

**A NOVEL CELL CULTURE MODEL
OF
MAMMALIAN ENDOCHONDRAL
OSSIFICATION**

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Abstract

Endochondral ossification (EO) is required for long bone formation during embryogenesis. It occurs in the growth plate where chondrocytes pass through discrete stages of proliferation, maturation, and hypertrophy, followed by the calcification and vascularisation of the extracellular matrix. While the expression of a number of novel genes has been localised in the growth plate, the molecular mechanisms that control chondrocyte differentiation in EO are not fully understood. The availability of a reliable cell culture system that recapitulates EO would facilitate greatly studies of chondrocyte differentiation. The aim of the work presented in this report was to develop and characterise a foetal bovine cell culture model of EO.

Chondrocytes derived from the epiphyses of long bones of foetal calves were treated with 15 $\mu\text{g/ml}$ of 5-azacytidine (aza-C), a potent DNA demethylating agent, for 48 hours. Cultures were maintained subsequently without aza-C for a period of 4 weeks and harvested at selected time-points for analyses of growth and differentiation status. Culturing foetal bovine epiphyseal chondrocytes at a density of 0.5×10^6 cells per cm^2 on plastic in the presence of 10% foetal calf serum (with and without aza-C) maintained a normal cell growth profile and favoured a chondrocytic phenotype as shown by the constitutive expression of type II collagen and absence of type I collagen. An extensive extracellular matrix rich in proteoglycans and collagen types II and VI was elaborated in aza-C treated and untreated cultures.

The cell culture system was characterised by studying the expression of several markers of chondrocyte differentiation. PTHrP and its receptor, both markers of maturation, were expressed in aza-C treated cultures (days 'd' 5-9). Type X collagen, which is restricted to hypertrophy, was expressed from day 11 onwards under aza-C treatment. Hypertrophy in aza-C treated cultures was confirmed by an increase in cell size reaching a 14-fold increase by day 15. A high expression of alkaline phosphatase was obtained during the hypertrophic period (d 14-28) following aza-C treatment. The pattern of expression of alkaline phosphatase coincides with the occurrence of calcification in aza-C treated cultures supplemented with calcium β -glycerophosphate (d 14-21). There is evidence for apoptosis in aza-C treated cultures after day 21 *in vitro*. The apoptosis suppressor, bcl-2, was expressed highly at days 7-12 with a slight decrease thereafter. The apoptotic inducer, bax, was not expressed until day 23 in aza-C treated cultures. The ratio of the level of expression of bcl-2 and bax showed a gradual shift in favour of bax with time in culture. The occurrence of apoptosis in aza-C treated cultures was confirmed by the characteristic presence of nuclear condensation and fragmentation (d 27-35), and the detection of exposed phosphatidylserine on the plasma membrane surface of apoptotic chondrocytes (d 26). The cell culture system was also used to define the expression of α -enolase, identified recently in the proliferative zone of the growth plate. α -Enolase was expressed highly at days 9-12 in aza-C treated cultures. In addition, exposing hypertrophic chondrocytes (initially treated with aza-C) to PTHrP (10^{-7}M) caused a marked suppression of type X collagen expression, thus showing that hypertrophy in the model is influenced by PTHrP, as is the case *in vivo*.

Taken together, these findings indicate that aza-C induced foetal bovine epiphyseal chondrocytes to differentiate in culture as *in vivo*. However, further experiments are required to confirm the validity of this cell culture system as a faithful recapitulation of EO.

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Abbreviations

A	Adenine
Ala	Alanine
AMV	Avian myeloblastosis virus
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine triphosphate
Aza-C	5-azacytidine
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
bp	Base pair
BSE	Bovine spongiform encephalopathy
C	Cytosine
Ca ²⁺	Calcium (II) ions
CaCl ₂	Calcium (II) chloride
CASP	Cartilage associated protein
CDMP	Cartilage-derived morphogenetic protein
CEC	Critical electrolyte concentrations
cDNA	Copy DNA
CD-RAP	Cartilage-derived retinoic acid-sensitive protein
cm	Centimetre
CMP	Cartilage matrix protein
CO ₂	Carbon dioxide
COMP	Cartilage oligomeric matrix protein
cpm	Counts per minute
CS	Chondroitin sulphate
DAB	3, 3'-diaminobenzidine
DAPI	4, 6-diamidino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	Diethylpyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
DNA-MeT	DNA methyltransferase
dNTP	Deoxynucleotide triphosphate
DS	Dermatan sulphate
dTTP	2'-deoxythymidine 5'-triphosphate

Abbreviations (continued)

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EO	Endochondral ossification
FACIT	Fibril-associated collagens with interrupted triple helices
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
G	Guanidine
GAG	Glycosaminoglycan
GDF	Growth differentiation factor
GH	Growth hormone
Glu	Glutamine
Gly	Glycine
HA	Hyaluronan
HCl	Hydrochloric acid
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])
H ₂ O ₂	Hydrogen peroxide
HS	Heparan sulphate
IGD	Interglobular domain
IGF	Insulin growth factor
Ihh	Indian hedgehog
IMS	Industrial methylated spirit
kDa	Kilodalton
KS	Keratan sulphate
Kv	Kilovolt
Leu	Leucine
M	Mole
MED	Multiple epiphyseal dysplasia
MEM	Minimal essential medium
mg	Milligramme
MgCl ₂	Magnesium (II) chloride
ml	Millilitre
mm	Millimetre
mM	Millimole
µg	Microgramme

Abbreviations (continued)

Mg ²⁺	Magnesium (II) ion
μl	Microlitre
μm	Micrometre (micron)
mRNA	Messenger RNA
NaCl	Sodium chloride
nm	Nanometre
MMP	Metalloproteinase
NaOH	Sodium hydroxide
ng	Nanogramme
nm	Nanometre
NP-40	Nonidet P-40
O ₂	Oxygen
°C	Degree Celsius
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Phe	Phenylalanine
PI	Propidium iodide
pmol	Picomole
PS	Phosphatidylserine
PSACH	Pseudoachondrodysplasia
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related peptide
PTR	Proteoglycan tandem repeat
RA	Retinoic acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
S	Sulphur
SAM	S-adenosylmethionine
SMCD	Metaphyseal chondrodysplasia type Schmid
T	Thymine
<i>Taq</i>	<i>Thermus aquaticus</i>
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinases
Tris	Tris[hydroxymethyl]aminomethane
V	Volt
Val	Valine

Abbreviations (continued)

v/v	Volume by volume
w/v	Weight by volume

To Mum

Chapter One

CHAPTER ONE

GENERAL INTRODUCTION

1.1. BONE AS A TISSUE

Bone is a living mineralised connective tissue with an extensive vascularisation. Its remarkable properties of hardness, resilience, and regenerative capacity make it the ideal basic component of the skeleton where it serves various essential purposes, including locomotion and protection of organs such as the brain and spinal cord by the skull and vertebral column respectively, and the heart and lungs by the ribcage. Bone is formed during the development of the embryo by the process of osteogenesis, which can occur by two mechanisms: (a) intramembranous ossification, and (b) endochondral ossification.

