone and not cartilage. Type I

collagen is the predominate collagen of bone, imparting strength and providing the site for mineralisation (Sandberg and Vuorio, 1987; Mundlos *et al.*, 1990). The extracellular matrix of the growth plate adjacent to the defect stained positively for type II collagen throughout the time course, while the chondrocytes began to stain positively for type I collagen. It is possible that the type I collagen would be secreted into the extracellular matrix and replace the type II collagen as the major collagen. The polarised light results suggest that this may have started to occur as there was a change from the ordered type II collagen fibre orientation in the growth plate adjacent to the defect. Osteopontin has been reported as important in regulating the mineralisation of cartilage and in the attachment of osteoclasts to bone (Reinholt *et al.*, 1990; Hultenby *et al.*, 1991). The presence of this molecule in the growth plate adjacent to the defect may alter the controlled mineralisation process of the growth plate. Decorin is important in the regulation of collagen fibril size and matrix assembly (Scott and Orford, 1981; Scott *et al.*, 1986; Scott, 1988; Santra *et al.*, 1995; De Luca *et al.*, 1996). If the matrix of the growth plate changed from type II to type I collagen, decorin may be up-regulated to assist in matrix reassembly.

Summary

The results presented in this chapter suggest that the normal repair process results in a switch from a chondrogenic to an osteogenic phenotype within the growth plate adjacent to the injury site. The occurrence of type I collagen, osteopontin and decorin within the growth plate indicates that an osteogenic phenotype is supported in growth plate cartilage repair. Treatment with rhOP-1 advances the appearance of type I collagen, osteopontin and decorin, and thus accelerates the process of endochondral bone formation.

Chapter 5

General Discussion and Conclusions

Review of Project Aims and Significance

Injuries to children's bones are complicated by the presence of the growth plates towards either end of the bones. The reported incidence of growth plate injuries ranges from 18% (Worlock and Stower, 1986; Mizuta *et al.*, 1987) to 30% (Mann and Rajmaira, 1990) of all children's fractures, with these injuries often leading to limb length discrepancies and angular deformities due to the formation of a bone bridge that spans the growth plate at the injury site. As the growth plate has a limited ability to repair itself, surgical practices such as the Langenskiöld procedure are used to remove the bone bridge and stabilise the injury area. However, reviews of the Langenskiöld procedure have reported fair and poor results in 15% to 43% of patients (Langenskiöld, 1981; Peterson, 1984; Williamson and Staheli, 1990; Hasler and Foster, 2002), with the technique often failing due to incomplete resection or reformation of the bone bridge. Therefore, the first aim of this study was to investigate the histological and molecular events that occur following the treatment of a growth plate injury using the Langenskiöld procedure.

Members of the BMP family were originally identified as proteins that induced ectopic bone formation when implanted subcutaneously (Urist, 1965; Sampath and Reddi, 1981). However, gene knockout and over expression models have demonstrated that the members of the BMP family are also involved in the process of embryogenesis and organogenesis (Kingsley *et al.*, 1992; King *et al.*, 1994; Dudley *et al.*, 1995; Luo *et al.*, 1995; Winnier *et al.*, 1995; Zhang and Bradley, 1996). There are now several *in vivo* studies demonstrating the strong chondrogenic potential of two members of the BMP family, BMP-2 and OP-1, for healing articular cartilage defects (Grgic *et al.*, 1997; Sellers *et al.*, 1997; Louwerse *et al.*, 2000; Sellers *et al.*, 2000). As the growth plate has a limited ability for self-repair, a BMP with chondrogenic potential was administered in conjunction with the Langenskiöld

procedure to evaluate the regenerative potential of the growth plate. OP-1 was selected for this study as it has been shown to induce the synthesis of the majority of extracellular matrix components and cartilage-specific growth factors (Flechtenmacher *et al.*, 1996; Huch *et al.*, 1997; Lietman *et al.*, 1997; Nishida *et al.*, 2000a; Nishida *et al.*, 2000b), and is the only BMP that does not induce catabolic events in adult articular cartilage (Huch *et al.*, 1997; Koepp *et al.*, 1999; Jennings *et al.*, 2001). It is also capable of reducing inflammation and down regulating type I collagen expression without the induction of type X collagen and chondrocyte proliferation *in vitro* (Flechtenmacher *et al.*, 1996; Huch *et al.*, 1997; Chubinskaya and Kuettner, 2003). Therefore, the second aim of this study was to evaluate the response of the growth plate and injury site following treatment using the Langenskiöld procedure in conjunction with the administration of rhOP-1.

Growth Plate Response Following the Langenskiöld Procedure

Following the Langenskiöld procedure, the untreated animals did not undergo substantial bone formation within the defect. The inserted fat remained viable and in position, and the overall growth of the operated limb continued at an equivalent rate to that of the normal animals. The growth plate adjacent to the defect increased in height 7 days after the surgery, possibly as the result of trauma to the area. By day 56, the untreated animals exhibited medial spur formation, with one animal showing a change in structure towards fibro-cartilage. All untreated animals also demonstrated the formation of a fibrous capsule around the fat implant. Immunolocation and polarised light studies indicated that there was a modification in the protein expression pattern of the growth plate chondrocytes adjacent to the defect towards an osteogenic phenotype, as they began to express the bone proteins type I collagen, osteopontin and decorin.

During the bone fracture repair process, type I collagen is expressed within the soft callus, the site of chondrogenesis, prior to endochondral ossification (Jingushi *et al.*, 1992). Several other studies have described the presence of type I collagen in the hypertrophic chondrocytes of the growth plate and in mineralised cartilage (von der Mark and von der Mark, 1977; Horton and Machado, 1988). Therefore, the presence of type I collagen in the chondrocytes adjacent to the defect may correlate with the chondrogenic stage of bone fracture repair, prior to ossification. The presence of decorin and osteopontin within the growth plate also supports this scenario, as decorin has been shown to be associated with type I collagen regulating matrix assembly (Scott and Orford, 1981; Scott *et al.*, 1986; Scott, 1988), whilst osteopontin has been reported to be important in the mineralisation process (Hultenby *et al.*, 1991). Both are important events in the endochondral ossification pathway. Osteopontin has also been reported to have roles in the influx of macrophages during wound healing (Giachelli *et al.*, 1998) and in regulating collagen fibrillogenesis (Liaw *et al.*, 1998). Therefore, the presence of these molecules suggests an up-regulation of the endochondral ossification pathway in the growth plate adjacent to the defect following the Langenskiöld procedure, leading to fibro-cartilage metaplasia.

Growth Plate Response Following rhOP-1 Administration

The administration of rhOP-1 to the injury site following the Langenskiöld procedure initiated a strong chondrogenic response within the growth plate adjacent to the defect. There was a significant increase in the height of the resting zone by day 56, which was accompanied by a significant decrease in the height of the proliferative and hypertrophic zones. As there were no changes in the number of chondrocytes per square millimetre, the increase in height was likely due to an up-regulation of matrix synthesis. The increase in growth plate height continued throughout the time course, however the rate of increase

slowed from 28.2 μm/day in the first 7 days to 5 μm/day for the remainder of the experimental period.

Histological analysis determined a greater degree of medial spur and fibro-cartilage formation in the animals treated with rhOP-1. The rhOP-1 treated animals had limited fibrous capsule formation when compared to the untreated animals. However, this may be a beneficial consequence as the majority of bone formation seen in the defects occurred within the fibrous capsules.

Type I collagen, osteopontin and decorin were also detected in the growth plate adjacent to the defect in the rhOP-1 treated animals, however these molecules were seen as early as day 7, compared to days 14 and 56 in the untreated animals. Therefore, the administration of rhOP-1 to the defect following the Langenskiöld procedure accelerated the endochondral ossification response and fibro-cartilage metaplasia seen in the untreated animals.

Alternate Delivery Regimes

The fat used as the interpositional material in this study proved to be successful in preventing bone bridge formation within the injury site. However, it was not the most ideal material to be used in conjunction with the rhOP-1, as it could not be impregnated with the rhOP-1, so the fat and the rhOP-1 had to be positioned and administered separately. The rhOP-1 was given as a single dose, and therefore may not have been present at the injury site for the whole experimental time course as the half-life of OP-1 in circulation has been reported to be approximately 30 minutes (Vukicevic *et al.*, 1998). An alternative method for delivering the rhOP-1 would be in the form of an impregnated bead or combined as a

paste or scaffolding which would allow a gradual release of the rhOP-1, and would ensure the rhOP-1 remained in the correct location. Type I collagen scaffolds infused with rhOP-1 are used to heal segmental bone defects, as the collagen can induce local bone formation. As type I collagen is a natural component of bone, its degradation and degradation products can be mediated by physiological means (Uludag *et al.*, 2001). The type I collagen scaffold is not suitable for growth plate defects, as it induces bone formation within the defect site without the presence of rhOP-1 (Johnstone *et al.*, 2002). A scaffold that resembles growth plate cartilage matrix (e.g. composed of type II collagen) may be more successful in promoting chondrogenesis and outgrowth of the remaining growth plate.

In this study, a single dose of rhOP-1 (350 μg) was injected at the interface of the defect and the remaining growth plate. This dosage was the same as that used in the study by Johnstone *et al.* (Johnstone *et al.*, 2002), which reported an increase in height of the growth plate adjacent to the defect. This dosage is within the range of that used in other *in vivo* studies to regenerate articular and thyroid cartilages, doses of which have ranged from 11 μg to 500 μg (Katic *et al.*, 2000; Louwerse *et al.*, 2000; Jelic *et al.*, 2001; Cook *et al.*, 2003). All of these studies have reported an induction of chondrogenesis, with cells taking on the chondrocyte phenotype and producing cartilage specific collagens and proteoglycans. The degree of integration between the old and new cartilage differed depending on the type of defect being examined. Both thyroid cartilage (Katic *et al.*, 2000) and chondral articular cartilage defects (Jelic *et al.*, 2001) showed integration of the newly synthesised cartilage with the existing cartilage after OP-1 administration (4 and 3 months, respectively), whereas contrasting results have been reported for subchondral defects (Grgic *et al.*, 1997; Louwerse *et al.*, 2000; Cook *et al.*, 2003).

A study by Piscione *et al.* (Piscione *et al.*, 1997) investigated the role of OP-1 in kidney development and determined that the administration of low levels of OP-1 stimulated growth of the ureteric bud/collecting ducts of the kidney, whereas higher doses had an inhibitory effect. Therefore, this indicates that the concentration of rhOP-1 is critical to its effect within the microenvironment in which it is deposited. It is also possible that the concentration required for chondrogenesis to occur within the remaining growth plate is critical, and a higher or lower concentration may result in the activation of the osteogenic pathway. Future studies should investigate the effects of rhOP-1 on the Langenskiöld procedure using both higher and lower concentrations of rhOP-1.

The Future of Growth Plate Repair

Current research for cartilage repair is focussed on two key areas: the addition of exogenous growth factors to promote healing; and tissue regeneration by addition of undifferentiated cells to the defect site.

The addition of growth factors (rhBMP-2, rhOP-1) to articular cartilage defects has proven to be successful (Grgic *et al.*, 1997; Sellers *et al.*, 1997; Louwerse *et al.*, 2000; Sellers *et al.*, 2000; Jelic *et al.*, 2001; Cook *et al.*, 2003). However, the repair of growth plate cartilage using these growth factors has been complicated (Johnstone *et al.*, 2002; Lee *et al.*, 2002). The adenoviral-mediated gene transfer of BMP-2 with muscle interposition in rabbits resulted in bone bridge formation across the growth plate and significant tibial shortening (Lee *et al.*, 2002). A chondrogenic response has been observed following administration of rhOP-1, however the interpositional material that has been inserted (type I collagen paste (Johnstone *et al.*, 2002) or fat in this study) has not allowed the tissue to regenerate. Future studies may look at a multifactorial approach to growth plate repair,

with the addition of a growth factor in conjunction with cells to support tissue regeneration.

Due to the limited availability of differentiated cells such as chondrocytes, research has moved towards the use of adult mesenchymal stem cells. These cells reside in the bone marrow and are supportive cells for haematopoiesis and a reservoir and regeneration pool for various mesenchymal tissues such as cartilage (Gerson, 1999). Mesenchymal stem cells have been documented for their use to repair articular cartilage defects (Wakitani *et al.*, 1994), and recently have been utilised to repair growth plate cartilage. A study by Chen *et al.* (Chen *et al.*, 2003) cultured stem cells from periosteum, embedded them in agarose and transferred them into an ablated growth plate model in rabbits. The mesenchymal stem cells showed chondrogenic potential, demonstrated by the columnar arrangement undertaken by the cells, as seen in the proliferative zone of a normal growth plate. There was no evidence of bone bridge formation. The regeneration of the growth plate in this study is promising, and the use of mesenchymal stem cells as a scaffold for growth plate repair warrants further research.

Conclusion

The administration of rhOP-1 in conjunction with the Langenskiöld procedure initiated a complex response in the growth plate adjacent to the defect. The histological and molecular evidence from this study confirms that the chondrocyte phenotype, although apparently widening the growth plate adjacent to the defect, is a mixture of (i) accelerated endochondral ossification, as evidenced by the appearance of bone-specific proteins, and (ii) fibro-cartilage transformation, as shown by histological analysis.

Although rhOP-1 has been successfully used to heal articular cartilage and segmental bone defects, its use in the repair of growth plate cartilage is contraindicated due to the stimulation of the chondrocytes to express molecules more typical of the osteogenic phenotype.

Appendices

Appendix A: Type I Collagen Alignment

Dog (AF035120): 4560 bp
Human (K01228): 3347 bp (partial mRNA)
Mouse (U08020): 4589 bp
Rat (Z78279): 5721 bp

* indicates bases that are conserved across all species listed.

The sequence in bold is the fragment used for riboprobe construction.

```
                    10        20        30        40        50        60
                     .         .         .         .         .         .
Dog                 ------------------------------------------------------------
Human               ------------------------------------------------------------
Mouse               ATGTTCAGCTTTGTGGACCTCCGGCTCCTGCTCCTCTTAGGGGCCACTGCCCTCCTGACG
Rat                 ATGTTCAGCTTTGTGGACCTCCGGCTCCTGCTCCTCTTAGGGGCCACTGCCCTCCTGACG

Dog                 ------------AGCACCAAGGCGGCAGGAGGGTTCAGCTAAGTTGGAGGTACTGGCCAC
Human               ------------------------------------------------------------
Mouse               CATGGCCAAGAAGACATCCCTGAAGTCAGCTGCATACACAATGGCCTAAGGGTCCCCAAT
Rat                 CATGGCCAAGAAGACATCCCTGAAGTCAGCTGCATACACAATGGCCTAAGGGTCCCTAAT

Dog                 GACTGCATGCCTGCGCCCGCCAGGTGATACCTCCGCCGGTGACCCAGGGGCTCTGCGACA
Human               ------------------------------------------------------------
Mouse               GGTGAGACGTGGAAACCCGAGGTATGCTTGATCTGTATCTGCCACAATGGCACGGCTGTG
Rat                 GGTGAGACGTGGAAACCTGATGTATGCTTGATCTGTATCTGCCACAATGGCACGGCTGTG

Dog                 CAAGGAGTCTGCATGTCTAAGTGGTA---GACATGCTCAGCTTTGTGGATACGCGGACTT
Human               ------------------------------------------------------------
Mouse               TGCGATGACGTGCAATGCAATGAAGAACTGGACTGTCCCAACCCCCAAAGACGGGAGGGC
Rat                 TGCGATGGCGTGCTATGCAAAGAAGACTTGGACTGTCCCAACCCCCAAAAACGGGAGGGC

Dog                 TGTTGCTGCTTGCAGTAACTTCG----TGCCTAGCAACATGCCAATCTTTACAGGAGGCA
Human               ------------------------------------------------------------
Mouse               GGGTGCTGTGCTTTCTGCCCGGAAGAATACGTATCACCAAACTCAGAAGATGTAGGAGTC
Rat                 GAGTGCTGTCCTTTCTGCCCAGAAGAATATGTATCACCAGACGCAGAAGTCATAGGAGTC

Dog                 ACTGCAAGAAAGGGCCCAACTGGAGATAGAGGACCACGTGGAGAAAGGGGTCCACCAGGA
Human               ------------------------------------------------------------
Mouse               GAGGGACCCAAGGGAGGCCCTGGCCCCCAAGGCCCAAGGGGACCCGTTGGCCCCCCTGGA
Rat                 GAGGGACCCAAGGGAGACCCTGGCCCCCAAGGCCCAAGGGGACCTGTTGGCCCCCCTGGA

Dog                 CCACCAGGCAGAGATGGTGACGATGGTATCCCAGGCCCTCCTGGTCCACCTGGTCCTCCT
Human               ------------------------------------------------------------
Mouse               CGAGATGGCATCCCTGGACAGCCTGGACTTCCTGGTCCTCCTGGTCCCCCTGGCCCCCCC
Rat                 CAAGATGGCATCCCTGGACAGCCTGGACTTCCTGGTCCTCCTGGTCCCCCCGGCCCCCCC

Dog                 GGCCCCCCTGGTCTTGGCGGGAACTTTGCAGCCCAG-----------TATGATGGAAAA
Human               ------------------------------------------------------------
Mouse               GGACCCCCTGGCCTTGGAGGAAACTTTGCTTCCCAGATGTCCTATGGCTATGATGAAAAA
Rat                 GGACCCCTTGGTCTTGGAGGAAACTTTGCTTCCCAGATGTCCTATGGCTATGATGAGAAA
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Dog     GGAGTTGGCCTT---GGCCCTGGACCAATGGGTTTGATGGGACCTAGAGGTCCACCTGGT
Human   ------------------------------------------------------------
Mouse   TCAGCTGGAGTTTCCGTGCCTGGCCCCATGGGTCCTTCTGGTCCTCGTGGTCTCCCTGGC
Rat     TCAGCTGGAGTTTCCGTGCCTGGCCCCATGGGTCCTTCTGGTCCTCGTGGTCTCCCTGGC

Dog     GCATCTGGCGCTCCTGGCCCTCAAGGTTTCCAAGGACCTGCTGGTGAGCCTGGTGAACCT
Human   ------------------------------------------------------------
Mouse   CCCCCTGGTGCACCTGGTCCACAAGGTTTCCAAGGCCCCCCTGGTGAACCTGGCGAGCCT
Rat     CCCCCTGGTGCACCTGGTCCTCAAGGTTTCCAAGGCCCCCCTGGTGAACCTGGCGAGCCT

Dog     GGTCAAACTGGTCCAGCGGGTGCTCGTGGTCCACCTGGGCCTCCTGGCAAGGCTGGTGAG
Human   ------------------------------------------------------------
Mouse   GGCGGTTCAGGTCCAATGGGTCCCCGAGGTCCCCCTGGCCCTCCTGGCAAGAATGGAGAT
Rat     GGCGGTTCAGGTCCAATGGGTCCCCCAGGTCCCCCTGGCCCTCCTGGCAAGAACGGAGAT

Dog     GATGGTCACCCTGGAAAACCTGGACGACCCGGTGAGAGAGGAGTTGTTGGACCACAGGGT
Human   ------------------------------------------------------------
Mouse   GATGGGGAAGCTGGCAAGCCCGGCCGTCCTGGTGAGCGTGGACCTCCTGGACCTCAGGGT
Rat     GATGGGGAAGCTGGTAAGCCTGGCCGCCCTGGTGAGCGTGGACCTCCTGGACCTCAGGGT

Dog     GCTCGTGGTTTCCCTGGGACTCCTGGACTTCCTGGTTTCAAGGGCATTAGGGGACACAAT
Human   ------------------------------------------------------------
Mouse   GCTCGTGGATTGCCTGGAACAGCTGGCCTCCCTGGAATGAAGGGACACCGAGGCTTCAGT
Rat     GCTCGTGGATTGCCTGGAACAGCTGGCCTCCCCGGAATGAAGGGACACAGAGGTTTCAGT

Dog     GGTCTGGATGGATTGAAGGGACAGCCCGGTGCTCCAGGCGTGAAGGGTGAACCTGGTGCC
Human   ------------------------------------------------------------
Mouse   GGTTTGGATGGTGCCAAAGGAGATGCTGGTCCTGCTGGTCCTAAGGGAGAGCCCGGCAGT
Rat     GGTTTGGATGGTGCCAAAGGAGATACTGGTCCTGCTGGTCCTAAGGGAGAGCCTGGCAGT

Dog     CCTGGTGAAAATGGAACGCCAGGTCAAACAGGAGCCCGTGGGCTTCCTGGTGAGAGAGGA
Human   ------------------------------------------------------------
Mouse   CCTGGTGAAAACGGAGCTCCTGGCCAGATGGGTCCCCGAGGTCTGCCCGGTGAGAGAGGT
Rat     CCTGGTGAAAATGGAACTCCCGGCCAGATGGGTCCCCGAGGTCTGCCTGGTGAGAGAGGT

Dog     CGTGTTGGTGCCCCTGGTCCAGCTGGTGCCCGTGGAAGTGATGGAAGTGTGGGTCCTGTG
Human   ------------------------------------------------------------
Mouse   CGCCCTGGACCTCCTGGCACTGCTGGTGCTCGCGGCAACGATGGTGCTGTTGGTGCTGCT
Rat     CGCCCTGGACCCCCTGGCACTGCTGGTGCTCGTGGTAACGATGGTGCTGTCGGTGCAGCT

Dog     GGTCCTGCTGGTCCTATCGGGTCTGCTGGCCCTCCAGGCTTCCCAGGTGCTCCTGGCCCC
Human   ------------------------------------------------------------
Mouse   GGACCCCCTGGTCCCACCGGCCCCACTGGCCCTCCTGGCTTCCCTGGTGCAGTTGGTGCT
Rat     GGGCCCCCCGGTCCCACCGGCCCTACTGGCCCTCCTGGCTTCCCTGGTGCAGCTGGTGCT

Dog     AAGGGTGAAATCGGACCTGTTGGTAACCCCGGTCCTGCTGGTCCCGCGGGTCCCCGTGGT
Human   ------------------------------------------------------------
Mouse   AAGGGTGAAGCTGGTCCCCAAGGAGCTAGAGGCTCTGAAGGTCCCCAGGGTGTGCGTGGT
Rat     AAGGGTGAAGCTGGTCCCCAGGGAGCCCGAGGCTCTGAAGGTCCCCAGGGTGTGCGTGGT

Dog     GAAGTGGGTCTTCCAGGTGTTTCCGGCCCCGTTGGACCCCCTGGTAACCCTGGAGCCAAC
Human   ------------------------------------------------------------
Mouse   GAGCCCGGACCCCCTGGCCCTGCTGGTGCTGCCGGCCCTGCTGGAAACCCTGGTGCTGAT
Rat     GAGCCCGGACCCCCTGGCCCTGCTGGTGCTGCTGGTCCTGCTGGAAACCCTGGTGCTGAT
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Dog     GGCCTGACTGGTGCTAAGGGTGCTGCTGGCCTGCCCGGTGTCGCTGGGGCTCCTGGCCTC
Human   ------------------------------------------------------------
Mouse   GGACAACCTGGCGCTAAAGGTGCCAATGGTGCTCCTGGTATTGCTGGTGCTCCTGGCTTC
Rat     GGACAACCTGGTGCTAAAGGTGCCAATGGTGCTCCTGGTATTGCTGGTGCTCCTGGCTTC

Dog     CCTGGACCCCGTGGGATTCCTGGTCCTGTTGGTGCCGCTGGTGCTACTGGTGCCAGAGGA
Human   ----------------------------------------GGCCCTCCTGGTCCCAAGGGT
Mouse   CCTGGTGCCCGAGGCCCCTCTGGACCCCAGGGCCCCAGCGGCCCTCCAGGTCCCAAGGGT
Rat     CCTGGTGCCCGAGGCCCCTCTGGACCTCAGGGCCCCAGCGGCGCTCCTGGTCCCAAGGGA
                                                **  ** * *** ***  **

Dog     ATTGTTGGTGAGCCTGGGCCAGCTGGTTCCAAAGGAGAAAGTGGCAACAAGGGTGAGCCT
Human   AACAGCGGTGAACCTGGTGCTCCTGGCAGCAAAGGAGACACTGGTGCTAAGGGAGAGCCT
Mouse   AACAGCGGTGAACCTGGTGCTCCTGGCAACAAAGGAGACACTGGTGCCAAAGGAGAACCC
Rat     ACCAGTGGTGAACCTGGTGCCCCTGGCAACAAAGGAGACACTGGTGCCAAAGGAGAACCC
        *     ***** *****  *  ****   ********* * ***    ** ** ** **

Dog     GGCTCTGCTGGTGCCCAAGGTCCTCCTGGTCCCAGTGGTGAAGAAGGAAAGAGAGGCCCC
Human   GGCCCTGTTGGTGTTCAAGGACCCCCTGGCCCTGCTGGAGAGGAAGGAAAGCGAGGAGCT
Mouse   GGTGCTACTGGAGTTCAAGGTCCCCCAGGCCCTGCCGGAGAAGAAGGAAAACGAGGAGCC
Rat     GGCCCTGCTGGAGTTCAAGGTCCCCCTGGCCCTGCTGGAGAAGAAGGAAAACGAGGAGCC
        **  **  *** *  ***** ** ** ** **    ** ** ********  ****  *

Dog     AATGGTGAAGCTGGATCTGCTGGGCCCTCTGGACCTCCTGGGCTGAGAGGAAGTCCTGGT
Human   CGAGGTGAACCCGGACCCACTGGCCTGCCCGGACCCCCTGGCGAGCGTGGTGGACCTGGT
Mouse   CGTGGTGAGCCTGGACCTTCCGGACTGCCTGGACCTCCTGGCGAGCGTGGTGGACCTGGT
Rat     CGTGGTGAGCCTGGACCTAGCGGCCTGCCCGGACCTCCTGGCGAGCGCGGTGGACCTGGT
           *****  * *** *    ** *   * ***** *****   * * **  * ******

Dog     TCTCGTGGTCTTCCTGGAGCTGATGGCCCAGCTGGTGTCATGGGTCCTCCCGGCCCTCGG
Human   AGCCGTGGTTTCCCTGGCGCAGATGGTGTTGCTGGTCCCAAGGGTCCCGCTGGTGAACGT
Mouse   AGCCGTGGTTTCCCTGGTGCTGATGGTGTTGCTGGCCCCAAGGGTCCTTCCGGTGAACGT
Rat     AGCCGTGGTTTCCCTGGTGCTGATGGTGTTGCTGGCCCCAAGGGTCCTTCTGGTGAACGT
           ****** * ***** ** *****    *****   ** ******  * **    **

Dog     GGTGCCACCGGCCCTGCTGGTGTCCGCGGTCCCAATGGAGATTCTGGTCGCCCTGGAGAG
Human   GGTTCTCCTGGCCCCGCTGGCCCCAAAGGATCTCCTGGTGAAGCTGGTCGTCCCGGTGAA
Mouse   GGTGCTCCCGGACCTGCTGGTCCCAAAGGTTCTCCTGGTGAAGCTGGTCGCCCCGGTGAA
Rat     GGTTCTCCTGGCCCTGCTGGTCCCAAAGGTTCTCCTGGTGAAGCTGGTCGCCCTGGTGAA
        *** *  * ** ** *****   *   **  *   *** **  ******* ** ** **

Dog     CCTGGCCTCATGGGACCCCGAGGTTTTCCTGGTGCCCCTGGTAATGTTGGCCCAGCTGGT
Human   GCTGGTCTGCCTGGTGCCAAGGGTCTGACTGGAAGCCCTGGCAGCCCTGGTCCTGATGGC
Mouse   GCTGGTCTCCCTGGTGCCAAGGGTCTCACTGGCAGTCCTGGCAGCCCTGGTCCTGATGGC
Rat     GCTGGTCTCCCTGGTGCCAAGGGTCTCACTGGCAGTCCTGGCAGCCCTGGTCCTGATGGC
         **** **    **  **   *** *  ****    ***** *    *** ** * ***

Dog     AAAGAAGGTCCCATGGGTCTCCCTGGTATTGATGGCAGGCCTGGACCAATTGGCCCAGCG
Human   AAAACTGGCCCCCCTGGTCCCGCCGGTCAAGATGGTCGCCCCGGACCCCCAGGCCCACCT
Mouse   AAAACCGGCCCCCCTGGTCCCGCTGGTCAAGATGGTCGCCCTGGACCCGCAGGTCCTCCT
Rat     AAAACCGGCCCCCCTGGTCCCGCTGGTCAAGATGGTCGCCCTGGACCCGCAGGTCCTCCT
        ***   ** ***   **** * * ***   *****  * ** *****    ** **  *

Dog     GGAGCAAGAGGAGAGCCCGGCAACATCGGATTCCCTGGACCCAAAGGCCCCACTGGTGAT
Human   GGTGCCCGTGGTCAGGCTGGTGTGATGGGATTCCCTGGACCTAAAGGTGCTGCTGGAGAG
Mouse   GGAGCCCGTGGCCAGGCTGGTGTGATGGGATTCCCTGGACCTAAGGGTACCGCTGGAGAA
Rat     GGAGCCCGTGGACAGGCTGGTGTGATGGGATTCCCTGGACCTAAGGGTACTGCTGGAGAA
        ** **  * **  ** * * **    ** ************** ** **  *  **** **

Dog     CCTGGCAAAAATGGTGATAAAGGTCATGCTGGTCTTGCTGGTGCTCGGGGTGCTCCAGGT
Human   CCCGGCAAGGCTGGAGAGCGAGGTGTTCCCGGACCCCCTGGCGCTGTCGGTCCTGCTGGC
Mouse   CCTGGAAAGGCTGGAGAGCGAGGCCTTCCCGGACCCCCTGGCGCTGTTGGTCCTGCTGGC
Rat     CCTGGAAAGGCTGGAGAGCGAGGTGTCCCCGGACCCCCTGGCGCTGTTGGTCCCGCTGGC
        ** ** **   *** **   ***     * ** *   **** ***   *** *  * **
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Dog     CCTGATGGAAACAATGGTGCTCAGGGACCTCCTGGACCACAAGGTGTCCAAGGTGGAAAA
Human   AAAGATGGAGAGGCTGGAGCTCAGGGACCCCCTGGCCCTGCTGGTCCCGCTGGCGAGAGA
Mouse   AAAGATGGAGAAGCTGGAGCTCAGGGAGCCCCTGGCCCTGCTGGTCCTGCTGGTGAGAGA