1.2. OSTEOGENESIS: BONE DEVELOPMENT

1.2.1. INTRAMEMBRANOUS OSSIFICATION

Intramembranous ossification is the process responsible for the formation of the flat bones of the skull and for the addition of new bone to the outer surfaces of long bones. It begins at specific centres of ossification where mesenchymal cells proliferate and condense around an extensive vascular network. These cells enlarge to become osteoblasts, which produce an organic, uncalcified matrix known as osteoid matrix. Matrix calcification rapidly follows. During this process, osteoblasts become trapped in the calcified matrix and become bone cells, called osteocytes. This sequence of matrix secretion, calcification, and entrapping of osteoblasts continues resulting in the deposition of many layers of bone. What initiates the differentiation of mesenchymal cells into osteoblasts is unknown.

1.2.2. ENDOCHONDRAL OSSIFICATION

During the early stages of embryonic development in higher vertebrates, long bone formation occurs by endochondral ossification (Figure 1.1). During this fundamental process, a cartilaginous template is first formed in the axial and appendicular skeleton of the embryo where undifferentiated mesenchymal cells initially condense and differentiate to form a core of chondrocytes. This cartilaginous model is surrounded by a highly vascular periosteum, which is composed of undifferentiated mesenchymal cells. Chondrocytes in the central part of the model enlarge considerably and become hypertrophic, secreting an extracellular matrix rich in various types of collagen. This matrix is more susceptible to vascular invasion from the periosteum. As this cartilage matrix is gradually degraded and is accompanied by the death of hypertrophic chondrocytes, osteoblasts are deposited on the partially degraded cartilage and they secrete bone matrix until all the cartilage is eventually replaced by bone. This process results in the radial growth of bone.

As the centre of the cartilage model is converted into bone, an ossification front is formed between the newly synthesised bone and the remaining cartilage, which moves towards the ends of the cartilage model, and growth plates are subsequently formed. At this stage, the lengthening bone is composed of a cylindrical shaft called the diaphysis, with dilated ends called the epiphyses (Figure 1.2). Longitudinal bone growth takes place in the narrow cartilaginous zones called the growth plates. Here, the chondrocytes enter a precisely regulated differentiation pathway which consists of distinctly different stages of development, including cellular proliferation, maturation, and hypertrophy (Poole, 1991; Brighton 1978). The hypertrophic cartilage is calcified and replaced by primary bone tissue while vascular invasion also takes place at the same time. Subsequent remodelling occurs whereby osteoblasts are deposited on the calcified cartilage scaffold where they produce osteoid (bone matrix) whereas osteoclasts are recruited to resorb bone tissue and excavate the bone marrow cavity. Consequently, primary bone is replaced by secondary bone and bone marrow, and secondary centres of ossification develop within the epiphyses later in development. A thin layer of cartilage tissue persists over the epiphyses and develops into articular

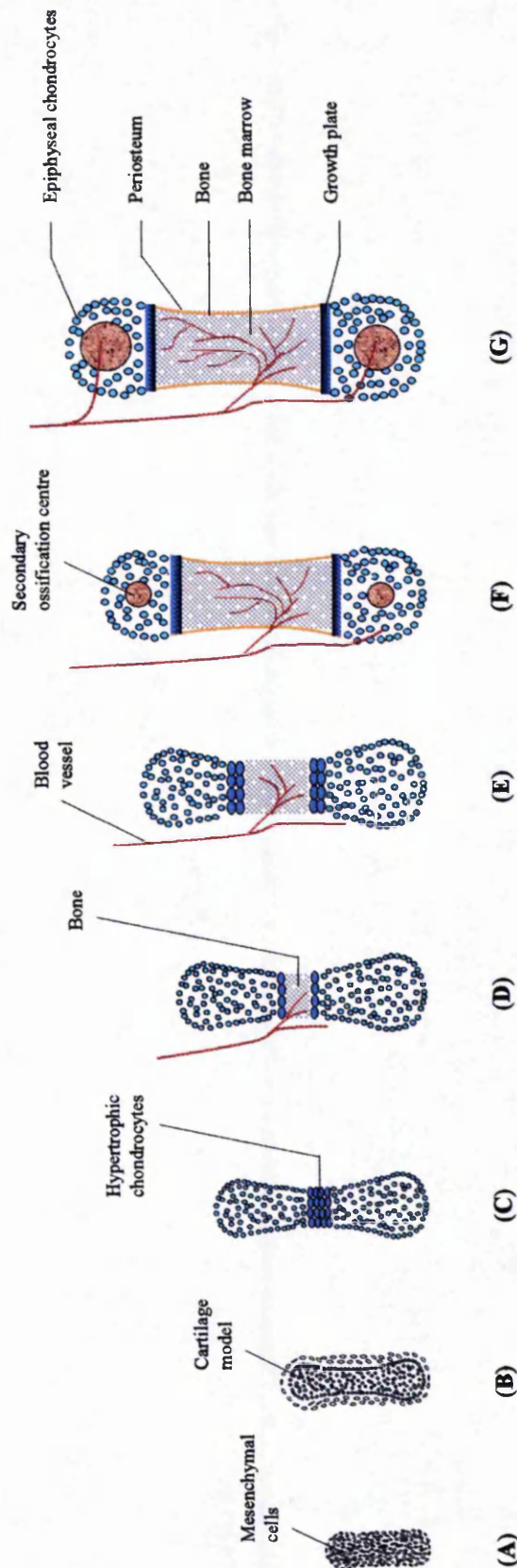


Figure 1.1. Endochondral ossification.