Rat     AAAGATGGCGAAGCTGGAGCTCAAGGAGCCCCCGGCCCTGCTGGTCCTGCTGGTGAGAGA
           *****  *   *** ***** *** * ** ** **    ***      ** *  * *

Dog     GGTGAACAGGGTCCTGCTGGTCCTCCAGGCTTCCAGGGTCTGCCTGGCCCCGCAGGTACA
Human   GGTGAACAAGGCCCTGCTGGCTCCCCCGGATTCCAGGGTCTCCCTGGTCCTGCTGGTCCT
Mouse   GGTGAACAAGGTCCCGCTGGCTCCCCTGGATTCCAGGGTCTTCCTGGTCCTGCCGGTCCT
Rat     GGTGAACAAGGTCCCGCTGGCTCCCCTGGATTCCAGGGTCTTCCTGGTCCCGCTGGTCCT
        ******** ** ** *****  * ** ** ********** ***** ** ** *** *

Dog     GCTGGTGAAGTTGGCAAACCAGGAGAAAGGGGTCTCCCTGGTGAATTTGGTCTTCCTGGT
Human   CCAGGTGAAGCAGGCAAACCTGGTGAACAGGGTGTTCCTGGAGACCTTGGCGCCCCTGGC
Mouse   CCTGGTGAAGCAGGCAAGCCTGGTGAACAGGGTGTTCCTGGAGACCTTGGTGCCCCTGGA
Rat     CCTGGTGAAGCAGGCAAGCCTGGTGAACAGGGTGTTCCTGGAGACCTTGGTGCCCCTGGA
         * *******  ***** ** ** ***  **** * ***** **  ****    *****

Dog     CCTGCTGGTCCAAGAGGGGAGCGTGGTCCCCCTGGAGAAAGTGGTGCTGCTGGTCCTTCT
Human   CCCTCTGGAGCAAGAGGCGAGAGAGGTTTCCCTGGCGAGCGTGGTGTGCAAGGTCCCCCT
Mouse   CCCTCTGGCGCAAGAGGCGAGAGAGGTTTCCCTGGTGAACGTGGTGTACAAGGTCCCCCA
Rat     CCCTCTGGCGCAAGAGGCGAGAGAGGTTTCCCTGGTGAACGTGGTGTACAAGGTCCCCCA
        **  ****  ******* *** * ***  ****** **  ******     *****  *

Dog     GGTCCTATTGGAAGCCGAGGTCCTTCTGGACCCCCTGGGCCCGATGGAAACAAGGGTGAA
Human   GGTCCTGCTGGACCCCGAGGGGCCAACGGTGCTCCCGGCAACGATGGTGCTAAGGGTGAT
Mouse   GGTCCTGCTGGTCCCCGAGGAAACAATGGTGCCCCCGGCAACGATGGTGCCAAGGGTGAT
Rat     GGTCCTGCTGGTCCCCGAGGAAACAATGGTGCCCCCGGCAACGATGGTGCCAAGGGTGAT
        ******  ***   ******        **  * ** **   ******    ********

Dog     CCTGGCGTGCTTGGCGCCCCGGGCACCGCTGGCGCGTCCGGTCCCGGTGGACTCCCAGGA
Human   GCTGGTGCCCCTGGAGCTCCCGGTAGCCAGGGCGCCCCTGGCCTTCAGGGAATGCCTGGT
Mouse   ACTGGTGCCCCCGGAGCTCCCGGTAGCCAGGGTGCCCCCGGTCTTCAGGGAATGCCTGGT
Rat     ACTGGTGCCCCCGGAGCTCCTGGTAGCCAGGGTGCCCCCGGTCTTCAGGGAATGCCTGGT
         **** *  *  ** ** ** ** * *   ** **  * ** *     *** * ** **

Dog     GAGAGGGGTGCTGCCGGCATTCCTGGAGGCAAGGGAGAAAAGGGTGAAACCGGTCTCAGA
Human   GAACGTGGTGCAGCTGGTCTTCCAGGGCCTAAGGGTGACAGAGGTGATGCTGGTCCCAAA
Mouse   GAACGTGGTGCAGCTGGTCTTCCAGGTCCTAAGGGTGACAGAGGTGATGCTGGTCCCAAA
Rat     GAACGTGGTGCAGCTGGTCTTCCAGGTCCTAAGGGTGACAGAGGTGATGCTGGTCCCAAA
        **  * ***** ** **  **** **    ***** ** *  *****  * **** ** *

Dog     GGCGAAATTGGTAACCCAGGCAGAGATGGCGCCCGTGGAGCTCCTGGTGCCATGGGTGCC
Human   GGTGCTGATGGCTCTCCTGGCAAAGATGGCGTCCGTGGTCTGACCGGCCCCATTGGTCCT
Mouse   GGTGCTGATGGTTCTCCTGGTAAAGATGGTGCCCGTGGTCTGACTGGTCCCATTGGTCCT
Rat     GGTGCTGATGGTTCTCCTGGCAAAGATGGCGTCCGTGGTCTGACTGGTCCCATTGGTCCT
        ** *    ***    ** ** * ****** * ******     * **  **** *** *

Dog     CCTGGTCCTGCCGGAGCCACTGGTGACCGGGGTGAAGCTGGTCCTGCCGGTCCCGCTGGC
Human   CCTGGCCCTGCTGGTGCCCCTGGTGACAAGGGTGAAAGTGGTCCCAGCGGCCCTGCTGGT
Mouse   CCTGGCCCTGCTGGTGCCCCTGGTGACAAGGGTGAAGCTGGTCCCAGTGGTCCTCCCGGT
Rat     CCTGGCCCTGCTGGTGCCCCTGGTGACAAGGGTGAAGCTGGTCCCAGTGGTCCTGCTGGC
        ***** ***** ** *** ********  *******  ******    ** **  * **

Dog     CCTGCTGGTCCTCGTGGTACCCCTGGTGAACGTGGTGAGGTTGGTCCCGCTGGCCCCAAT
Human   CCCACTGGAGCTCGTGGTGCCCCCGGAGACCGTGGTGAGCCTGGTCCCCCCGGCCCTGCT
Mouse   CCCACCGGAGCCCGTGGTGCTCCCGGAGACCGTGGTGAGGCTGGTCCCCCTGGTCCTGCT
Rat     CCCACCGGAGCCCGTGGTGCCCCCGGAGACCGTGGTGAGGCTGGTCCCCCTGGTCCTGCT
        **  * **  * ****** * ** ** ** *********  ******* * ** **   *

Dog     GGATTTGCTGGTCCTGCTGGTGCTGCTGGTCAACCTGGTGCTAAGGGAGAGAGAGGAACC
Human   GGCTTTGCTGGCCCCCCTGGTGCTGACGGCCAACCTGGTGCTAAAGGCGAACCTGGTGAT
Mouse   GGCTTTGCCGGCCCCCCTGGTGCTGATGGCCAACCTGGTGCGAAAGGTGAACCTGGTGAT
Rat     GGCTTCGCTGGCCCCCCTGGTGCTGATGGCCAACCTGGTGCGAAAGGTGAACCTGGTGAT
        ** ** ** ** **  *********  ** ********** ** ** **    **
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Dog     AAAGGGCCCAAGGGTGAAAATGGCCCCGTTGGTCCCACAGGCCCCATTGGATCTGCCGGC
Human   GCTGGTGCCAAAGGCGATGCTGGTCCCCCTGGGCCTGCCGGACCCGCTGGACCCCCTGGC
Mouse   ACTGGTGTTAAAGGTGATGCTGGTCCTCCTGGCCCTGCTGGTCCTGCTGGACCCCCCGGC
Rat     ACTGGTGTGAAAGGTGACGCTGGTCCTCCTGGCCCTGCTGGTCCCGCTGGACCCCCTGGC
           **    ** ** **   *** **   *** **  * ** **   **** *  * ***

Dog     CCATCTGGTCCAAATGGTCCCCCTGGTCCTGCTGGAAGTCGTGGTGATGGTGGCCCCCCT
Human   CCCATTGGTAATGTTGGTGCTCCTGGAGCCAAAGGTGCTCGCGGCAGCGCTGGTCCCCCT
Mouse   CCCATTGGTAACGTTGGTGCTCCTGGACCCAAAGGTCCTCGTGGTGCTGCTGGTCCCCCT
Rat     CCCATTGGTAACGTTGGTGCTCCTGGACCCAAAGGTTCTCGTGGTGCTGCTGGTCCCCCT
        **   ****     **** * *****  *    **   *** **    * *** ******

Dog     GGTGCTACTGGTTTCCCTGGTGCTGCTGGGCGGACTGGTCCTCCTGGACCCTCTGGTATC
Human   GGTGCTACTGGTTTCCCTGGTGCTGCTGGCCGAGTCGGTCCTCCTGGCCCCTCTGGAAAT
Mouse   GGTGCTACTGGCTTCCCTGGTGCTGCTGGCCGTGTCGGTCCCCCTGGTCCCTCTGGAAAT
Rat     GGTGCTACTGGTTTCCCTGGTGCTGCTGGTCGTGTTGGTCCCCCTGGTCCCTCTGGAAAT
        ********** ***************** **    ***** ***** ******** *

Dog     ACTGGCCCTCCTGGTCCCCCTGGTGCTGCTGGTAAAGAAGGACTCCGTGGGCCTCGAGGT
Human   GCTGGACCCCCTGGCCCTCCTGGTCCTGCTGGCAAAGAAGGCGGCAAAGGTCCCCGTGGT
Mouse   GCTGGACCCCCTGGCCCTCCCGGTCCCGTTGGCAAAGAAGGGGGCAAAGGTCCCCGTGGT
Rat     GCTGGACCCCCTGGCCCTCCCGGTCCCGTTGGCAAAGAAGGGGGCAAAGGTCCCCGTGGT
         **** ** ***** ** ** *** * * *** ********   *   ** ** ** ***

Dog     GACCAAGGTCCAGTTGGCCGAACGGGAGAAACAGGTGCCTCTGGTCCCCCTGGCTTTACT
Human   GAGACTGGCCCTGCTGGACGTCCTGGTGAAGTTGGTCCCCCTGGTCCCCCTGGCCCTGCT
Mouse   GAGACTGGCCCTGCTGGACGTCCTGGTGAAGTTGGTCCCCCAGGTCCCCCCGGTCCTGCT
Rat     GAGACTGGTCCCGCTGGACGTCCTGGTGAAGTTGGTCCCCCAGGTCCCCCTGGCCCTGCT
        **    ** ** * *** **  * ** ***   *** ** * ******** **   * **

Dog     GGTGAGAAGGGTCCCTCTGGAGAGCCTGGTACCGCTGGACCTCCTGGCACACCAGGTCCT
Human   GGCGAGAAAGGATCCCCTGGTGCTGATGGTCCTGCTGGTGCTCCTGGTACTCCCGGGCCT
Mouse   GGTGAGAAAGGATCTCCTGGTGCTGATGGACCTGCTGGCTCTCCTGGTACCCCTGGACCT
Rat     GGTGAGAAAGGATCTCCTGGTGCTGATGGACCTGCTGGCTCTCCTGGTA<b>CCCCTGGACCT</b>
        ** ***** **  *  **** *    ***  * *****  ******* ** ** ** ***

Dog     CAAGGTCTCCTTGGTGCTCCTGGCATTCTGGGTCTCCCAGGCTCTCGGGGTGAGCGTGGT
Human   CAAGGTATTGCTGGACAGCGTGGTGTGGTCGGCCTGCCTGGTCAGAGAGGAGAGAGAGGC
Mouse   CAGGGTATTGCTGGACAACGTGGTGTGGTCGGTCTTCCCGGTCAGAGAGGAGAAAGAGGC
Rat     <b>CAGGGTATTGCTGGACAGCGTGGTGTGGTCGGTCTTCCCGGTCAGAGAGGAAAAAGAGGC</b>
        ** *** *   ***    * ***  *  * ** ** ** **     * **  *  * **

Dog     CTACCAGGTGTTGCTGGATCCGTGGGTGAACCCGGACCTCTTGGCATTGCTGGTCCACCT
Human   TTCCCTGGTCTTCCTGGCCCCTCTGGTGAACCTGGCAAACAAGGTCCCTCTGGAGCAAGT
Mouse   TTCCCTGGTCTTCCTGGCCCCTCTGGTGAACCTGGCAAACAAGGTCCTTCTGGATCAAGT
Rat     <b>TTCCCTGGTCTTCCTGGACCCTCTGGTGAACCCGGCAAACAAGGTCCTTCTGGAGCAAGT</b>
         * ** *** ** ****  **   ******** **    *  **     ****  **  *

Dog     GGGGCTCGTGGTCCTCCTGGAGCTGTGGGTGCGCCTGGAGTCAACGGTGCTCCTGGTGAA
Human   GGTGAACGTGGTCCCCCCGGTCCCATGGGCCCCCCTGGATTGGCTGGACCCCCTGGTGAA
Mouse   GGTGAACGCGGTCCCCCTGGCCCCATGGGGCCCCCTGGATTGGCTGGTCCCCCTGGTGAA
Rat     <b>GGTGAACGTGGTCCCCCTGGCCCTATGGGCCCCCCTGGATTGGCTGGCCCCCCTGGTGAA</b>
        ** *  ** ***** ** **  *  ****  * ****** *    **  * *********

Dog     GCTGGTCGTGATGGCAACCCTGGGAATGATGGTCCCCCAGGCCGCGATGGTCAAGCCGGA
Human   TCTGGACGTGAGGGGGCTCCTGCTGCCGAAGGTTCCCCTGGACGAGACGGTTCTCCTGGC
Mouse   TCTGGACGTGAGGGATCCCCTGGTGCTGAAGGCTCCCCTGGAAGGGATGGTGCTCCCGGG
Rat     <b>TCTGGTCGTGAGGGATCCCCTGGTGCTGAAGGCTCCCCTGGAAGAGATGGTGCTCCTGGT</b>
         **** ***** **    ****     ** **  **** **  * ** ***    * **

Dog     CACAAGGGAGAGCGTGGTTACCCTGGCAACATTGGTCCCGTTGGCGCTGTGGGTGCACCT
Human   GCCAAGGGTGACCGTGGTGAGACCGGCCCCGCTGGACCCCCTGGTGCTCNTGGTGCTCNT
Mouse   GCCAAGGGTGACCGTGGTGAGACTGGCCCCGCTGGCCCCCCTGGTGCCCCTGGTGCTCCC
Rat     <b>GCCAAGGGTGACCGTGGTGAGACTGGCCCTGCTGGCCCCCCTGGTGCTCCTGGTGCTCCT</b>
          ****** ** ****** *  * ***     *** ***  *** **    ***** *
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Dog     GGTCCTCATGGCCCTGTGGGTCCCACTGGCAAACACGGAAACCGTGGTGAACCTGGTCCT
Human   GGTGCCCCTGGCCCCGTTGGCCCTGCTGGCAAGAGTGGTGATCGTGGTGAGACTGGTCCT
Mouse   GGTGCTCCCGGCCCTGTTGGTCCCGCTGGCAAGAATGGCGATCGTGGTGAGACTGGTCCT
Rat     GGTGCTCCCGGCCCTGTTGGTCCTGCTGGCAAGAATGGCGACCGTGGTGAGACTGGTCCT
        *** * *  ***** ** ** **  *******    **  * ********  ********

Dog     GCTGGTTCTGTTGGTCCCGTCGGCGCTGTTGGTCCAAGAGGTCCTAGTGGCCCACAAGGT
Human   GCTGGTCCCGCCGGTCCCGTCGGCCCCGCTGGCGCCCGTGGCCCCGCCGGACCCCAAGGC
Mouse   GCTGGTCCTGCTGGTCCCATTGGCCCTGCTGGTGCCCGTGGCCCTGCTGGACCCCAAGGC
Rat     GCTGGTCCTGCTGGTCCCATTGGCCCTGCTGGTGCCCGTGGTCCTGCTGGACCCCAAGGC
        ****** * *  ****** * *** * * ***  *  * ** **    ** ** *****

Dog     ATTCGAGGTGATAAGGGAGAGCCTGGTGAGAAGGGGCCCAGAGGTCTTCCTGGCTTAAAG
Human   CCCCGTGGTGACAAGGGTGAGACAGGCGAACAGGGCGACAGAGGCATAAAGGGTCACCGT
Mouse   CCCCGTGGTGACAAGGGTGAGACAGGCGAACAAGGTGACAGAGGCATAAAGGGTCATCGT
Rat     CCCCGTGGTGACAAGGGTGAGACAGGCGAACAAGGTGACAGAGGCATAAAGGGTCATCGT
           ** ***** ***** *** * ** **  * **   ******  *    **

Dog     GGACACAATGGACTGCAAGGTCTTCCTGGTCTTGCTGGTCAACATGGCGATCAAGGTGCA
Human   GGCTTCTCTGGCCTCCAGGGTCCCCCTGGCCCTCCTGGCTCTCCTGGTGAACAAGGTCCC
Mouse   GGCTTCTCTGGTCTCCAGGGTCCTCCTGGTTCTCCTGGTTCTCCTGGTGAACAAGGCCCC
Rat     GGCTTCTCTGGTCTCCAGGGTCCTCCTGGCTCTCCTGGCTCTCCTGGTGAACAAGGCCCC
        **   *  *** ** ** ****  *****   * ****    * *** ** *****  *

Dog     CCTGGTTCTGTGGGTCCTGCTGGTCCTAGGGGTCCTGCTGGTCCTTCTGGCCCTGCTGGC
Human   TCTGGAGCCTCTGGTCCTGCTGGTCCCCGAGGTCCCCCTGGCTCTGCTGGTGCTCCTGGC
Mouse   TCTGGAGCTTCAGGTCCTGCAGGCCCCCGGGGTCCCCCTGGCTCTGCTGGTTCTCCTGGC
Rat     TCTGGAGCTTCTGGTCCTGCAGGTCCCCGGGGTCCCCCTGGCTCTGCTGGTTCTCCTGGC
         ****  *    ******** ** **  * *****  ****  ** ****  ** *****

Dog     AAAGATGGTCGCACTGGGCAACCTGGTACAGTCGGACCTGCTGGCATTCGTGGCTCTCAG
Human   AAAGATGGACTCAACGGTCTCCCTGGCCCCATTGGGCCCCCTGGTCCTCGCGGTCGCACT
Mouse   AAAGACGGACTCAACGGTCTCCCTGGCCCCATTGGTCCCCCTGGTCCTCGAGGTCGCACT
Rat     AAAGATGGACTCAACGGTCTCCCTGGCCCCATTGGTCCCCCTGGTCCTCGAGGTCGCACT
        ***** ** * **  ** *  *****  *  * ** **  ****   *** **

Dog     GGTAGCCAAGGTCCTGCTGGCCCTCCTGGTCCCCCTGGCCCTCCTGGCCCTCCTGGCCCA
Human   GGTGATGCTGGTCCTGTTGGTCCCCCCGGCCCTCCTGGACCTCCTGGTCCCCCTGGTCCT
Mouse   GGTGACAGCGGCCCTGCTGGTCCCCCCGGCCCTCCTGGACCCCCTGGCCCTCCTGGACCT
Rat     GGCGATAGTGGTCCTGCTGGTCCCCCCGGACCTCCTGGACCCCCTGGCCCTCCCGGTCCT
        **       ** **** *** ** ** ** ** ***** ** ***** ** ** ** **

Dog     AGTGGTGGTGGCTATGACTTTGGTTA--------------TGAAGGGGACTTCTACAGG
Human   CCCAGCGCTGGTTTCGACTTCAGCTTCCTCCCCCAGCCACCTCAAGAGAAGGCTCACGAT
Mouse   CCCAGTGGCGGTTATGACTTCAGCTTCCTGCCTCAGCCACCTCAAGAGAAGTCTCAAGAT
Rat     CCCAGCGGTGGTTATGACTTCAGCTTCCTGCCTCAGCCACCTCAAGAGAAGTCTCAAGAT
           * *  ** *  *****  * *                  * *** * *     *

Dog     GCTGACCA-----GCCTCGCTCACCACCTTCTCTCAGACC----CAAGGACTATGAAGTT
Human   GGTGGCCGCTACTACCGGGCTGATGATGCCAATGTGGTTCGTGACCGTGACCTCGAGGTG
Mouse   GGTGACCGCTACTACCGGGCCGATGATGCTAACGTGGTTCGTGACCGTGACCTTGCGGTG
Rat     GGTGGCCGTTACTACCGGGCCGATGATGCCAACGTGGTCCGTGACCGTGACCTTGAGGTG
        * ** **       **  **  *  *          *  *    *   ***   *  **

Dog     GATGCTACTCTGAAATCCCTCAACAACCAGATTGAGACCCTTCTTACTCCTGAAGGCTCT
Human   GACACCACCCTCAAGAGCCTGAGCCAGCAGATCGAGAACATCCGGAGCCCAGAGGGCNCC
Mouse   GACGCCACCCTCAAGAGCCTGAGTCAGCAGATTGAGAACATCCGCAGCCCCGAAGGCAGC
Rat     GACACTACCCTCAAGAGCCTGAGCCAGCAGATTGAGAACATCCGCAGCCCTGAGGGCAGC
        **  * ** ** **   *** *   * ***** **** * * *  *  ** ** ***

Dog     AGGAAGAACCCAGCTCGCACATGCCGAGACCTGAGACTCAGCCACCCAGAGTGGAGCAGT
Human   CGCAAGAACCCCGCCCGCACCTGCCGTGACCTCAAGATGTGCCACTCTGACTGGAAGAGT
Mouse   CGCAAGAACCCTGCCCGCACATGCCGCGACCTCAAGATGTGCCACTCTGACTGGAAGAGC
Rat     CGCAAGAACCCCGCCCGCACATGCCGTGACCTCAAGATGTGCCACTCTGACTGGAAGAGC
         * ******** ** ***** ***** ***** *   *  ***** * ** ****  **
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Dog     GGTTACTACTGGATTGACCCTAACCAAGGATGCACTATGGATGCTATCAAAGTATACTGT
Human   GGAGAGTACTGGATTGACCCCAACCAAGGCTGCAACCTGGATGCCATCAAAGTCTTCTGC
Mouse   GGAGAGTACTGGATCGACCCTAACCAAGGCTGCAACCTGGACGCCATCAAGGTCTACTGC
Rat     GGAGAGTACTGGATCGACCCTAACCAAGGCTGCAACCTGGATGCCATCAAGGTCTACTGC
        **  * ******** ***** ******** ****   **** ** ***** ** * ***

Dog     GATTTCTCTACTGGCGAAACCTGCATTCGGGCTCAACCTGAAAACATCCCAGCCAAGAAC
Human   AACATGGAGACTGGTGAGACCTGCGTGTACCCCACTCAGCCCAGTGTGGCCCAGAAGAAC
Mouse   AACATGGAGACAGGACAGACCTGTGTGTTCCCTACTCAGCCGTCTGTGCCTCAGAAGAAC
Rat     AACATGGAGACAGGTCAGACCTGTGTGTTCCCCACTCAGCCCTCTGTGCCTCAGAAGAAC
         *  *    ** **  * *****  *     *    *          *  *    ******

Dog     TGGTACA------GAAACTCCAAGGTCAAGAAGCACATCTGGTTAGGAGAAACTATCAAT
Human   TGGTACATCAGCAAGAACCCCAAGGACAAGAGGCATGTCTGGTTCGGCGAGAGCATGACC
Mouse   TGGTACATCAGCCCGAACCCCAAGGAAAAGAAGCACGTCTGGTTTGGAGAGAGCATGACC
Rat     TGGTACATCAGCCCAAACCCCAAGGAGAAAAAGCATGTCTGGTTTGGAGAGAGCATGACC
        *******        *** ******  ** * ***  ******* ** ** *  ** *

Dog     GGTGGTACCCAGTTTGAATATAATGTTGAAGGAGTAACCACCAAGGAAATGGCTACTCAA
Human   GATGGATTCCAGTTCGAGTATGGCGGCCAGGGCTCCGACCCTGCCGATGTGGCCATCCAG
Mouse   GATGGATTCCCGTTCGAGTACGGAAGCGAGGGCTCCGACCCGACCGATGTCGCTATCCAG
Rat     GATGGATTCCAGTTCGAGTATGGAAGCGAAGGTTCCGATCCTGCCGATGTCGCTATCCAG
        * ***   ** *** ** **         * **        *    **  * ** *  **

Dog     CTCGCCTTCATGCGCCTGCTGGCCAACCATGCCTCTCAAAACATCACCTACCACTGCAAG
Human   CTGACCTTCCTGCGCCTGATGTCCACCGAGGCCTCCCAGAACATCACCTACCACTGCAAG
Mouse   CTGACCTTCCTGCGCCTAATGTCCACCGAGGCCTCCCAGAACATCACCTATCACTGCAAG
Rat     CTGACCTTCCTGCGCCTGATGTCCACCGAGGCCTCCCAGAACATCACCTATCACTGCAAG
        **  ***** *******  ** *** * * ***** ** ************ *********

Dog     AACAGCATTGCCTATATGGATGAGGAGACTGGCAACCTGAAAAAGGCTGTCATTCTGCAA
Human   AACAGCGTGGCCTACATGGACCAGCAGACTGGCAACCTCAAGAAGGCCCTGCTCCTCNAG
Mouse   AACAGCGTAGCCTACATGGACCAACAGACTGGCAACCTCAAGAAGGCCCTGCTCCTCCAG
Rat     AACAGCGTAGCCTACATGGACCAACAGACTGGCAACCTCAAGAAGTCCCTGCTCCTCCAG
        ****** * ***** *****  *  ************** ** *** *  *  * **  *

Dog     GGTTCCAATGATGTTGAACTGGTTGCCGAGGGCAACAGCAGGTTCACCTACACTGTTCTT
Human   GGCTCCAACGAGATCGAGATCCGCGCCGAGGGCAACAGCCGCTTCACCTACAGCGTCACT
Mouse   GGATCCAACGAGATCGAGCTCAGAGGCGAAGGCAACAGTCGCTTCACCTACAGCAGGGTT
Rat     GGCTCCAACGAGATCGAGCTCAGGGGCGAAGGCAACAGTCGATTCACCTACAGCACGCTT
        ** ***** **  * **  *    * *** ********  * **********       *

Dog     GTAGACGGCTGCTCTAAAAAGACAAATGAATGGAGAAAGACAATCATTGAATACAAAACA
Human   GTCGATGGCTGCACGAGTCACACCGGAGCCTGGGGCAAGACAGTGATTGAATACAAAACC
Mouse   GTGGACGGCTGCACGAGTCACACCGGAACTTGGGGCAAGACAGTCATCGAATACAAAACC
Rat     GTGGATGGCTGCACGAGTCACACCGGAACTTGGGGCAAGACAGTCATCGAATACAAAACC
        ** ** ****** * *   * **       *** * ****** * ** ***********

Dog     AATAAGCCATCCCGCCTGCCTATCCTTGATATTGCACCTTTGGACATCGGTGATGCTGAC
Human   ACCAAGTCCTCCCGCCTGCCCATCATCGATGTGGCCCCCTTGGACGTTGGTGCCCCAGAC
Mouse   ACCAAGACCTCCCGCCTGCCCATCATCGATGTGGCTCCCTTGGACATTGGTGCCCCAGAC
Rat     ACCAAGACCTCCCGCCTGCCCATCATCGATGTGGCTCCCTTGGACATTGGTGCCCCAGAC
        *  *** * *********** *** * *** * ** ** ****** * ****   * ***

Dog     CAAGAATTCAGGGTGGACGTTGGCCCAGTCTGTTTCAAATAAATGAACTCAACCTAAATT
Human   CAGGAATTCGGCTTCGACGTTGGCCCTGTCTGCTTCCTGTAAACTCCCTCCATCCCAACT
Mouse   CAGGAATTCGGACTAGACATTGGCCCTGCCTGCTTCGTGTAAACTCCCTCCACCCCAATC
Rat     CAGGAATTCGGAATGGACATTGGCCCTGCCTGCTTCGTGTAAACTCCCTCCATCCCAATC
        ** ****** *  * *** ******* * *** ***   ****    *** * *  **

Dog     AAAAAAAAAAAAGAAATCTGAAAAAACTTTCTCTCTTTGCCATTTCTTTTTCTTCTTTTT
Human   TGG-------------------------CTCCCTCCCACCCAACCAACTTTCCCCCCAAC
Mouse   TGG-------------------------TTCCCTCCCACCCAGCCCACTTTTCCCCAACC
Rat     TGG-------------------------TTCCCTCCCACCCAGCCCACTTTCCCCCAACC
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Dog     TTTTTAACTGAAAGTTGAGTCCTTCCATTTCCTCTGCACATCTACTTGCTTAAATTGTGG
Human   CCGGAA-CAGACAAGCAACCCAAACTGAACCCCCTC-AAA-----------------AG
Mouse   CTGGAAACAGACGAACAACCCAAACTCAATTTCCCCCAAA-----------------AG
Rat     CTGGAAACAGACCAACAACCCAAACTCAATTTCCCC-AAA-----------------AG
             * * **      *  *   *        *   * *                  *

Dog     GCAAAAGAGAAGGAGAAGGATTGATCAGAGCATAGTGCAATACGATTTCATTCATTCCCC
Human   CCAAAAAA-TGGGAGACAATTTCACATGGACTT--TGGAAAATATTTTTTT-CCTTTGCA
Mouse   CCAAAAATATGGGAGATAATTTCACATGGACTT--TGGAAAACATTTTTTTTCCTTTGCA
Rat     CCAAAAAT-TGGGAGACAATTTCACATGGACTT--TGGAAAACATTTTTTT-CCTTTGCA
         *****     *****    ** *   *  * *  ** ** *   ***  * * **  *

Dog     CTCCCTGCCCCCAAAAATTTGGAATTTTTTTTCAACACTCTTACATCCGTTGTGGAAAAT
Human   TTCATCTCTCAAACTTAGTTTTTATCTTTGACCAACCGAACATGACCAAAAACCAAAAGT
Mouse   TTCACCTTTCAAACTTAGTTTTTACCAAAGACCAACTGAACGTGACCAAAAACCAAAAGT
Rat     TTCATCTCTCAAACTTAGTTTTTATCTTTGACCAACTGAACGTGACCAAAAACCAAAAGT
         **      *  *   * **   *        ****       * *        *** *

Dog     GTCAACCTTTGTTAGAAAACCAAAATAAAAATTGAAAAATAAAATAAAAACCATGAACAT
Human   G-CATTCAACCTTACC-----------------------------------------------
Mouse   G-CATTCAACCTTAC-----------------------------------------------
Rat     G-CATTCAACCTTACCAAAAAGAAAAAAAAATAAGAATAAATAAATAACTTTTTAAAAAA
        * **  *    ***

Dog     TTGC-------------------------------------------------------------
Human   -------------------------------------------------------------
Mouse   -------------------------------------------------------------
Rat     GGAAGCTTGGTCCTCTTGCTTGAAGACCTATGTGGGTATAAGTCCCTTTCTGCCCACTGG

Dog     -------------------------------------------------------------
Human   -------------------------------------------------------------
Mouse   -------------------------------------------------------------
Rat     GCTTATGATACCCCAAATGCTGCCTTTTCTGTTCCTTTCTCCACCCCCTCTTGGGGCCTC

Dog     -------------------------------------------------------------
Human   -------------------------------------------------------------
Mouse   -------------------------------------------------------------
Rat     TCCTCCATTGCTCCCCAAATTTAAGTCTCCCCCAAAGACACAGGAAATAATGCATTGTCT

Dog     -------------------------------------------------------------
Human   -------------------------------------------------------------
Mouse   -------------------------------------------------------------
Rat     GCCCAGCCAGCAAAGGCAATGCTGAATCGTCCCACCAGCCCCTCAACCCCCAGCTTACTT

Dog     -------------------------------------------------------------
Human   -------------------------------------------------------------
Mouse   -------------------------------------------------------------
Rat     CCCTACCCAGCACCTTCAAATCCTGCCGGGACATTGGGTTCTCGGACTATTGAAGGAGCC

Dog     -------------------------------------------------------------
Human   -------------------------------------------------------------
Mouse   -------------------------------------------------------------
Rat     TAACCATCTGGCATCTCCATGGCCTCTGCAACAAATCCCCACACACACTTTGTTTTTGAG

Dog     -------------------------------------------------------------
Human   -------------------------------------------------------------
Mouse   -------------------------------------------------------------
Rat     GGCCTGTGCTGGGGGAGCCACCTGCCCCTCGCAGGGGTTTGGAGCCAGGCAGGGTCACAG
```

```
Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     CAGACTGGAAACATCGGCCACACATGTGCAGGCTGGGTGGGAGAGACTGTTCTGTTCCTT

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     GTGTAATTGTGTTGCTGAAAGACTACCTCGTTCTTGTCTTTGTGTGTCACCGGGGCAACT

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     GTGTGGGGGCGGGGATGGGGGCAGGGTGGCATCGCGCCCAGTTTGGTATCAAAGGTGCTA

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     CATCTCTGTGAAGGGGTGGGGTGGGAAGGAATTTCTGGTGCTATAGAATCTGAGATGCTC

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     CCCTACACCAGCAAATGTTCCTTTTGTTCAAAGTATTTTTTTATTCTTTTTTTTTTTTTA

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     ATGGATAGGGACTTGTGTGAATTTTCTTTTCCTGACGGTGCTATTTAACAAGGGAGGAGA

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     GAGTGCCAACTCCAGCCTGCTCTCTCTCTACCCCCCTCTTCACTCTTCCAGCTCCTGGGC

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     CTATCTGATGATCTCTCTCTCTTCTGAAACCCTCCCCTCTTGCTGCTGCTCCCTACCCTC

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     AGCTTCTCTCTCTCTCTGTCCTGCATCAGGGTTTCAGAGCACCATTTTCCAAAGCACAAA

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     GCAGTTTTTATCCCTGGGGTGGGAGGAAGCAAGAGACTCTGTACCTATTTTGTATGTGTA

Dog     ------------------------------------------------------------
Human   ------------------------------------------------------------
Mouse   ------------------------------------------------------------
Rat     TAATAATTTGAGATGTTTTTAATTATTTTGATTGCTGGCATAAAGCAGGGGGAAATGATC

Dog     ----------
Human   ----------
Mouse   ----------
Rat     CAGTCCACAG
```