A, B. Undifferentiated mesenchymal cells condense to form a cartilaginous template of chondrocytes.

B, C. Chondrocytes in the centre of the cartilage model enlarge and become hypertrophic. An extracellular matrix rich in collagens is secreted, allowing blood vessels to enter.

D, E. Osteoblasts secrete bone matrix. This process is accompanied by cartilage matrix degradation and hypertrophic cell death.

F, G. Growth plates are formed which move towards the ends of the cartilage model. Chondrocyte differentiation within the growth plates leads to bone elongation. Secondary centres of ossification develop later in development and primary bone is replaced by secondary bone and bone marrow.

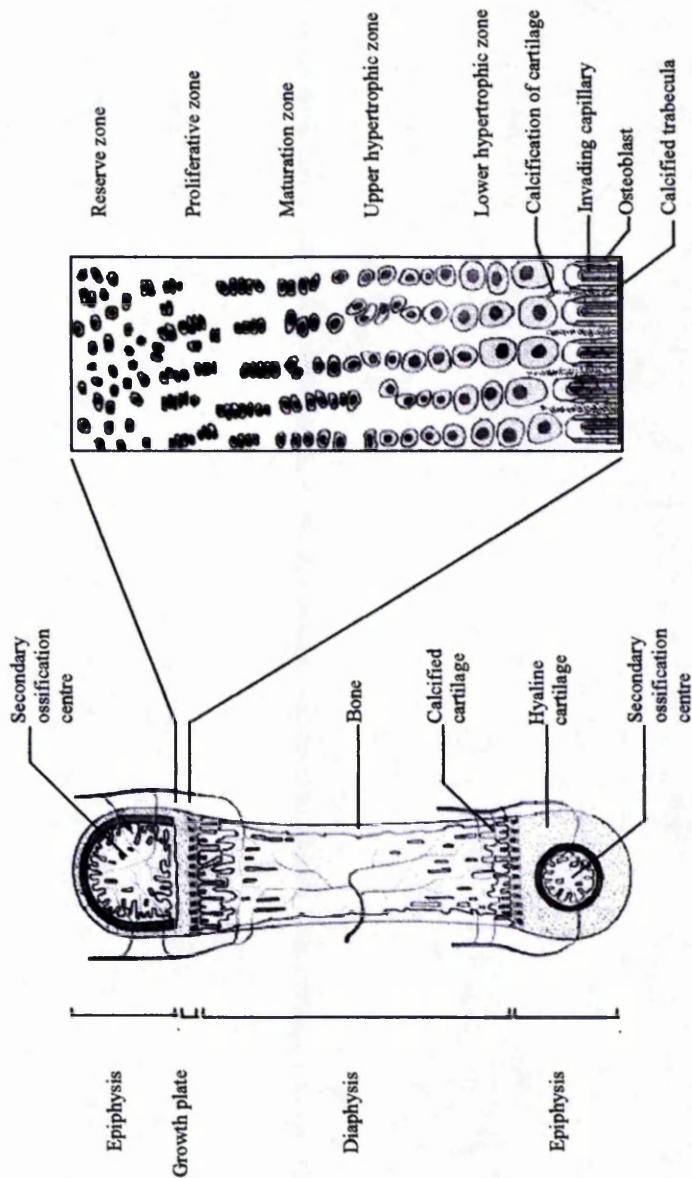


Figure 1.2. Structure of the growth plate. The left diagram shows a lengthening bone in later stages of embryonic development. Secondary centres of ossification have developed within the epiphyses and vascularisation of the calcified cartilage matrix has started. On the right is a diagrammatic representation of the growth plate showing the organisation and differentiation of chondrocytes. (*Adapted from Wallis, 1993*).

cartilage (Hunziker, 1988) to protect the epiphyseal regions from abrasive contact in the joint cavity (Price *et al.*, 1994). Endochondral ossification thus starts at embryogenesis and is completed normally at puberty with the subsequent fusion of the diaphyseal and epiphyseal bone. It is a developmentally regulated process, which allows longitudinal bone growth and it may be reactivated in cases of bone fracture repair and osteoarthritis.

1.3. THE EXTRACELLULAR MATRIX OF CARTILAGE

The extracellular matrix is the structurally stable material that surrounds cells in connective tissues. It is organised into a complex and ordered network composed of different macromolecules secreted by the cells they surround. Each type of extracellular matrix differs in amount and composition in accordance with the specific functions of the tissues. For instance, cartilage is particularly rich in proteoglycans to withstand compressive forces. Matrix assembly is important since the arrangement of the matrix constituents has a significant bearing on the physical properties of the tissues. The extracellular matrix of cartilage is composed of three major classes of macromolecules: (1) collagens, (2) proteoglycans, and (3) glycoproteins. Each class of macromolecules consists of a number of members, each differing in chemical composition, structural organisation, and physical as well as functional properties. The binding of cells to the extracellular matrix is mediated by integrins (Fernandez *et al.*, 1998). It has been shown that integrins bind to matrix components at a number of ligand binding sites, including Arg-Gly-Asp sequence of fibronectin and vitronectin, and Leu-Asp-Val-Pro sequence of fibronectin (Newham and Humphries, 1996).

One of the main functions of the extracellular matrix is to provide mechanical support and physical strength to tissues. However, whilst growth factors, hormones, vitamins, and cell-to-cell contacts are known to control the biological behaviour of the cell, the extracellular matrix is also involved in the regulation of cell growth, shape, state of differentiation, development, and metabolic responses (Adams and Watt, 1993; Blau and Baltimore, 1991). The roles played by and the interactions between both

regulatory factors such as growth factors and the extracellular matrix in the differentiation of chondrocytes in the growth plate cartilage are discussed in Section 1.6.

A rapid turnover of the extracellular matrix is important particularly in tissue regeneration and development. The extracellular matrix of cartilage during embryogenesis and early adulthood is under continuous degradation followed by its production and reassembly. During the skeletal development of the embryo, the extracellular matrix in the growth plate is degraded and reorganised continuously as the chondrocytes go through each stage in their differentiation pathway as well as during subsequent bone remodelling. The extracellular matrix is degraded mainly by a family of related enzymes known as matrix metalloproteinases (MMPs). In general, the MMPs show a certain degree of substrate specificity: the collagenases (MMP-1, -3, -13) degrade collagen types I, II, and III; the gelatinases (MMP-2, -9) act on types IV, V, VII, and X collagens; and the stromelysins (MMP-3, -10) act on proteoglycans, laminins, and fibronectin (Birkedal-Hansen *et al.*, 1993). The activity of MMPs is regulated by the presence of their inhibitors, tissue inhibitor of metalloproteinase proteins (TIMPs).