Appendix B: Type II Collagen Alignment

Horse (ECU62528): 4257 bp
Human (HSC1A2CS): 4257 bp
Mouse (MUSPACOLL): 5453 bp
Rat (RATCOLLII): 4538 bp
Xenopus (XELCOL2A1A): 4476 bp

* indicates bases that are conserved across all species listed.

The sequence in bold is the fragment used for riboprobe construction.

```
                    10        20        30        40        50        60
                     .         .         .         .         .         .
Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       ------------------------------------------------------------
Rat         ------------------------------------------------------------
Xenopus     ATGTTTAGCTTTGTGGATTCAAGGACTCTAGTGCTGTTCGCAGCCACACAAGTCATTTTA

Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       ----------------ATGATCCGCCTCGGGGCTCCCCAGTCGCTGGTGCTGCTGACGC
Rat         ----------------GGCTGCCGGGTCTCCTGCCTCCTCCTCCTGCTCCTAGAGC
Xenopus     TTAGCGGTTGTACGATGCCAAGATGAAGAAGATGTCCTGGATACAGGCAGCTGCGTGCAG

Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       TGCTCATCGCCGCGGTCCTACGGTGTCAGGGCCAGG---ATGCCCAGGAGGCTGGCAGCT
Rat         CTCCTGCATGAGGGCGCGGTAGAGACCCGGACCCGCTCCGTGCTCTGCCGCCTCGCCGAG
Xenopus     GATGGGCAGAGGTATAGTGATAAAGATGTGTGGAAACCCGAGCCCTGCCAAATCTGTG-T

Horse       ----------------------------------------ATGATACGCCTCGGGGCTC
Human       ----------------------------------------ATGATTCGCCTCGGTGCTC
Mouse       GTCTGCAGAATGGGCAGAGGTATAAAGATAAG-GATGTATGGAAGCCCTCATCTTGCCGC
Rat         CTTCGCCCGGGCCAAGGCTCTGCCAGGCCTCGCGGTGAGCCATGATCCGCCTCGGGGCTC
Xenopus     CTGTGACACTGGGACTGTTCTCTGCGATGAAATAATCTGCGAAGAGTCCAAAGATTGCCC
                                                          *         * *

Horse       CCCAGACGCTGGTGCTGCTGACGCTGCTCGTCGCCGCTGTCCTTCGGTGTCACGGCCAGG
Human       CCCAGTCGCTGGTACTGCTGACGCTGCTCGTCGCCGCTGTCCTTCGGTGTCAGGGCCAGG
Mouse       ATCTGT-GTGTGTGACACTGGGAATGTCCTCTGCGATGACATTATCTGTGAAGACCCAGA
Rat         CCCAGTCGCTGGTGCTGCTGACGCTGCTCATCGCCACGGTCCTACAATGTCAGGGCCAGG
Xenopus     CAATGCTGAGATCCCCTTCGGAGAGTGCTGTCCCATCTGCCCAACTGAGCAATCTTCTAC
              * *           *           *                   *     *

Horse       ATGT--CCGGCAGCCAGGACCGAAGGGACAGAAAGGAGAACCTGGAGACATCAAGGATAT
Human       ATGT--CCGGCAACCAGGACCAAAGGGACAGAAAGGAGAACCTGGAGACATCAAGGATAT
Mouse       CTGC--CTCAACCCCGAGATCCCCTTCGGAGAGTGCTGT-CCCATCTGCCCAGCTGACCT
Rat         ATGC--CCGAAAATTAGGGCCAAAGGGGCAGAAAGGAGAACCTGGAGATATCAAAGATAT
Xenopus     CTCCAGTGGGCAAGGAGTGGCCAAGGGACAGAAAGGTGAACCTGGTGATATTAAAGATGT
             *                 *        ***  *  *  **                **  *

Horse       TGTAGGACCCAAAGGACCTCCTGGACCTCAGGGACCTGCAGGTGAACA-AGGACCCAGAG
Human       TGTAGGACCCAAAGGACCTCCTGGGCCTCAGGGACCTGCAGGGGAACA-AGGACCCAGAG
Mouse       CGCCACTGCCAGTGGTC-----GAAAATTAGGG-----CCAAAGATCATAGGACCCAGAG
Rat         CATAGGACCTAAAGGACCTCCTGGCCCTCAGGGACCTGCCGGTGAACA-AGGACCCAGAG
Xenopus     TGTAGGACCAAGAGGACCACCTGGACCACAGGGACCTTCTGGTGAACA-AGGACCTAGAG
                  * *  ** *     *       ****      *    ** ** ****** ****
```

```
Horse       GTGATCGTGGTGACAAAGGAGAAAAAGGTGCCCCTGGACCTCGTGGCAGAGATGGAGAGC
Human       GGGATCGTGGTGACAAAGGTGAAAAAGGTGCCCCTGGACCTCGTGGCAGAGATGGAGAAC
Mouse       GACCTCCTGGCCCTCAGGG---------TGCGCCTGGACCCCGTGGCAGAGATGGAGAAC
Rat         GTGACCGTGGTGACAAGGGAGAGAGGGGTGCACCTGGACCCCGTGGCAGAGATGGAGAAC
Xenopus     GAGAACGTGGAGATAAGGGCGAGACTGGTGCTCCCGGACCCCGTGGAAGAGATGGCGAAC
            *    * ***     * **          *** ** ***** ***** ******** ** *

Horse       CTGGGACCCCTGGAAATCCTGGCCCCCCTGGTCCTCCTGGCCCCCCTGGTCCCCCTGGCC
Human       CTGGGACCCTTGGAAATCCTGGCCCCCCTGGTCCTCCCGGCCCCCCTGGTCCCCCTGGTC
Mouse       CTGGTACCCCTGGAAATCCTGGCCCCGCTGGCCCTCCAGGTCCCCCTGGTCCCCCTGGCC
Rat         CTGGTACCCCTGGAAATCCTGGTCCCCCTGGCCCTCCAGGCCCCCCTGGTCCCCCTGGCC
Xenopus     CCGGTACTCCTGGGAACCCAGGACCTGCTGGCCCGCCTGGACCTCCTGGACCCCCTGGCC
            * ** ** * *** ** ** ** **  **** ** ** ** ** ***** ******** *

Horse       TTGGTG---GAAACTTTGCTGCCCAGATGGCTGGAGGATTTGATGAGAAGGCTGGTGGCG
Human       TTGGTG---GAAACTTTGCTGCCCAGATGGCTGGAGGATTTGATGAAAAGGCTGGTGGCG
Mouse       TTAGTGCAGGAAACTTCGCGGCTCAGATGGCTGGAGGGTATGACGAGAAGGCTGGTGGTG
Rat         TTGGTGGAGGAAACTTTGCAGCCCAGATGGCTGGAGGATTTGACGAGAAGGCTGGTGGTG
Xenopus     TTGGCG---GAAACTTTGCTGCTCAAATGACAGGAGGATTTGATGAAAAAGCTGGCGGTG
            ** * *   ******* ** ** ** *** * ***** * *** ** ** ***** ** *

Horse       CCCAGATGGGAGTAATGCAAGGACCAATGGGCCCTATGGGACCACGTGGACCACCAGGCC
Human       CCCAGTTGGGAGTAATGCAAGGACCAATGGGCCCCATGGGACCTCGAGGACCTCCAGGCC
Mouse       CCCAGATGGGAGTCATGCAAGGGCCCATGGGCCCCATGGGACCCCGTGGACCCCCAGGCC
Rat         CCCAGATGGGAGTCATGCAAGGCCCCATGGGCCCCATGGGACCTCGTGGACCCCCAGGCC
Xenopus     CACAAATGGGAGTTATGCAAGGACCAATGGGCCCTATGGGACCAAGAGGACCTCCCGGCC
            * **  ******* ******** ** ******** ********  * ***** ** ****

Horse       CTGCTGGCGCTCCTGGACCTCAAGGATTTCAAGGCAACCCTGGTGAACCTGGGGAACCCG
Human       CTGCAGGTGCTCCTGGGCCTCAAGGATTTCAAGGCAATCCTGGTGAACCTGGTGAACCTG
Mouse       CTGCCGGTGCCCCCGGCCCTCAAGGATTTCAAGGCAATCCTGGCGAACCTGGCGAGCCTG
Rat         CCGCCGGTGCCCCCGGCCCTCAAGGATTTCAAGGCAATCCTGGTGAACCTGGCGAGCCTG
Xenopus     CATCAGGAGCTCCTGGACCTCAAGGTTTCCAAGGGAATCCCGGTGAACCTGGTGAATCTG
            *  * ** ** ** ** ******** ** ***** ** ** ** ******** **  * *

Horse       GCGTCTCTGGTCCCATGGGTCCCCGTGGTCCTCCTGGCCCCCCTGGAAAACCTGGTGATG
Human       GTGTCTCTGGTCCCATGGGTCCCCGTGGTCCTCCTGGTCCCCCTGGAAAGCCTGGTGATG
Mouse       GTGTCTCTGGTCCCATGGGTCCCCGAGGTCCTCCTGGCCCTGCTGGAAAACCTGGTGACG
Rat         GTGTCTCTGGTCCCATTGGTCCCCGAGGTCCTCCAGGTCCTGCTGGAAAACCTGGTGATG
Xenopus     GTGCTGGTGGTCCTATGGGTCCTCGTGGTCCTCCTGGTCCTTCGGGAAAGCCTGGTGATG
            * *    ****** ** ***** ** ******** ** **  * ***** ******** *

Horse       ATGGTGAAGCTGGAAAGCCTGGAAAATCTGGTGAAAGAGGCCCTCCTGGCCCTCAGGGTG
Human       ATGGTGAAGCTGGAAAACCTGGAAAAGCTGGTGAAAGGGGTCCGCCTGGTCCTCAGGGTG
Mouse       ACGGTGAAGCTGGGAAGCCCGGAAAGTCTGGGGAAAGAGGCCTCCCTGGCCCTATGGGTG
Rat         ATGGTGAAGCTGGAAAGCCCGGAAAGGCCGGAGAAAGAGGCCTCCCTGGTCCTCAGGGTG
Xenopus     ATGGTGAAGCTGGCAAACCTGGCAAATCTGGTGAACGTGGACCCCCAGGACCTCAGGGTG
            * ************ ** ** ** **  * ** *** * ** *  ** ** ***  *****

Horse       CTCGCGGCTTCCCGGGAACCCCAGGCCTTCCTGGTGTCAAAGGTCACAGAGGTTACCCAG
Human       CTCGTGGTTTCCCAGGAACCCCAGGCCTTCCTGGTGTCAAAGGTCACAGAGGTTATCCAG
Mouse       CTCGTGGATTCCCAGGAACCCCGGGTCTCCCCGGTGTCAAGGGTCACAGAGGTTACCCAG
Rat         CTCGTGGATTCCCGGGAACCCCCGGTCTTCCTGGTGTCAAGGGTCACAGAGGTTACCCAG
Xenopus     CTAGAGGTTTCCCAGGAACCCCAGGACTTCCAGGAGTAAAAGGACACAGGGGTTACCCAG
            ** * ** ***** ******** ** ** ** ** ** ** ** ***** ***** ****

Horse       GTCTGGATGGTGCTAAGGGAGAAGCCGGTGCTCCAGGTGTGAAGGGCGAGAGTGGTTCCC
Human       GCCTGGACGGTGCTAAGGGAGAGGCGGGTGCTCCTGGTGTGAAGGGTGAGAGTGGTTCCC
Mouse       GCCTCGACGGTGCTAAGGGGGAAGCTGGTGCTCCGGGTGTGAAGGGTGAGAGTGGTTCCC
Rat         GCCTCGATGGTGCTAAGGGAGAAGCTGGTGCTCCAGGTGTGAAGGGTGAGAGCGGTTCCC
Xenopus     GTCTTGATGGTGGAAAGGGTGAAGCTGGTGCTGCAGGTGCCAAGGGAGAAAGTGGAGCTT
            * ** ** ****  ***** ** ** ****** * ****  ***** ** ** **  *
```

```
Horse     CGGGTGAGAATGGTTCTCCGGGCCCAATGGGTCCCCGCGGCCTGCCTGGTGAGAGAGGAC
Human     CGGGTGAGAACGGATCTCCGGGCCCAATGGGTCCTCGTGGCCTGCCTGGTGAAAGAGGAC
Mouse     CTGGTGAGAACGGATCCCCGGGCCCAATGGGTCCCCGTGGCCTGCCTGGCGAGAGAGGAC
Rat       CTGGTGAGAACGGATCCCCAGGCCCAATGGGTCCCCGTGGCCTGCCTGGCGAGAGAGGAC
Xenopus   CTGGTGAAGCCGGAGCTCCTGGTCCAATGGGTCCCCGTGGTCTTCCAGGTGAGAGAGGAC
          * *****    **  * ** ** ********** ** ** ** ** ** ** *******

Horse     GGACTGGCCCTGCTGGCGCTGCAGGTGCCCGGGGTAACGATGGTCAACCAGGCCCCGCAG
Human     GGACTGGCCCTGCTGGCGCTGCGGGTGCCCGAGGCAACGATGGTCAGCCAGGCCCCGCAG
Mouse     GGACTGGCCCTGCTGGTGCTGCTGGTGCTCGGGGTAACGATGGCCAGCCAGGCCCCGCTG
Rat       GGACTGGCCCTGCTGGTGCTGCTGGTGCTCGAGGCAACGACGGCCAGCCAGGCCCTGCTG
Xenopus   GTCCTGGATCTTCTGGTGCTGCTGGTGCTCGTGGTAATGATGGTCTCCCTGGTCCCGCTG
          *  ****  ** **** ***** ***** ** ** ** ** ** *  ** ** ** ** *

Horse     GGCCTCCGGGTCCAGTAGGTCCTGCTGGCGGTCCTGGATTCCCTGGTGCTCCGGGTGCCA
Human     GGCCTCCGGGTCCTGTCGGTCCTGCTGGTGGTCCTGGCTTCCCTGGTGCTCCTGGAGCCA
Mouse     GACCTCCGGGTCCTGTGGGTCCCGCAGGTGGTCCTGGCTTCCCTGGTGCTCCTGGTGCCA
Rat       GACCTCCGGGTCCTGTTGGTCCTGCAGGTGGTCCTGGCTTCCTTGGTGCTCCTGGTGCCA
Xenopus   GTCCACCAGGACCTGTTGGCCCTGCTGGTGCTCCTGGTTTCCCTGGTGCCCCTGGCTCAA
          * ** ** ** ** ** ** ** ** ** ** * ****** **** ****** ** **  * *

Horse     AGGGTGAAGCTGGCCCCACTGGTGCTCGTGGTCCTGAAGGTGCTCAAGGTCCTCGTGGCG
Human     AGGGTGAAGCCGGCCCCACTGGTGCCCGTGGTCCTGAAGGTGCTCAAGGTCCTCGCGGTG
Mouse     AGGGCGAAGCTGGTCCCACTGGTGCTCGCGGTCCTGAAGGTGCTCAAGGTTCTCGTGGCG
Rat       AGGGCGAAGCTGGTCCCACTGGTGCCCGTGGTCCTGAAGGTGCTCAAGGTTCTCGTGGCG
Xenopus   AGGGTGAAGCTGGCCCAACTGGAGCTCGTGGCCCTGAGGGTGCTCAAGGACCCAGAGGAG
          **** ***** ** ** ***** ** ** ** ***** **********  *  * ** *

Horse     AACCTGGTACTCCTGGGTCCCCAGGGCCTGCTGGTGCTGCTGGTAACCCTGGAACTGATG
Human     AACCTGGTACTCCTGGGTCCCCTGGGCCTGCTGGTGCCTCCGGTAACCCTGGAACAGATG
Mouse     AGCCTGGCAATCCTGGGTCCCCTGGGCCTGCAGGTGCTTCTGGTAACCCAGGGACTGATG
Rat       AACCCGGCAATCCCGGATCCCCCGGGCCCGCAGGCGCTTCTGGTAACCCAGGGACTGATG
Xenopus   AATCTGGTACCCCTGGATCCCCTGGACCTTCTGGAGCTTCTGGTAACCCTGGTACTGATG
          *  * ** *  ** ** ***** ** **  * ** **  * ******** ** ** ****

Horse     GAATTCCTGGAGCCAAAGGATCTGCTGGTGCTCCTGGCATTGCTGGTGCTCCCGGCTTCC
Human     GAATTCCTGGAGCCAAAGGATCTGCTGGTGCTCCTGGCATTGCTGGTGCTCCTGGCTTCC
Mouse     GTATTCCTGGAGCCAAAGGATCCGCTGGTGCTCCTGGAATTGCTGGTGCCCCTGGCTTCC
Rat       GTATTCCCGGAGCCAAAGGATCTGCTGGCGCTCCTGGAATTGCTGGTGCCCCTGGCTTCC
Xenopus   GTATTCCTGGCGCCAAAGGTTCATCTGGTGCTTCTGGTATTGCTGGTGCCCCTGGTTTCC
          * ***** ** ******** **  **** *** **** ********** ** ** ****

Horse     CTGGTCCCCGTGGTCCTCCCGGCCCTCAAGGTGCAACTGGTCCTCTGGGCCCGAAAGGTC
Human     CTGGGCCACGGGGCCCTCCTGACCCTCAAGGTGCAACTGGTCCTCTGGGCCCGAAAGGTC
Mouse     CTGGGCCCCGTGGCCCTCCCGGTCCTCAAGGTGCAACTGGTCCCCTTGGCCCCAAAGGTC
Rat       CTGGGCCCCGTGGCCCTCCCGGTCCTCAAGGTGCAACTGGTCCTCTGGGCCCCAAAGGTC
Xenopus   CTGGACCACGTGGTCCACCAGGACCTCAAGGAGCTACTGGTCCTCTTGGTCCCAAAGGCG
          **** ** ** ** ** ** *  ******** ** ******** ** ** ** *****

Horse     AGACGGGTGAACCTGGTATTGCTGGCTTCAAAGGCGAACAAGGCCCCAAGGGAGAACCTG
Human     AGACGGGTAAACCTGGTATTGCTGGCTTCAAAGGTGAACAAGGCCCCAAGGGAGAACCTG
Mouse     AGGCGGGTGAACCTGGCATTGCTGGCTTTAAAGGTGATCAAGGCCCCAAGGGAGAGACTG
Rat       AGACGGGTGAGCCCGGCATCGCTGGCTTCAAAGGTGAACAAGGCCCCAAGGGAGAGACTG
Xenopus   AGACTGGTGACCCTGGTATAGCAGGTTTCAAGGGTGAACATGGTCCCAAAGGTGAAATCG
          ** * *** * ** ** ** ** ** ** ** ** ** ** ** ** ***** ** **    *

Horse     GCCCTGCTGGTCCCCAAGGAGCCCCTGGTCCTGCTGGTGAAGAAGGCAAAAGAGGTGCTC
Human     GCCCTGCTGGCCCCCAGGGAGCCCCTGGACCCGCTGGTGAAGAAGGCAAGAGAGGTGCCC
Mouse     GACCTGCTGGGCCCCAAGGAGCCCCTGGCCCCGCTGGTGAAGAAGGCAAACGAGGTGCTC
Rat       GACCTGCTGGGCCCCAGGGAGCCCCTGGCCCTGCTGGTGAAGAAGGAAAACGAGGTGCTC
Xenopus   GGTCTGCAGGTCCTCAGGGTGCTCCTGGCCCAGCTGGAGAAGAAGGCAAAAGAGGAGCTC
          *  **** ** ** ** ** ** ***** ** ***** ******** **  **** ** *
```

```
Horse     GTGGAGAGCCTGGCGGTGCTGGGCCTGTCGGTCCCCCTGGAGAAAGAGGTGCTCCTGGCA
Human     GTGGAGAGCCTGGTGGCGTTGGGCCCATCGGTCCCCCTGGAGAAAGAGGTGCTCCCGGCA
Mouse     GAGGAGAGCCGGGTGGTGCTGGACCAATCGGACCCCCTGGAGAGAGAGGTGCTCCTGGCA
Rat       GAGGAGAGCCCGGTGGCGCTGGACCTATCGGCCCCCCTGGAGAGAGAGGTGCTCCTGGCA
Xenopus   GTGGTGAACCTGGTGCTGCTGGGCCACTTGGTCCTCCTGGTGAGAGAGGTGCTCCTGGTA
          * ** ** ** ** *  * *** **  * ** ** ***** ** ********** ** *

Horse     ACCGTGGTTTCCCAGGTCAAGATGGTCTGGCCGGTCCCAAGGGAGCCCCTGGAGAGCGAG
Human     ACCGCGGTTTCCCAGGTCAAGATGGTCTGGCAGGTCCCAAGGGAGCCCCTGGAGAGCGAG
Mouse     ACCGTGGATTCCCAGGTCAAGATGGTCTGGCAGGTCCCAAGGGTGCCCCTGGAGAGCGAG
Rat       ACCGCGGTTTCCCAGGTCAAGATGGTCTGGCAGGTCCCAAGGGTGCCCCTGGAGAGCGAG
Xenopus   ATCGTGGTTTCCCTGGTCAAGATGGTCTTGCTGGTCCTAAGGGTGCTCCTGGTGAACGTG
          * ** ** ***** ************** ** ***** ***** ** ***** ** ** *

Horse     GGCCCAGTGGCCTTGCTGGCCCCAAGGGAGCCAATGGTGACCCTGGCCGTCCCGGCGAGC
Human     GGCCCAGTGGTCTTGCTGGCCCCAAGGGAGCCAACGGTGACCCTGGCCGTCCTGGAGAAC
Mouse     GGCCCAGTGGCTTGGCTGGTCCCAAGGGAGCCAACGGTGACCCGGGTCGTCCTGGAGAAC
Rat       GGCCTAGTGGCCTGGCTGGTCCTAAGGGTGCCAATGGTGACCCGGGTCGTCCTGGAGAAC
Xenopus   GTGTTCCAGGTCTTGGTGGACCAAAGGGTGGTAATGGTGATCCTGGCCGTCCCGGTGAAC
          *       **  * * *** ** ***** *  ** ***** ** ** ***** ** ** *

Horse     CTGGCCTTCCTGGAGCCCGGGGTCTCACTGGTCGCCCTGGTGATGCTGGTCCTCAAGGCA
Human     CTGGCCTTCCTGGAGCCCGGGGTCTCACTGGCCGCCCTGGTGATGCTGGTCCTCAAGGCA
Mouse     CTGGTCTTCCTGGAGCCAGGGGTCTTACCGGTCGCCCTGGTGACGCTGGTCCTCAAGGCA
Rat       CTGGTCTTCCTGGAGCCAGGGGTCTTACTGGCCGCCCTGGTGATGCTGGTCCTCAAGGCA
Xenopus   CCGGTCTCCCTGGTGCTAGAGGTCTTACTGGCCGTCCTGGTGATGCTGGTCCTCAGGGAA
          * ** ** ***** **  * ***** ** ** ** ******** ********** ** *

Horse     AAGTTGGTCCCTCTGGTGCCCCTGGTGAAGATGGTCGCCCTGGACCTCCAGGTCCTCAGG
Human     AAGTTGGCCCTTCTGGAGCCCCTGGTGAAGATGGTCGTCCTGGACCTCCAGGTCCTCAGG
Mouse     AAGTTGGTCCTTCTGGAGCCCCTGGTGAAGACGGTCGCCCTGGACCTCCTGGTCCTCAGG
Rat       AAGTTGGTCCTTCTGGAGCCCCTGGAGAAGACGGTCGCCCTGGTCCTCCTGGTCCTCAGG
Xenopus   AAGTTGGGCCCTCTGGTGCTGCTGGTGAAGATGGTCGTCCAGGACCTCCTGGGCCACAAG
          ******* ** ***** **  **** ***** ***** ** ** ***** ** ** ** *

Horse     GGGCTCGTGGGCAGCCTGGTGTCATGGGTTTCCCTGGCCCCAAAGGTGCCAATGGCGAGC
Human     GGGCTCGTGGGCAGCCTGGTGTCATGGGTTTCCCTGGCCCCAAAGGTGCCAACGGTGAGC
Mouse     GAGCTCGTGGGCAGCCTGGCGTCATGGGTTTCCCTGGCCCCAAAGGTGCCAACGGCGAGC
Rat       GAGCTCGTGGGCAGCCTGGCGTCATGGGTTTCCCTGGCCCCAAAGGAGCCAATGGCGAGC
Xenopus   GTGCTCGCGGTCAGCCTGGTGTTATGGGATTCCCTGGACCTAAGGGTGCCAATGGTGAAC
          * ***** ** ******** ** ***** ******** ** ** ** ***** ** ** *

Horse     CTGGCAAAGCTGGTGAAAAGGGACTGCCTGGTGCTCCTGGTCTGAGAGGTCTTCCTGGCA
Human     CTGGCAAAGCTGGTGAGAAGGGACTGCCTGGTGCTCCTGGTCTGAGGGGTCTTCCTGGCA
Mouse     CTGGCAAAGCTGGTGAGAAGGGTCTGGCTGGCGCTCCTGGTCTGAGAGGTCTTCCTGGAA
Rat       CTGGCAAAGCTGGTGAGAAAGGACTGGCTGGTGCTCCTGGTCTGAGAGGTCTTCCTGGCA
Xenopus   CTGGCAAATCTGGTGAGAAAGGACTTGGTGGTGCTCCTGGTTTGAGGGGTTTGCCTGGAA
          ******** ******* ** ** **   *** ********* **** *** * ***** *

Horse     AAGATGGTGAGACAGGTGCTGCAGGACCCCCCGGACCTGCTGGACCTGCTGGAGAACGAG
Human     AAGATGGTGAGACAGGTGCTGAAGGCCCCCCTGGCCCTGCTGGACCTGCTGGTGAACGAG
Mouse     AAGACGGTGAGACGGGAGCCGCAGGACCCCCCGGCCCCAGTGGACCTGCTGGTGAACGAG
Rat       AAGATGGTGAGACAGGAGCCGCAGGACCCCCCGGCCCCAGCGGACCTGCTGGTGAACGAG
Xenopus   AAGATGGTGAAACTGGTGCTCAAGGTCCCAATGGTCCAGCTGGACCTGCTGGTGAGAGAG
          **** ***** ** ** **   *** ***   ** **    *********** **  ***

Horse     GCGAGCAGGGTGCTCCTGGACCGTCTGGGTTCCAGGGACTTCCTGGCCCTCCCGGTCCCC
Human     GCGAGCAGGGTGCTCCTGGGCCATCTGGGTTCCAGGGACTTCCTGGCCCTCCTGGTCCCC
Mouse     GCGAGCAGGGCGCTCCTGGACCATCAGGGTTCCAGGGACTTCCTGGCCCTCCCGGTCCCC
Rat       GCGAGCAGGGTGCTCCTGGGCCATCAGGGTTCCAGGGACTTCCTGGCCCTCCTGGTCCCC
Xenopus   GTGAACAGGGACCTCCTGGTCCATCTGGCTTCCAGGGACTTCCTGGACCTCCCGGTTCTC
          * ** *****  ******* ** ** ** ***************** ***** *** * *
```

```
Horse       CAGGCGAAGGTGGAAAACCAGGTGACCAGGGTGTTCCCGGAGAAGCTGGAGCCCCCGGCC
Human       CAGGTGAAGGTGGAAAACCAGGTGACCAGGGTGTTCCCGGTGAAGCTGGAGCCCCTGGAC
Mouse       CAGGTGAAGGTGGAAAGCAAGGTGACCAGGGTATTCCTGGTGAAGCTGGAGCTCCTGGCC
Rat         CGGGTGAAGGTGGAAAGCAAGGTGACCAGGGTATTCCTGGTGAAGCTGGAGCCCCTGGCC
Xenopus     CCGGAGAAGGTGGCAAACCAGGAGATCAGGGTGTGCCCGGAGAAGCAGGTGCAGCAGGTC
            * ** ******** ** * *** ** ****** * ** ** ***** ** **  * ** *

Horse       TCGTGGGTCCCAGGGGTGAACGAGGTTTCCCAGGTGAACGTGGCTCTCCCGGCGCCCAGG
Human       TAGTGGGTCCCAGGGGTGAACGAGGTTTCCCAGGTGAACGTGGCTCTCCCGGTGCCCAGG
Mouse       TTGTGGGTCCTCGGGGCGAGCGAGGTTTCCCAGGTGAACGTGGCTCTCCTGGTGCTCAGG
Rat         TCGTTGGTCCCCGGGGTGAACGAGGTTTCCCAGGTGAACGTGGCTCTCCCGGTGCTCAGG
Xenopus     TTGTTGGACCAAGAGGTGAACGTGGTTTCCCAGGTGAGCGTGGGTCTTCCGGTCCTCAAG
            * ** ** **  * ** ** ** ********** ***** *** * **  * ** *

Horse       GCCTCCAGGGTGCCCGTGGCCTCCCCGGCACTCCTGGCACTGATGGTCCTAAAGGTGCAT
Human       GCCTCCAGGGTCCCCGTGGCCTCCCCGGCACTCCTGGCACTGATGGTCCCAAAGGTGCAT
Mouse       GCCTTCAGGGTCCCCGAGGCCTCCCTGGCACTCCTGGTACTGATGGTCCCAAAGGTGCAG
Rat         GCCTCCAGGGTCCCCGAGGCCTCCCTGGCACTCCTGGTACTGATGGTCCCAAAGGTGCGG
Xenopus     GTCTTCAGGGTTCTCGTGGTCTTCCTGGAACTCCTGGTACTGATGGTCCCAAGGGTGCAA
            * ** ****** * ** ** ** ** ** ******** ********** ** *****

Horse       CTGGCCCAGCTGGCCCCCCTGGGGCTCAGGGTCCTCCAGGTCTGCAGGGGATGCCTGGTG
Human       CTGGCCCAGCAGGCCCCCCTGGGGCTCAGGGCCCTCCAGGTCTTCAGGGAATGCCTGGCG
Mouse       CTGGCCCAGATGGCCCCCCTGGGGCTCAGGGGCCTCCAGGTCTACAGGGAATGCCTGGTG
Rat         CTGGCCCAGATGGTCCCCCTGGGGCTCAGGGCCCTCCAGGTCTACAGGGAATGCCTGGTG
Xenopus     CTGGTCCATCTGGTCCCAATGGTGCCCAAGGTCCTCCTGGTCTTCAAGGTATGCCTGGTG
            **** ***   ** ***  *** ** ** ** ***** ***** ** ** ******** *

Horse       AGAGGGGAGCAGCTGGTATCGCCGGGCCCAAGGGAGACAGGGGTGATGTTGGTGAGAAAG
Human       AGAGGGGAGCAGCTGGTATCGCTGGGCCCAAAGGCGACAGGGGTGACGTTGGTGAGAAAG
Mouse       AGAGAGGAGCCGCTGGCATTGCTGGGCCCAAGGGAGACAGAGGCGATGTTGGCGAGAAAG
Rat         AGAGGGGAGCAGCTGGTATTGCTGGACCCAAGGGAGACAGAGGTGATGTTGGCGAGAAAG
Xenopus     AGAGAGGAGCTGCTGGTATTTCTGGACCCAAAGGTGATAGAGGTGATACTGGTGAGAAAG
            **** ***** ***** **  * ** ***** ** ** ** ** **   *** *******

Horse       GCCCTGAGGGAGCCCCCGGCAAAGACGGTGGACGAGGTCTGACTGGTCCCATTGGCCCCC
Human       GCCCTGAGGGAGCCCCTGGAAAGGATGGTGGACGAGGCCTGACAGGTCCCATTGGCCCCC
Mouse       GCCCAGAGGGAGCTCCTGGGAAGGATGGCGGCCGAGGTCTGACTGGGCCCATCGGACCCC
Rat         GCCCAGAGGGAGCTCCTGGAAAAGATGGTGGCCGAGGTCTTACTGGGCCCATCGGGCCCC
Xenopus     GCCCAGAGGGTGCTCCTGGCAAAGATGGTTCAAGAGGTTTGACAGGTCCACTTGGTCCCC
            **** ***** ** ** ** ** ** **     ****  * ** ** **  * ** ****

Horse       CTGGCCCGGCTGGCGCCAACGGTGAGAAGGGAGAAGTTGGACCTCCTGGTCCTGCAGGAA
Human       CTGGCCCAGCTGGTGCTAATGGCGAGAAGGGAGAAGTTGGACCTCCTGGTCCTGCAGGAA
Mouse       CAGGCCCAGCAGGGGCCAACGGCGAGAAGGGAGAAGCCGGACCTCCTGGCCCGTCAGGAA
Rat         CAGGACCAGCAGGTGCCAATGGCGAGAAGGGAGAAGTCGGACCTCCTGGTCCTTCAGGAA
Xenopus     CTGGTCCAGCTGGTCCTAATGGTGAGAAGGGTGAATCTGGTCCTTCTGGTCCACCTGGTA
            * ** ** ** **  * ** ** ******** ***   ** *** **** **  * ** *

Horse       CTGCTGGTGCTCGTGGTGCCCCGGGCGAACGTGGAGAGACTGGACCCCCCGGACCCGCTG
Human       GTGCTGGTGCTCGTGGCGCTCCGGGTGAACGTGGAGAGACTGGGCCCCCCGGGACCAGCG
Mouse       GTACCGGAGCTCGAGGAGCCCCGGGTGAACCTGGAGAGACCGGGCCACCTGGACCTGCTG
Rat         GTACTGGAGCTCGAGGCGCCCCGGGTGAGCGCGGAGAGACTGGGCCACCTGGACCTGCTG
Xenopus     TTGTTGGTGCCCGTGGTGCCCCTGGTGATCGTGGTGAGAATGGCCCTCCAGGTCCTGCTG
             *   ** ** ** ** ** ** ** ** *  ** ****  ** ** ** **  *    *

Horse       GATTCGCAGGTCCTCCCGGTGCTGATGGCCAGCCTGGTGCTAAGGGTGAGCAAGGAGAGG
Human       GGATTGCTGGGCCTCCTGGTGCTGATGGCCAGCCTGGGGCCAAGGGTGAGCAAGGAGAGG
Mouse       GATTCGCTGGCCCTCCTGGTGCTGATGGCCAGCCTGGTGCCAAGGGTGATCAAGGAGAAG
Rat         GATTCGCTGGCCCTCCTGGTGCTGATGGCCAGCCTGGTGCCAAGGGCGATCAAGGAGAAG
Xenopus     GCTTTGCTGGTCCTCCAGGTTCTGATGGTCAAGCTGGTCTTAAGGGTGATCAAGGTGAAT
            *  * ** ** ***** *** ******* **  ****    ***** ** ***** **
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Horse       CCGGCCAGAAAGGTGATGCTGGTGCCCCAGGTCCTCAGGGCCCCTCTGGAGCTCCTGGGC
Human       CCGGCCAGAAAGGCGATGCTGGTGCCCCTGGTCCTCAGGGCCCCTCTGGAGCACCTGGGC
Mouse       CCGGACAGAAAGGAGATGCTGGTGCCCCCGGCCCACAAGGCCCCTCGGGAGCCCCTGGGC
Rat         CTGGACAGAAAGGTGACGCTGGTGCCCCTGGCCCACAAGGCCCCTCAGGAGCTCCCGGGC
Xenopus     CTGGCCAGAAGGGTGATGCTGGTGCTCCTGGTCCTCAGGGTCCATCTGGTGCTCCTGGCC
            * ** ***** ** ** ******** ** ** ** ** ** ** ** ** ** ** * *

Horse       CTCAGGGTCCTACTGGTGTGACTGGTCCTAAAGGAGCCCGAGGTGCTCAAGGGCCCCCGG
Human       CTCAGGGTCCTACTGGAGTGACTGGTCCTAAAGGAGCCCGAGGTGCCCAAGGCCCCCCGG
Mouse       CACAGGGTCCTACTGGAGTGACTGGTCCTAAGGGAGCCCGAGGTGCCCAAGGTCCCCCGG
Rat         CACAGGGTCCTACTGGAGTGACTGGTCCTAAGGGAGCCCGGGGCGCCCAAGGCCCACCGG
Xenopus     CACAGGGCCCAACTGGTGTTAATGGTCCTAAAGGAGCTCGTGGTGCTCAAGGTCCTGCTG
            * ***** ** ***** ** * ********* ***** ** ** ** ***** **  * *

Horse       GAGCCACCGGATTCCCCGGAGCTGCTGGCCGTGTCGGACCCCCAGGCTCCAATGGCAACC
Human       GAGCCACTGGATTCCCTGGAGCTGCTGGCCGCGTTGGACCCCCAGGCTCCAATGGCAACC
Mouse       GAGCCACCGGATTCCCTGGAGCTGCTGGCCGAGTTGGACCCCCAGGTGCTAATGGCAATC
Rat         GAGCCACCGGATTCCCTGGAGCTGCTGGCCGAGTTGGACCCCCAGGTTCTAATGGCAACC
Xenopus     GTGCTACTGGATTCCCTGGCGCTGCTGGAAGAGTTGGCACCCCAGGTCCCAATGGTAACC
            * ** ** ******** ** ********  * ** **  *******  * ***** ** *

Horse       CTGGACCCCCCGGTCCCCCTGGTCCTTCTGGAAAAGATGGTCCCAAAGGTGCTCGAGGAG
Human       CTGGACCCCCTGGTCCCCCTGGTCCTTCTGGAAAAGATGGTCCCAAAGGTGCTCGAGGAG
Mouse       CTGGACCCGCCGGTCCCCCTGGTCCTGCTGGAAAAGATGGTCCCAAAGGTGTTCGAGGAG
Rat         CTGGGCCCGCCGGTCCCCCTGGTCCTGCTGGAAAAGATGGTCCCAAAGGTGCTCGAGGAG
Xenopus     CTGGACCTCCTGGTCCTCCCGGATCTGCTGGAAAAGAAGGACCAAAGGGTGTACGTGGTG
            **** **  * ***** ** **  ** ********** ** ** ** ****  ** ** *

Horse       ACAGCGGTCCCCCCGGCCGAGCTGGTGACCCTGGCCTTCAAGGTCCTGCTGGACCCCCTG
Human       ACAGCGGCCCCCCTGGCCGAGCTGGTGAACCCGGCCTCCAAGGTCCTGCTGGACCCCCTG
Mouse       ACAGTGGCCCCCCTGGCAGAGCTGGTGACCCCGGTCTTGAAGGTCCTGCAGGAGCTCCTG
Rat         ACACTGGTGCCCCTGGCAGAGCTGGTGACCCTGGACTTCAAGGACCCGCAGGAGCTCCTG
Xenopus     ATGCTGGTCCTACTGGTCGTGCTGGTGATCCAGGTCTTCAGGGTCCTGCTGGTGCTCCTG
            *    **  *  * **  * ******** ** ** **  * ** ** ** **  * ****

Horse       GCGAGAAGGGAGAGCCTGGAGATGATGGTCCCTCTGGTCCTGACGGTCCTCCAGGTCCCC
Human       GCGAGAAGGGAGAGCCTGGAGATGACGGTCCCTCTGGTGCCGAAGGTCCACCAGGTCCCC
Mouse       GCGAGAAAGGAGAACCTGGAGATGATGGTCCCTCTGGTCTTGATGGTCCTCCAGGTCCCC
Rat         GAGAGAAAGGCGAACCTGGAGATGACGGTCCCTCTGGTTCTGATGGTCCCCCAGGTCCCC
Xenopus     GAGAGAAGGGAGAGCCTGGTGAAGATGGTCCTTCTGGTCCAGATGGTCCCAGTGGTCCTC
            * ***** ** ** ***** ** ** ***** ******   ** *****    ***** *

Horse       AGGGTCTGGCTGGACAGAGGGGCATCGTTGGTCTGCCTGGGCAGCGTGGTGAGAGAGGAT
Human       AGGGTCTGGCTGGTCAGAGAGGCATCGTCGGTCTGCCTGGGCAACGTGGTGAGAGAGGAT
Mouse       AGGGGCTGGCTGGTCAAAGGGGCATTGTTGGTCTGCCTGGTCAGCGTGGTGAGAGAGGAT
Rat         AGGGGCTGGCTGGTCAAAGGGGCATTGTTGGTCTGCCTGGTCAGCGTGGTGAGAGAGGAT
Xenopus     AAGGTTTGTCTGGAAATCGTGGTATTGTTGGTCTACCAGGTCAGCGTGGTGAAAGAGGAT
            * **  ** ****  *  * ** ** ** ***** ** ** ** ******** *******

Horse       TCCCCGGCCTGCCTGGCCCATCGGGCGAGCCTGGCAAGCAGGGAGCTCCTGGAGCATCTG
Human       TCCCTGGCTTGCCTGGCCCGTCGGGTGAGCCCGGCCAGCAGGGTGCTCCTGGAGCATCTG
Mouse       TCCCCGGCCTTCCCGGCCCATCGGGTGAGCCCGGCAAGCAGGGTGCACCTGGCGCGTCTG
Rat         TCCCAGGCCTTCCCGGCCCATCGGGTGAGCCCGGCAAGCAGGGTGCACCTGGTGCATCTG
Xenopus     TCCCTGGACTTCCTGGCCCATCTGGTGAACCTGGCAAACAAGGAGGCCCTGGATCTTCAG
            **** **  * ** ***** ** ** ** ** *** * ** ** *  *****  * ** *

Horse       GAGACCGAGGTCCCCCTGGACCCGTGGGTCCTCCTGGCCTGACTGGTCCTGCTGGTGAAC
Human       GAGACAGAGGTCCTCCTGGCCCCGTGGGTCCTCCTGGCCTGACGGGTCCTGCAGGTGAAC
Mouse       GAGACAGAGGTCCTCCTGGTCCTGTGGGGCCTCCTGGCCTGACAGGGCCTGCAGGTGAAC
Rat         GAGACAGAGGTCCTCCTGGTCCTGTGGGGCCTCCTGGCTTGACAGGACCTGCAGGTGAAC
Xenopus     GAGACCGTGGACCCCCTGGCCCTGTTGGCCCACCTGGTTTGACTGGACCTGCTGGAGAGA
            ***** * ** ** ***** ** ** ** ** *****  **** ** ***** ** **
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Horse       CTGGACGAGAGGGAACGCCTGGTGCTGATGGCCCCCCTGGCAGAGATGGTGCAGCTGGAG
Human       CTGGACGAGAGGGAAGCCCCGGTGCTGATGGCCCCCCTGGCAGAGATGGCGCTGCTGGAG
Mouse       CTGGACGAGAGGGCAGCCCTGGTGCTGATGGACCCCCTGGAAGAGATGGTGCAGCTGGAG
Rat         CTGGACGAGAGGGCAGCCCTGGTGCTGATGGACCCCCTGGCAGAGATGGTGCGGCTGGAG
Xenopus     CTGGACGAGAAGGTAATCCCGGATCTGATGGCCCACCTGGTCGAGATGGTGCTACTGGAA
            ********** ** *  ** **  ******* ** *****  ******* **  *****

Horse       TCAAGGGTGATCGTGGTGAGGCTGGTGCCCTGGGTGCTCCCGGAGCCCCTGGACCCCCTG
Human       TCAAGGGTGATCGTGGTGAGACTGGTGCTGTGGGAGCTCCTGGAGCCCCTGGGCCCCCTG
Mouse       TCAAGGGAGATCGTGGTGAGACTGGAGCACTGGGTGCCCCTGGAGCTCCTGGGCCCCCAG
Rat         TCAAGGGTGATCGTGGTGAGACTGGTGCACTTGGTGCTCCTGGAGCTCCTGGGCCCCCAG
Xenopus     TTAAGGGTGATCGTGGTGAAACTGGACCAATTGGCGCTCCTGGTGCTCCAGGTTCCCCTG
            * ***** **********  ****  *  * ** ** ** ** ** ** **  **** *

Horse       GCTCTCCTGGCCCTGCCGGCCCAACTGGCAAGCAAGGAGACAGAGGAGAAGCTGGTGCAC
Human       GCTCCCCTGGCCCCGCTGGTCCAACTGGCAAGCAAGGAGACAGAGGAGAAGCTGGTGCAC
Mouse       GCTCTCCTGGTCCTGCTGGCCCAACTGGCAAACAAGGAGACAGAGGAGAGGCTGGTGCAC
Rat         GCTCTCCTGGTCCTGCTGGCCCAACTGGCAAACAAGGAGACAGAGGAGAGGCTGGTGCAC
Xenopus     GTGCTCCTGGACCTGTTGGCCCAACTGGAAAACAAGGAGACAGAGGAGAATCTGGATCCC
            *  * ***** ** *  ** ******** ** ****************  ****  * *

Horse       AAGGCCCCATGGGACCTGCAGGACCAGCTGGAGCCCGAGGATTGCCAGGCCCTCAAGGTC
Human       AAGGCCCCATGGGACCCTCAGGACCAGCTGGAGCCCGGGGAATCCAGGGTCCTCAAGGCC