1.3.1. COLLAGENS

Collagens represent a family of proteins and are the major constituents of all extracellular matrices. They constitute collectively about thirty percent of all proteins in tissues. Collagens are structurally related molecules that contain at least one domain with a triple helical conformation (van der Rest and Garrone, 1991). However, other molecules that also contain similar triple helical domains are excluded from the collagen family because they do not participate in the assembly of the extracellular matrix. Examples include the complement component C1q (Acton *et al.*, 1993), acetylcholine esterase (Mays and Rosenberry, 1981), and the mannose-binding protein (Drickamer *et al.*, 1986). To date, nineteen distinct types of collagen have been identified (Table 1.1), each differing in its molecular structure and

TABLE 1.1
Collagens^(a)

Collagen type	Chain(s)	Molecular species	Tissue distribution	Function(s)	References
I	$\alpha 1(I)$ $\alpha 2(I)$	$[\alpha 1(I)]_2 \alpha 2(I)$ $[\alpha 1(I)]_3$	All connective tissues (e.g. skin, tendon, ligament, bone, cornea, etc) except basement membrane and hyaline cartilage	Provides support and tensile strength to connective tissues	Jimenez <i>et al.</i> , 1977
II	$\alpha 1(II)$	$[\alpha 1(II)]_3$	Hyaline cartilage, intervertebral discs, vitreous body	Provides tensile strength to tissues	Cheah <i>et al.</i> , 1991
III	$\alpha 1(III)$	$[\alpha 1(III)]_3$	Distensible connective tissue (e.g skin, vessels, lung, kidney, nerve, uterus, cardiac muscle, intestine)	Associates with type I, provides support to extensible tissues	Janecko and Ramirez, 1989
IV	$\alpha 1(IV)$ $\alpha 2(IV)$ $\alpha 3(IV)$ $\alpha 4(IV)$ $\alpha 5(IV)$ $\alpha 6(IV)$	$\alpha 1[(IV)]_2 \alpha 2(IV)$ $\alpha 3[(IV)]_2 \alpha 4(IV) (?)$ $\alpha 5[(IV)]_2 \alpha 6(IV) (?)$ $(?)$	Basement membranes	Self-assembles into a meshwork; provides scaffold for formation of, and structural support to basement membranes; also contributes to filtration property of basement membranes	Hudson <i>et al.</i> , 1993
V	$\alpha 1(V)$ $\alpha 2(V)$ $\alpha 3(V)$	$[\alpha 1(V)]_3$ $[\alpha 1(V)]_2 \alpha 2(V)$ $\alpha 1(V) \alpha 2(V) \alpha 3(V)$	Connective tissues (bone, tendon, cornea, skin, blood vessels, liver, lung, placenta) except basement membranes	Acts as small supporting pericellular fibres; Associates with type I; mixed molecule comprising both type V and XI α -chains known	Niyibizi <i>et al.</i> , 1984
VI	$\alpha 1(VI)$ $\alpha 2(VI)$ $\alpha 3(VI)$	$\alpha 1(VI) \alpha 2(VI) \alpha 3(VI)$	Practically all connective tissues (cartilaginous and non-cartilaginous) except basement membranes	Maintains structural integrity of connective tissue by linking cells to their surrounding matrix	Bonaldo <i>et al.</i> , 1990
VII	$\alpha 1(VII)$	$[\alpha 1(VII)]_3$	Epidermis, intestinal submucosa, oral mucosa, cervix	Anchors epithelial basement membrane to underlying stromal tissue	Burgeson <i>et al.</i> , 1990
VIII	$\alpha 1(VIII)$ $\alpha 2(VIII)$	$[\alpha 1(VIII)]_2 \alpha 2(VIII)$	Descemet's membrane, endothelial cells	Unknown (hexagonal network in Descemet's membrane)	Muragaki <i>et al.</i> , 1992
IX	$\alpha 1(IX)$ $\alpha 2(IX)$ $\alpha 3(IX)$	$\alpha 1(IX) \alpha 2(IX) \alpha 3(IX)$	Hyaline cartilage, intervertebral discs, vitreous humour	Associates with type II; allows formation of type II fibrils and may regulate fibril diameter; may also link type II fibrils with cartilage proteoglycans	Wu <i>et al.</i> , 1992
X	$\alpha 1(X)$	$[\alpha 1(X)]_3$	Hypertrophic mineralising cartilage during endochondral ossification	Associates with type II in growth plate during endochondral bone formation; may provide mechanical support to hypertrophic chondrocytes	Schmid and Linsenmayer, 1987
XI	$\alpha 1(XI)$ $\alpha 2(XI)$ $\alpha 3(XI)$	$\alpha 1(XI) \alpha 2(XI) \alpha 3(XI)$	Hyaline cartilage, intervertebral disc	Forms heterotypic fibrils with types II and IX; may regulate fibril diameter; mixed molecules comprising both type V and XI α -chains known	Sandberg <i>et al.</i> , 1993
XII	$\alpha 1(XII)$	$[\alpha 1(XII)]_3$	Embryonic tendon and skin, periodontal ligament, cartilage	Associates with type I, possibly mediates interactions of different extracellular matrix components	Watt <i>et al.</i> , 1992
XIII	$\alpha 1(XIII)$	$(?)$	Endothelial cells, skin, intestine, placenta, bone, cartilage growth plate, striated muscle, hair follicles, bone marrow mesenchymal cells	Membrane-intercalated	Sandberg <i>et al.</i> , 1989

TABLE 1.1 (continued)
Collagens^(a)

<i>Collagen type</i>	<i>Chain(s)</i>	<i>Molecular species</i>	<i>Tissue distribution</i>	<i>Function(s)</i>	<i>References</i>
XIV	$\alpha 1(\text{XIV})$	$[\alpha 1(\text{XIV})]_3$	Foetal skin and tendon, developing cartilage	Possibly similar functions to type XII	Watt <i>et al.</i> , 1992
XV	$\alpha 1(\text{XV})$	(?)	Fibroblasts, smooth muscle	Unknown	Myers <i>et al.</i> , 1992
XVI	$\alpha 1(\text{XVI})$	$[\alpha 1(\text{XVI})]_3$	Heart and lung fibroblasts, keratinocytes, endothelial cells in smooth muscles, placenta	Unknown	Pan <i>et al.</i> , 1992
XVII	$\alpha 1(\text{XVII})$	$[\alpha 1(\text{XVII})]_3$	Dermal-epidermal junction of skin	Connects basal cells to stroma; membrane-intercalated; component of hemidesmosomes	Li <i>et al.</i> , 1993c
XVIII	$\alpha 1(\text{XVIII})$	(?)	Vascularised tissues (lung, liver, kidney)	Unknown	Oh <i>et al.</i> , 1994
XIX	$\alpha 1(\text{XIX})$	(?)	Low expression in cultured skin fibroblasts and tumour cells	Unknown	Inoguchi <i>et al.</i> , 1995

^(a) Adapted from Ayad *et al.*, (1998), Comper (1996), and Haralson and Hassell (1995).

supramolecular assembly (Bateman *et al.*, 1996; van der Rest and Garrone, 1991). Such variations in the molecular structure and organisation of collagens reflect largely the physical properties and functions of the tissues in which they are found. For example, tendon is rich in type I collagen fibres which are aligned into a rope-like structure, thus allowing the tissue to withstand large tensional forces with minimal deformation.

1.3.1.1. THE COLLAGEN TRIPLE HELIX

The basic structural unit of a collagen molecule is a right-handed triple helix, consisting of three polypeptide chains twisted around each other in a rope-like fashion (Traub and Piez, 1971). Each polypeptide chain has a left-handed helical conformation with a characteristic amino acid sequence that contains the triplet repeat sequence Gly-X-Y (Ramachandran and Ramakrishnan, 1976). Glycine represents about thirty-three percent of the total amino acids per collagen molecule whereas approximately twenty percent of X and Y residues are proline and hydroxyproline, respectively. Since every third residue of each polypeptide chain resides in the centre of the triple helix, this residue must be glycine as it is the only residue with a side chain small enough to be accommodated into the crowded centre of the helix. The bulky rings of proline and hydroxyproline residues are located on the outside of the helix.

The collagen molecule undergoes extensive post-translational modification including hydroxylation of proline and lysine, glycosylation of hydroxylysine, and disulphide bond formation between the C-terminal ends of every three polypeptide chains prior to the assembly of the triple helix. The triple helical conformation is stabilised by hydrogen bonds holding the three polypeptide chains together. The hydroxyl group of hydroxyproline participates extensively in hydrogen bonding (Grant and Prockop, 1972). In addition, stable covalent intramolecular and intermolecular cross-links are formed, involving lysine and hydroxylysine (Kielty *et al.*, 1993). The triple helix acts as a molecular rod that physically separates globular domains in the collagen

molecule. The existence of the triple helical structure allows the various collagen types to perform different functions to meet the specialised needs of tissues.

1.3.1.2. SUPRAMOLECULAR ASSEMBLIES OF COLLAGENS

Collagens can be classified according to their molecular structures and the supramolecular arrangements they form (van der Rest and Garrone, 1991): (1) fibrillar collagens (types I, II, III, V, and XI), (2) fibril-associated collagens with interrupted triple helices or FACITs (types IX, XII, and XIV), (3) sheet-forming collagens (types IV, VIII, and X), (4) collagen forming beaded filaments (type VI), and (5) collagen forming anchoring fibrils (type VII). The molecular structures and aggregates of these collagens are depicted schematically in figure 1.3. In this report, the principal collagens present in cartilage are described, namely types II, IX, X, and XI. Type VI collagen, which is not specifically found in cartilage, is also discussed since its expression is investigated in this report.

1.3.1.2.1. **Type II collagen**

Type II collagen is the major component of the extracellular matrix of cartilage. It consists of three genetically identical $\alpha 1(\text{II})$ chains. It is classified as a fibrillar collagen since it participates in the formation of “quarter-staggered” fibrils (Figure 1.3). Type II collagen is composed of long uninterrupted triple helical domains. In its supramolecular assembly, the molecules align in a parallel fashion but are staggered by about 67 nm (or D period), giving rise to regions of maximal density (overlaps) and regions with holes (gaps) (Brodsky and Eikenberry, 1982). In cartilage, type II collagen forms heterotypic fibrils with collagen types IX and XI. The heterotypic assembly of fibrils is discussed in Sections 1.3.1.2.1 and 1.3.1.2.2.

Type II collagen is synthesised as a triple-helical procollagen, which is processed extracellularly to give a triple helical molecule that can participate in the “staggered” arrangement. Type II procollagen can be expressed in two forms by differential splicing of the primary gene transcript: type IIA procollagen, which includes, and type






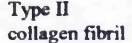














Collagen type	Molecule	Aggregate
<i>Collagens forming "quarter-staggered" fibrils</i>		
I, II, III, V, XI		
<i>Fibril-associated collagen with interrupted helices (FACITs)</i>		
IX, XII, XIV	<div>Types XII & XIV</div>  <div>Type IX</div>  <div>Type IX</div>  <div>Type II collagen fibril</div> 	
<i>Collagens forming sheets</i>		
IV		<div>Monomer</div>  <div>Dimer</div>  <div>Tetramer ("spider")</div> 
VIII, X		<div>Hexagonal lattice</div> 
<i>Collagen forming beaded filaments</i>		
VI		<div>Monomer</div>  <div>Dimer</div>  <div>Tetramer</div>  <div>Beaded filament</div> 
<i>Collagen forming anchoring fibrils</i>		
VII		<div>Dimer</div>  <div>Anchoring fibril</div> 

Figure 1.3. Collagen molecular structures and supramolecular assemblies. This figure shows schematic representations of molecules and aggregates of the existing collagen types, wherever known. The triple helical domains are represented by black lines while the non-triple helical domains are drawn as blue lines or red circles. (Adapted from van der Rest and Garrone, 1990).