Mouse       AAGGTCCTATGGGTCCCTCAGGACCTGCTGGAGCCCGTGGGATTGCAGGCCCTCAAGGCC
Rat         AAGGTCCTATGGGCCCCTCAGGACCTGCTGGAGCCCGTGGAATTGCTGGCCCTCAAGGCC
Xenopus     AAGGTCCACTTGGTCCTTCTGGTCCTGCTGGTGCTCGAGGTTTGAATGGTCCTCAAGGTC
            **** **  * ** **  * ** ** ***** ** ** **  *    ** ******** *

Horse       CCCGAGGTGACAAAGGAGAAGCTGGAGAGGCTGGCGAGAGGGGACTGAAGGGACACCGTG
Human       CCAGAGGTGACAAAGGAGAGGCTGGAGAGCCTGGCGAGAGAGGCCTGAAGGGACACCGTG
Mouse       CCCGAGGTGACAAAGGAGAATCTGGAGAGCAGGGCGAGAGGGGACTGAAGGGACACCGAG
Rat         CCCGAGGTGACAAAGGAGAAGCTGGAGAGCCTGGCGAGAGAGGACTGAAGGGGCACCGAG
Xenopus     CACGTGGTGATAAGGGTGAAGCTGGTGAGGCAGGAGAGAGAGGACAGAAAGGTCACAGAG
            *  * ***** ** ** **  **** ***   ** ***** ** * *** ** *** * *

Horse       GCTTCACTGGTCTGCAGGGTCTGCCTGGCCCTCCTGGTCCTTCTGGAGACCAAGGTGCTT
Human       GCTTCACTGGTCTGCAGGGTCTGCCCGGCCCTCCTGGTCCTTCTGGAGACCAAGGTGCTT
Mouse       GTTTCACTGGACTGCAGGGTCTGCCTGGCCCTCCGGGTCCTTCTGGAGATCAGGGTGCTT
Rat         GTTTCACTGGACTGCAGGGTCTGCCTGGCCCCCCGGGTCCTTCTGGAGATCAGGGTACTT
Xenopus     GATTCACTGGTCTTCAGGGTCTACCCGGTCCCCCTGGTACTGCTGGTGATCAAGGTGCTA
            * ******** ** ******** ** ** ** ** *** ** **** ** ** *** **

Horse       CTGGTCCTGCTGGTCCTTCTGGCCCTAGAGGTCCTCCTGGCCCCGTCGGTCCCTCTGGCA
Human       CTGGTCCTGCTGGTCCTTCTGGCCCTAGAGGTCCTCCTGGCCCCGTCGGTCCCTCTGGCA
Mouse       CTGGCCCTGCTGGTCCTTCTGGCCCTAGAGGTCCACCTGGCCCTGTTGGTCCCTCTGGCA
Rat         CTGGCCCTGCTGGTCCTTCCGGCCCTAGAGGTCCACCTGGCCCTGTTGGTCCCTCTGGCA
Xenopus     CTGGTTCTGCCGGTCCTTCTGGTCCTAGAGGTCCCCCTGGCCCTGTTGGTCCTTCTGGAA
            ****  **** ******** ** ********** ******** ** ***** ***** *

Horse       AAGATGGCGCTAATGGAATCCCTGGCCCCATCGGACCTCCTGGCCCCCGTGGACGTTCAG
Human       AAGATGGTGCTAATGGAATCCCTGGCCCCATTGGGCCTCCTGGTCCCCGTGGACGATCAG
Mouse       AAGATGGCTCTAATGGAATCCCTGGCCCCATCGGGCCTCCAGGTCCCCGTGGACGCTCAG
Rat         AAGATGGCTCTAATGGAATCCCTGGCCCCATCGGGCCTCCAGGTCCCCGTGGACGCTCAG
Xenopus     AAGATGGCTCTAATGGTATCCCTGGTCCAATTGGTCCACCTGGTCCACGCGGTCGTAGTG
            *******  ******* ******** ** ** ** ** ** ** ** ** ** **    *

Horse       GCGAAACTGGCCCCGCTGGTCCTCCCGGAAATCCTGGACCCCCTGGCCCTCCAGGTCCCC
Human       GCGAAACCGGCCCTGCTGGTCCTCCTGGAAATCCTGGACCCCCTGGTCCTCCAGGTCCCC
Mouse       GAGAAACAGGCCCTGTTGGTCCCCCTGGAAGTCCCGGTCCTCCTGGCCCTCCAGGTCCTC
Rat         GAGAAACTGGCCCTGCTGGTCCTCCTGGAAATCCTGGTCCCCCTGGCCCTCCGGGTCCTC
Xenopus     GTGAAACTGGCCCAGCTGGTCCACCTGGTCAATCTGGCCCACCTGGCCCCCCTGGTCCTC
            * ***** ***** * ****** ** **     * ** ** ***** ** ** ***** *
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Horse       CTGGCCCTGGCATCGACATGTCCGCCTTTGCTGGCCTGGGCCCGAGAGAGAAGGGCCCTG
Human       CTGGCCCTGGCATCGACATGTCCGCCTTTGCTGGCTTAGGCCCGAGAGAGAAGGGCCCCG
Mouse       CTGGTCCTGGCATCGACATGTCAGCCTTTGCTGGCTTAGGGCAGAGAGAGAAGGGCCCCG
Rat         CTGGTCCTGGCATCGACATGTCAGCCTTTGCTGGCTTAGGACAGAGAGAGAAGGGCCCCG
Xenopus     CTGGGCCTGGAATTGACATGTCTGCTTTTGCTGGCCTATCTCAACCTGAAAAAGGACCTG
            **** ***** ** ******** ** ********* *    *      ** ** ** ** *

Horse       ACCCCCTGCAGTACATGCGGGCTGACGAGGCGGCTGGCGGCCTGAGACCGCATGACGAGG
Human       ACCCCCTGCAGTACATGCGGGCCGACCAGGCAGCCGGTGGCCTGAGACAGCATGACGCCG
Mouse       ACCCCATGCAGTACATGCGGGCCGACGAGGCAGACAGTACCTTGAGACAGCACGACGTGG
Rat         ATCCCCTGCAGTACATGCGGGCCGACGAGGCAGACAGTACCTTGAGACAGCATGACGTCG
Xenopus     ACCCAATGCGCTACATGCGTGCTGACCAAGCCTCTAGTTCTGTC---CTTCAGACCGCTG
            * **  ***  ******** ** *** * **     *     *    * **   **  *

Horse       AGGTGGAGGCTACACTCAAGTCCCTCAACAACCAGATCGAGAGCATCCGCAGCCCCGAGG
Human       AGGTGGATGCCACACTCAAGTCCCTCAACAACCAGATTGAGAGCATCCGCAGCCCCGAGG
Mouse       AGGTGGACGCTACACTCAAGTCACTGAACAACCAGATTGAGAGCATCCGCAGCCCCGACG
Rat         AGGTGGACGCCACGCTCAAGTCGCTGAACAACCAGATCGAGAGCATCCGCAGCCCTGATG
Xenopus     ATGTTGAAGCCAATTTGAAAACCCTGAATAACCAGATCGAAAGCATTCGCAGCCCAGATG
            * ** ** ** *   * **  * ** ** ******** ** ***** ******** ** *

Horse       GCTCCCGCAAGAACCCTGCTCGCACCTGCCGGGACCTGAAACTCTGCCACCCTGAATGGA
Human       GCTCCCGCAAGAACCCTGCTCGCACCTGCAGAGACCTGAAACTCTGCCACCCTGAGTGGA
Mouse       GCTCCCGCAAGAACCCTGCTCGCACTTGCCAAGACCTGAAACTCTGCCACCCCGAGTGGA
Rat         GCTCCCGCAAGAATCCCGCTCGCACCTGCCAGGACCTGAAACTCTGCCACCCAGAGTGGA
Xenopus     GTACCAAAAAGAACCCAGCCCGCACATGCCGTGACCTGAAACTCTGCCATCCTGAATGGA
            *  **   ***** ** ** ***** ***   ****************** ** ** ****

Horse       AGAGCGGAGACTACTGGATTGACCCCAACCAGGGCTGCACCTTAGACGCCATGAAGGTTT
Human       AGAGTGGAGACTACTGGATTGACCCCAACCAAGGCTGCACCTTGGACGCCATGAAGGTTT
Mouse       AGAGCGGAGACTACTGGATTGATCCCAACCAGGGCTGCACCTTGGACGCCATGAAAGTTT
Rat         AGAGCGGAGACTACTGGATTGATCCCAACCAGGGCTGCACCTTGGACGCCATGAAAGTCT
Xenopus     AGAGTGGTGAATATTGGATTGATCCCAACGAGGGCTGCACTGTAGATGCTATCAAAGTCT
            **** ** ** ** ******** ****** * ********  * ** ** ** ** ** *

Horse       TCTGCAACATGGAGACTGGCGAGACCTGCGTCTACCCCAACCCAGCGAACGTTCCCAAGA
Human       TCTGCAACATGGAGACTGGCGAGACTTGCGTCTACCCCAATCCAGCAAACGTTCCCAAGA
Mouse       TCTGCAACATGGAGACCGGCGAGACTTGCGTCTACCCCAACCCAGCGACTGTCCCTCGGA
Rat         TCTGCAACATGGAGACTGGCGAGTCTTGCGTCTACCCCAACCCAGCGACTGTGCCTCGGA
Xenopus     TCTGCAACATGGAGACTGGAGAGTCCTGTGTATACCCCAGCCCATCAAAGATCCCCAAGA
            ***************** ** *** * ** ** *******  *** * *   * **   **

Horse       AGAACTGGTGGAGCAGCAAGAGCAAGGACAAGAAACATATCTGGTTTGGAGAAACCATCA
Human       AGAACTGGTGGAGCAGCAAGAGCAAGGAGAAGAAACACATCTGGTTTGGAGAAACCATCA
Mouse       AAAACTGGTGGAGCAGCAAGAGCAAGGAAAAGAAACACATCTGGTTTGGAGAGACCATGA
Rat         AGAACTGGTGGAGCAGCAAGAGCAAGGAGAAGAAGCACATCTGGTTTGGAGAGACCATGA
Xenopus     AGAACTGGTGGAGCGCCAAGGGCAAGGAAAAGAAGCACATTTGGTTTGGAGAGACAATCA
            * ************  **** ******* ***** ** ** ************ ** ** *

Horse       ACGGTGGCTTCCACTTCAGCTATGGAGATGACAACCTGGCTCCCAACACTGCCAACGTCC
Human       ATGGTGGCTTCCATTTCAGCTATGGAGATGACAATCTGGCTCCCAACACTGCCAACGTCC
Mouse       ACGGTGGCTTCCACTTCAGCTATGGCGATGGCAACCTGGCTCCCAACACCGCTAACGTCC
Rat         ACGGCGGCTTCCACTTCAGCTACGGCGACGGCAACCTGGCTCCCAACACCGCTAACGTCC
Xenopus     ATGGTGGTTTCCAATTCAGCTATGGAGATGACAGCTCAGCACCCAACACTGCTAACATTC
            * ** ** ***** ******** ** ** * **      ** ******** ** *** * *

Horse       AGATGACCTTCCTGCGTCTGCTGTCCACCGAGGGCTCCCAGAACATCACCTACCACTGCA
Human       AGATGACCTTCCTACGCCTGCTGTCCACGGAAGGCTCCCAGAACATCACCTACCACTGCA
Mouse       AGATGACTTTCCTCCGTCTACTGTCCACTGAGGGCTCCCAGAACATCACCTACCACTGTA
Rat         AGATGACTTTCCTCCGTCTACTGTCCACTGAGGGCTCCCAGAACATCACCTACCACTGTA
Xenopus     AGTTGACCTTCCTGCGTCTGCTGTCCACTGATGCATCCCAGAACATCACCTATCACTGCA
            ** **** ***** ** ** ******** ** *  ****************** ***** *
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Horse       AGAACAGCATTGCCTACCTGGACGAAGCAGCTGGCAACCTCAAGAAGGCCCTGCTCATCC
Human       AGAACAGCATTGCCTATCTGGACGAAGCAGCTGGCAACCTCAAGAAGGCCCTGCTCATCC
Mouse       AGAACAGCATCGCCTACCTGGACGAAGCGGCTGGCAACCTCAAGAAGGCCTTGCTCATCC
Rat         AGAACAGCATTGCCTACCTGGACGAAGCAGCCGGCAACCTCAAGAAGGCCTTGCTCATCC
Xenopus     AGAATAGCATTGCATTTATGGATGAAGCTTCAGGCAACCTTAAGAAGGCTGTTCTACTCC
            **** ***** ** *   **** *****  * ******** ********  * **  ***

Horse       AGGGCTCCAATGACGTGGAGATCCGGGCTGAGGGCAACAGCAGGTTCACGTATACTGTCC
Human       AGGGCTCCAATGACGTGGAGATCCGGGCAGAGGGCAATAGCAGGTTCACGTACACTGCCC
Mouse       AGGGCTCCAATGATGTAGAGATGAGGGCCGAGGGCAACAGCAGGTTCACATACACTGCCC
Rat         AGGGCTCCAATGATGTGGAGATGAGGGCCGAGGGCAACAGCAGGTTCACGTACACTGCCC
Xenopus     AAGGATCCAATGATGTAGAAATCAGAGCTGAAGGCAACAGCAGATTCACTTACAATGCCT
            * ** ******** ** ** **  * ** ** ***** ***** ***** ** * ** *

Horse       TGAAGGATGGCTGCACGAAACACACCGGTAAGTGGGGCAAGACTACGATTGAGTACCGGT
Human       TGAAGGATGGCTGCACGAAACATACCGGTAAGTGGGGCAAGACTGTTATCGAGTACCGGT
Mouse       TGAAGGATGGCTGCACGAAACACACTGGTAAGTGGGGCAAGACCGTCATCGAGTACCGAT
Rat         TGAAGGATGGCTGCACGAAACACACCGGTAAGTGGGGCAAGACCATCATCGAGTACCGAT
Xenopus     TGGAAGATGGCTGCAAGAAACACACTGGCAAATGGAGCAAGACAGTTATTGAATATAGGA
            ** * ********** ****** ** ** ** *** *******    ** ** **  *

Horse       CACAGAAGACCTCGCGCCTGCCCATCATTGACATTGCACCCATGGACATAGGAGGGCCTG
Human       CACAGAAGACCTCACGCCTCCCCATCATTGACATTGCACCCATGGACATAGGAGGGCCCG
Mouse       CACAGAAGACCTCCCGCCTTCCCATTATTGACATCGCACCCATGGACATTGGAGGGGCTG
Rat         CACAGAAGACCTCACGCCTTCCCATTGTTGACATTGCACCCATGGACATCGGAGGGCCTG
Xenopus     CACAGAAAACATCTCGCCTGCCCATCGTAGACATTGCACCTATGGATATTGGTGGCGCTG
            ******* ** ** ***** *****  * ***** ***** ***** ** ** **  * *

Horse       AGCAGGAATTTGGTGTGGACATAGGGCCCGTCTGCTTCTTGTAA----------------
Human       AGCAGGAATTCGGTGTGGACATAGGGCCGGTCTGCTTCTTGTAA----------------
Mouse       AACAGGAATTTGGTGTGGACATAGGGCCTGTCTGCTTCTTGTAAAACCCCCGAACCCTGA
Rat         ATCAGGAATTTGGTGTGGACATAGGGCCTGTCTGTTTCTTGTAAAACCCTC-AACCCCAA
Xenopus     ATCAGGAATTTGGTGTTGACATTGGCCCAGTCTGCTTCTTGTAA----------------
            * ******** ***** ***** ** ** ***** *********

Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       AACAACACAATCCATTGCGAACCCAAAGGACCCAAACACTTTCCAACCGCAGTCACTCCA
Rat         AACAACACAATCCATTGCGAACCCAAAGGACCCAAATACTTTCTAACCGCAGTCACTCTA
Xenopus     ------------------------------------------------------------

Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       GGATCTGCACTGAATGGCTGACCTGACCTGATGATACCCAACCGTCCTCCCCTCACAGCC
Rat         GGATCTGCACTGAATGGCTGACCTGACCTGA---TATTCAACTATCCTAACCTCACAGTC
Xenopus     ------------------------------------------------------------

Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       CGGACTGTGCTCCCCTTTCTAAGAGACCTGAACTGGGCAGACTGCAAAATAAAATCTCGG
Rat         CGGAC---------------------------------------------------------
Xenopus     ------------------------------------------------------------

Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       TGTTCTATTTATTTATTGTCTTCCTGTAAGACCTCTGGGTCCAGGCGGAGACAGGAACTA
Rat         ------------------------------------------------------------
Xenopus     ------------------------------------------------------------

Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       TCTGGTGTGAGTCAGACGCCCCCCGAGTGACTGTTCCCAGCCCCAGCCAGAAGACCCCTA
Rat         ------------------------------------------------------------
Xenopus     ------------------------------------------------------------
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Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    CAGATGCTGGGCGCAGGGACTGCGTGTCCTACACAATGGTGCTATTCTGTGTCAAACACC
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------

Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    TCCTGCTATTTTTTAAAACATCAATTGATATTAAAAACCAAAAAAAAAAAAAAATCATTGG
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------

Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    AAAGGATATGGTGACTTGTGTTTTGTTCTTTGTTTTGTTCTTCCGGTTGTATTTACTAGT
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------

Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    CCTTGGTTCTATAAGGCATGCCCAAATATGGTCCCAGGACTGTCTGTGGACCTTACAATC
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------

Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    CAGGATGCTGAAGTCACACTGCCTGGTTTGGTGATGTCACTCACACACACAAAAGACCTG
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------

Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    CCTCTCTTGTAAACTTGGGCACTTTGTGGAATCCACTGGCGACCAGAAATCAAACAGTAT
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------

Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    CAGCCTTGTGCTATGGAAGTGAAACACCTGACGTAGGTTACATCATCAGGAAAGCAAGGT
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------

Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    TTATTTGGTCTCATGGTTTTGAGGGCTGAAATGATGAACTAACACAGAGGAGGATCAGAA
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------

Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    AATGGCTCCTGTTTTGGAGTTTTCACAAAATTAGGTAGTTGTATTGACTTGGACCTTAGG
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------

Horse    ------------------------------------------------------------
Human    ------------------------------------------------------------
Mouse    GGAAGGCAGCAGGTCACAGCAGGAGCACAGGTCAAAGCTCATGCCCTAAGCTAGGAAGAA
Rat      ------------------------------------------------------------
Xenopus  ------------------------------------------------------------
```