IIB procollagen which excludes, exon 2 (Sandell *et al.*, 1991). It has been found that type IIB is expressed in differentiated chondrocytes while type IIA is localised in non-cartilaginous tissues such as the skin and brain, and in chondroprogenitor cells (Oganesian *et al.*, 1997, 1996; Sandell *et al.*, 1991). Such a differential expression during embryogenesis implies a potential difference in the function of the two procollagen types. These findings also suggest that the exclusion of exon 2, which encodes part of the amino-propeptide of type II collagen, may be important in the process of chondrogenesis.

It is well established that the main function of type II collagen in cartilage is to provide tensile strength, thus maintaining the structural integrity of the tissue. Mutations within the helical domain caused a disruption in the assembly of the homotrimeric collagen molecule with a severe disorganisation of the growth plate, a marked reduction in the proliferative zone, and lethality (Metsaranta *et al.*, 1992). Equally important is the balance among the constituents of cartilage collagen fibrils, namely type II, IX, and XI collagens, to ensure correct heterotypic fibril formation, which can otherwise cause lethality at birth (Garofalo *et al.*, 1993). Moreover, it was shown that chondrocytes died by apoptosis in transgenic mice lacking type II collagen (Yang *et al.*, 1997), which shows that the expression of type II collagen in cartilage is essential for the viability of chondrocytes. During endochondral ossification (EO), type II collagen is degraded by collagenase in the hypertrophic zone of the growth plate (Alini *et al.*, 1992; Dean *et al.*, 1989). The exact significance of such a cleavage is still unknown, but it is possible that a disruption of the collagen network may help remove constraints on cell swelling that occurs during hypertrophy (Alini *et al.*, 1992). In addition, type II collagen has been implicated in the initiation of cartilage mineralisation since it has been shown to be attached to matrix vesicles, stimulating the uptake of calcium ions (Ca^{2+}) into the vesicles (Kirsch *et al.*, 1994).

Type II collagen mutations have been implicated in various hereditary skeletal disorders characterised by an abnormal EO (Horton and Hecht, 1993). It is believed that any interruption in the Gly-X-Y repeat sequence within the triple helical domain

leads to very severe and often lethal consequences, such as achondrogenesis type II (Mortier *et al.*, 1995), and hypochondrogenesis (Bogaert *et al.*, 1992). In contrast, moderate disease phenotypes such as spondyloepiphyseal dysplasia congenita (Tiller *et al.*, 1995) and Kniest dysplasia (Weis *et al.*, 1998), are caused by mutations leading to alterations in the length of the α -chains.

1.3.1.2.2. Type IX collagen

Type IX collagen belongs to the family of fibril-associated collagens with interrupted triple helices (FACITs) (van der Rest and Garrone, 1991) and is a minor component of hyaline cartilage. It comprises three genetically distinct polypeptide chains designated $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ (van der Rest *et al.*, 1985). The structure of the type IX collagen molecule is depicted in figure 1.4 (Abedin *et al.*, 1982; Ayad *et al.*, 1981; Shimokomaki *et al.*, 1980). It should be noted that type IX collagen in the cornea and vitreous lacks the globular NC4 domain (Bishop *et al.*, 1992; Svoboda *et al.*, 1988) due to the use of an alternative promoter and transcription sites in the $\alpha 1(\text{IX})$ gene (Nishimura *et al.*, 1989). The unique feature of type IX collagen is that it exists both in a proteoglycan and a non-proteoglycan form in cartilage (Ayad *et al.*, 1991; Bruckner *et al.*, 1988).

Due to its structural properties including its proteoglycan character, type IX collagen has been regarded as the ideal mediator of interactions between type II collagen and the components of the surrounding cartilage (Bruckner *et al.*, 1988). The current model proposed for the association of type IX collagen with type II collagen fibrils is depicted in figure 1.4. There is also evidence showing the existence of intermolecular bonding between type IX molecules (Wu *et al.*, 1992). It is believed that the function of type IX collagen is to bridge collagen fibrils and other matrix components, thereby conferring resistance to the swelling of the collagen network and maintaining mechanical stability in cartilage. The pivotal role of type IX collagen in skeletal development is demonstrated by the occurrence of multiple epiphyseal dysplasia as a result of a mutation in the *COL9A2* gene (Muragaki *et al.*, 1996). An abnormal structure of type IX collagen was associated with diastrophic dysplasia