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Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       GAGGAGGAGGGAGAGACCAGTGTCCTACAGACCCTTCTGAGAACCTGTCCCCGTGACCTG
Rat         ------------------------------------------------------------
Xenopus     ------------------------------------------------------------

Horse       ------------------------------------------------------------
Human       ------------------------------------------------------------
Mouse       AGGACCACCCCTAGGCCCTACCTCTCCAGGTTCCACCACCTAAGAGCATCACCCTGGGAG
Rat         ------------------------------------------------------------
Xenopus     ------------------------------------------------------------

Horse       -----------------------------------
Human       -----------------------------------
Mouse       CAAGCCTTTGAGGGACACTGCACTCAGACATGGATCC
Rat         -----------------------------------
Xenopus     -----------------------------------
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Amendments

Page 2, Line 9 Replace references with "(Recker, 1992; Price *et al.*, 1994)".
Page 36, Lines 6 and 7 Replace references with "(Langenskiöld and Edgren, 1950; Heikel, 1960; Österman, 1972; Langenskiöld anhd Österman, 1979; Langenskiöld *et al.*, 1986)".
Page 41, Paragraph 2 Insert the following at the end of the paragraph: "The positive effects of OP-1 on cartilage repair are well documented, and together with evidence that OP-1 does not have any catabolic effects (Chubinskaya and Kuettner, 2003), makes this molecule a good candidate for growth plate repair above other molecules."
Page 52, Line 13 Replace sentence with "Titanium Kirschner wires (K-wires) were inserted 20 mm apart proximally and distally to the defect, as measured using callipers (Fig. 2.1)."
Page 56, Line 14 and Page 63, Line 16 Replace "Chondroitenase ABC" with "Chondroitinase ABC".
Table 3.5 Insert "%" after each value in the table.
Page 71, Line 9 Replace reference with (Stadelmaier *et al.*, 1995).
Page 78, Lines 15 to 17 Replace sentence with "The average number of cells in the rhOP-1 treated animals was 1296.4 ± 582.2 cells/mm^2 in the resting zone, 2066.1 ± 390.1 cells/mm^2 in the proliferative zone and 1447.6 ± 265.5 cells/mm^2 in the hypertrophic zone. The average number of cells in the untreated animals was 1248.4 ± 262.0 cells/mm^2 in the resting zone, 2019.8 ± 380.5 cells/mm^2 in the proliferative zone and 1581.0 ± 466.2 cells/mm^2 in the hypertrophic zone."
Table 3.6 Insert "cells/mm^2" after all values in table.
Page 79, Line 16 Replace sentence with "The occurrence of a medial spur did not become apparent until day 56, except for a single untreated animal at day 7 (#103) that exhibited a slight tethering (Fig. 3.4B, Table 3.7)."
Page 80, Lines 18 and 19 Replace sentence with "Only one of the rhOP-1 treated animals (#122) showed collagen fibres and fibroblasts around the fat implant at day 7."
Page 81, Line 12 Replace sentence with "Due to sectioning and limited tissue availability, only 13 of the 18 animals could be analysed using this method, restricting the conclusions that could be drawn from these results (data not shown)."
Page 82, Line 17 Replace sentence with "At the time of dissection of the tibia, it was noted that a large outgrowth protruded from the bone (data not shown)."
Figures 4.2, 4.6, 4.7, 4.8, 4.12, 4.13, 4.14 and 4.15 The defect is now indicated in these figures.
Page 113, Paragraph 1 Insert the following at the end of the paragraph: "The use of a lower dose of rhOP-1 may decrease the osteogenic response of the growth plate, whereas an increase in rhOP-1 may result in the acceleration of the osteogenic phenotype."
Page 114, Paragraph 2 Insert the following at the end of the paragraph: "The use of mesenchymal stem cells in conjunction with growth factor administration may prove to be a beneficial treatment for growth plate injuries."
Page 115, Paragraph 1 Insert the following at the end of the paragraph: "The use of rhOP-1 to heal other types of growth plate injuries is limited. Growth plate injuries that require an increase in matrix synthesis (for example, after a crushing injury) may benefit from rhOP-1 treatment, however, ablation injuries requiring regeneration of the growth plate through cellular proliferation will not be assisted with rhOP-1 treatment."
Page 154, Line 8 Insert reference in list: "Stadelmaier, D.M., Arnoczky, S.P., Dodds, J. and Ross, H. (1995). The effect of drilling and soft tissue grafting across open growth plates. A histologic study. Am J Sports Med *23(4)*, 431-435.

Insert the following table after page 97

Table 4.1 Glycosaminoglycan Epitope Antibody Specificity

Antibody	Specificity	References
2-B-6	Recognises an unsaturated, disulphated disaccharide which is produced by chondroitinase digestion of native chondroitin sulphate and dermatan sulphate chains.	Caterson *et al.*, 1985; Byers *et al.*, 1992.
5-D-4	Recognises native chains of skeletal keratan sulphate.	Caterson *et al.*, 1983; Byers *et al.*, 1992.
7-D-4	Recognises an epitope within native chondroitin sulphate chains that is thought to be oversulphated and is usually destroyed by chondroitinase digestion.	Caterson *et al.*, 1990; Byers *et al.*, 1992.

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