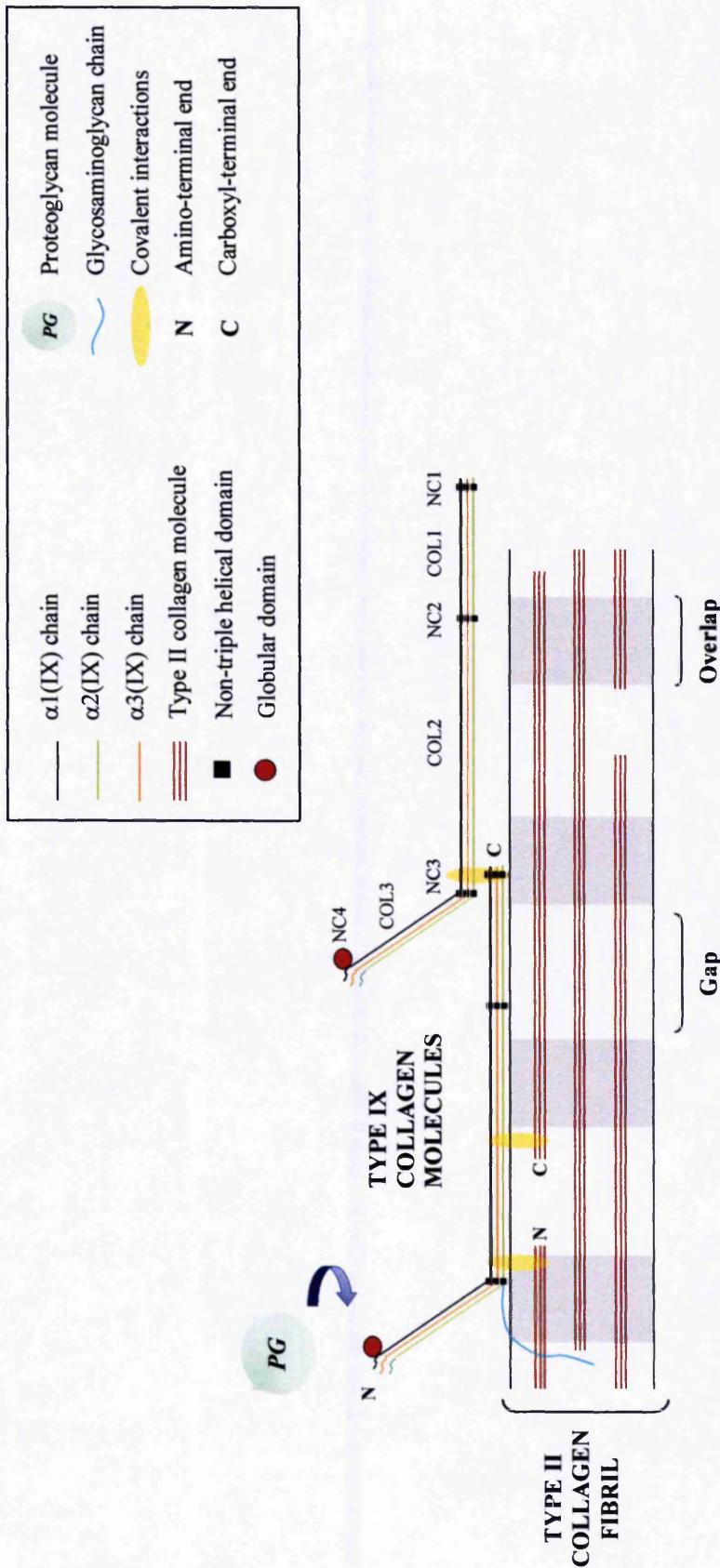


Figure 1.4. Schematic representation of the association of type IX collagen with type II collagen fibril. Type IX collagen is composed of three genetically distinct chains, $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$, and $\alpha 3(\text{IX})$. Type IX collagen is believed to decorate the surface of the type II collagen fibril (Muller-Glauser *et al.*, 1986) in an antiparallel fashion (Wu *et al.*, 1992). It is thought that the glycosaminoglycan chain attached to the NC3 domain of the $\alpha 2(\text{IX})$ chain targets the type IX molecule to the correct site for cross-linking to type II collagen fibril by fitting in the gap zone of the fibril (Eyre *et al.*, 1987). The large globular NC4 domain is contributed mainly by the $\alpha 1(\text{IX})$ chain, and possibly binds proteoglycans due to its basic nature (Vasios *et al.*, 1988). There is also evidence for the intermolecular association between type IX molecules (Wu *et al.*, 1992) (Adapted from Diab *et al.*, 1996 and Nishimura *et al.*, 1989).

characterised by short-limbed dwarfism and spinal deformation (Diab *et al.*, 1994). In this case, collagen II fibres were abnormally thicker, which supports the hypothesis that type IX collagen is important in regulating the diameter of collagen fibres.

1.3.1.2.3. Type XI collagen

Type XI collagen is a fibrillar collagen found predominantly in cartilage, although also expressed in non-cartilaginous tissues such as the kidney, skin, and muscle (Lui *et al.*, 1995; Sandberg *et al.*, 1993). Type XI collagen is composed of three polypeptides, namely $\alpha 1(XI)$, $\alpha 2(XI)$, and $\alpha 3(XI)$ chains (Morris and Bachinger, 1987). While $\alpha 1(XI)$ and $\alpha 2(XI)$ chains are encoded by *COL11A1* and *COL11A2* genes respectively, the $\alpha 3(XI)$ chain is encoded by the *COL2A1* gene, but differs from the $\alpha 1(II)$ chain by being overglycosylated (Furuto and Miller, 1983).

It is well established that type XI collagen participates in the formation of heterotypic fibrils together with collagen types II and IX (Mendler *et al.*, 1989). There is also evidence for collagen types XI and IX as well as XI and II covalent interactions (Eyre and Wu, 1995; Mendler *et al.*, 1989). It has been shown that type XI collagen associates almost exclusively with thin fibrils in cartilage (Keene *et al.*, 1995). This observation led to the suggestion that type XI collagen may perform a role in controlling fibril diameter (Li *et al.*, 1995). It was found that a loss of type XI collagen in the cartilage matrix as a result of a deletion mutation in one of the genes for type XI collagen in *cho* mice caused death at birth with abnormally thick collagen fibrils (Li *et al.*, 1995).

Mutations in type XI collagen have been found to cause skeletal disorders, including Stickler syndrome (Richards *et al.*, 1996; Vikkula *et al.*, 1995) and Marshall syndrome, a rare autosomal dominant disease characterised by craniofacial abnormalities and deafness (Griffith *et al.*, 1998). In all the above cases, it has been proposed that the mutant type XI collagen were formed with an incorrect triple helical structure. These findings support the hypothesis that in addition to its role in

maintaining the structural integrity of cartilage, type XI collagen also plays an essential role in skeletal development.

1.3.1.2.4. Type X collagen

Type X collagen has been classified as a member of the family of collagen forming sheets (van der Rest and Garrone, 1991). It is a short chain collagen with a distribution restricted to the hypertrophic zone of the growth plate during endochondral bone formation (Schmid and Linsenmayer, 1987). Type X collagen comprises three identical $\alpha 1(X)$ chains. Each chain consists of three domains: a short non-helical region at the amino-terminus (NC2), a short central triple-helical region with several imperfections in the Gly-X-Y repeat structure, and a large globular domain at the carboxyl-terminus (NC1) (Schmid *et al.*, 1984). The supramolecular structure formed by type X collagen has not been fully resolved (Figure 1.3). Immunoelectron microscopic studies have shown type X collagen organised as pericellular mats of filaments surrounding hypertrophic chondrocytes (Schmid and Linsenmayer, 1990), while it has also been localised in association with type II collagen fibrils (Poole and Pidoux, 1989). These observations suggest that newly synthesised type X collagen molecules diffuse from the surface of chondrocytes into the extracellular matrix to bind to preexisting collagen fibrils (Chen *et al.*, 1990). In addition, type X collagen molecules aggregate to form hexagonal lattice structures *in vitro* (Kwan *et al.*, 1991), possibly via their NC1 globular domains (Barber and Kwan, 1996). The possible functions and importance of type X collagen in the growth plate cartilage during endochondral ossification are discussed in Section 1.4.2.4.1.

1.3.1.2.5. Type VI collagen

Type VI collagen is present in the extracellular matrix of virtually all connective tissues, including cartilage (Timpl and Engel, 1987). It is composed of three genetically distinct α -chains, namely $\alpha 1(VI)$, $\alpha 2(VI)$ and $\alpha 3(VI)$ chain (Ayad *et al.*, 1989; Trueb and Winterhalter, 1986). Heterotrimeric monomers associate in an anti-parallel manner to form dimers, which align laterally to form tetramers. In turn, the

tetramers aggregate in an end-to-end fashion to form multimers, bringing the globular domains in close contact (Figure 1.3). This organisation results in the formation of the so-called beaded filaments, which consist of alternating filamentous and beaded regions (Engvall *et al.*, 1986). It is still not clear how the supramolecular assemblies are stabilised. There is some evidence that interactions between the terminal globular domains may play a role since these domains have been found to have a high affinity for each other as well as for the triple helical domains (Kuo *et al.*, 1995).

It is generally believed that type VI collagen is involved mainly in maintaining the structural integrity of the connective tissue by linking the cells to their surrounding matrix (Bonaldi *et al.*, 1990). Several studies have demonstrated interactions between type VI collagen and other extracellular matrix components, including collagen fibrils (Keene *et al.*, 1998), decorin (Bidanset *et al.*, 1992), and hyaluronan (Kielty *et al.*, 1992). Electron microscopy showed type VI collagen anchored to the chondrocyte membrane as well as linked to the surrounding collagen network in the chondron (Poole *et al.*, 1992). This observation suggests that type VI collagen plays a role in stabilising the various components of the extracellular matrix in the pericellular microenvironment, thereby maintaining the integrity of the chondron. Mutations affecting either the triple-helical domain (Jobsis *et al.*, 1996) or the globular domain (Pan *et al.*, 1998) of type VI collagen cause Bethlem myopathy, which is characterised by multiple joint contractures, muscular weakness and wasting. These findings support the critical role of type VI collagen in cell attachment with the extracellular matrix.

1.3.2. PROTEOGLYCANS

Proteoglycans represent a diverse family of molecules characterised by a protein core to which is bound covalently one or more glycosaminoglycan (GAG) side chains. The GAG chains include four types: (1) chondroitin sulphate (CS), (2) dermatan sulphate (DS), (3) heparan sulphate (HS), and (4) keratan sulphate (KS). Hyaluronan is another important GAG, but unlike the other GAG molecules, it is not sulphated

and is not attached covalently to protein. All these molecules consist characteristically of a disaccharide repeat unit, which (except for KS) is composed of a hexuronate and a hexosamine (Table 1.2). The large structural diversity of proteoglycans arises from variations in the core protein, and the type, number and size of GAG chains attached. This structural diversity leads to a wide variety of biological functions of proteoglycans. In cartilage, the major proteoglycans in the extracellular matrix include aggrecan, decorin, and biglycan. Hyaluronan is also a crucial constituent of the cartilage matrix. Due to their high content of sulphate and carboxylate groups, GAGs bear a high negative charge and are very acidic and therefore, have a high affinity for a large variety of ligands. In addition, the hydrophilicity of the GAG chains confers strong water-binding properties on them. Proteoglycans contribute considerably to cartilage hydration, thus providing major resistance to compressive forces that arise, particularly during chondrocyte maturation and mechanical loading.

1.3.2.1. AGGRECAN

Aggrecan is the most abundantly expressed proteoglycan in the extracellular matrix of cartilage. It is a large molecule comprising several domains, as shown in figure 1.5. The primary role of aggrecan in cartilage is to swell and hydrate the network of collagen fibrils, allowing cartilage to withstand compressive load with minimal tissue deformation. Aggrecan plays an essential role in the organisation of cartilage matrix during skeletal development. A complete absence of the aggrecan core protein in the homozygote *cmd* mice caused cartilage matrix deficiency (*cmd*) characterised by skeletal deformities and death at birth (Watanabe *et al.*, 1994).

Aggrecan exists in cartilage as multimolecular aggregates composed of aggrecan monomers non-covalently bound to hyaluronan and stabilised by link protein (Faltz *et al.*, 1979) (Figure 1.5). The short IGD domain separating G1 and G2 contains sites for the proteolytic cleavage of aggrecan, which occurs during extracellular matrix turnover and cartilage degradation (Fosang *et al.*, 1993, 1992, 1991). The enzyme with “aggrecanase” activity in cartilage is still unknown. A recent report showed the

TABLE 1.2
Glycosaminoglycans

Glycosaminoglycan	Repeating disaccharide unit	Glycosidic linkage within disaccharide unit	Linkage to core protein	Position of sulphate substitution on sugar residue(s)
Chondroitin sulphate	Glucuronate <i>N</i> -acetylglactosamine	β 1-3	<i>O</i> -linked to serine	4 (chondroitin-4-sulphate) 6 (chondroitin-6-sulphate) (both on <i>N</i> -acetylglactosamine residue)
Dermatan sulphate	Glucuronate/ Iduronate <i>N</i> -acetylglactosamine	α 1-3	<i>O</i> -linked to serine	4, 6 (as in chondroitin sulphate) 2 (iduronate residue)
Heparan sulphate	Glucuronate <i>N</i> -acetylglucosamine	β 1-4	<i>O</i> -linked to serine	6 (<i>N</i> -acetylglucosamine residue) <i>N</i> -linked (<i>N</i> -acetylglucosamine residue) <i>O</i> -linked (glucuronate residue)
Keratan sulphate	Galactose <i>N</i> -acetylglucosamine	β 1-4	<i>O</i> -linked to serine / threonine (cartilage) <i>N</i> -linked to asparagine (cornea)	6 (either or both sugar residues)
Hyaluronan	Glucuronate <i>N</i> -acetylglucosamine	β 1-3	—	—

Note. Dermatan sulphate is a structural isomer of chondroitin sulphate in which some glucuronate residues are epimerised to iduronate. Heparan sulphate contains a proportion of its *N*-acetyl groups removed and replaced by sulphates, thus giving rise to *N*-sulphated glucosamines. The glucuronate residues in either heparan sulphate or heparin can also undergo epimerisation to iduronate, which can be 2-sulphated. Unlike the other glycosaminoglycans, hyaluronan is neither linked to any core protein nor sulphated.

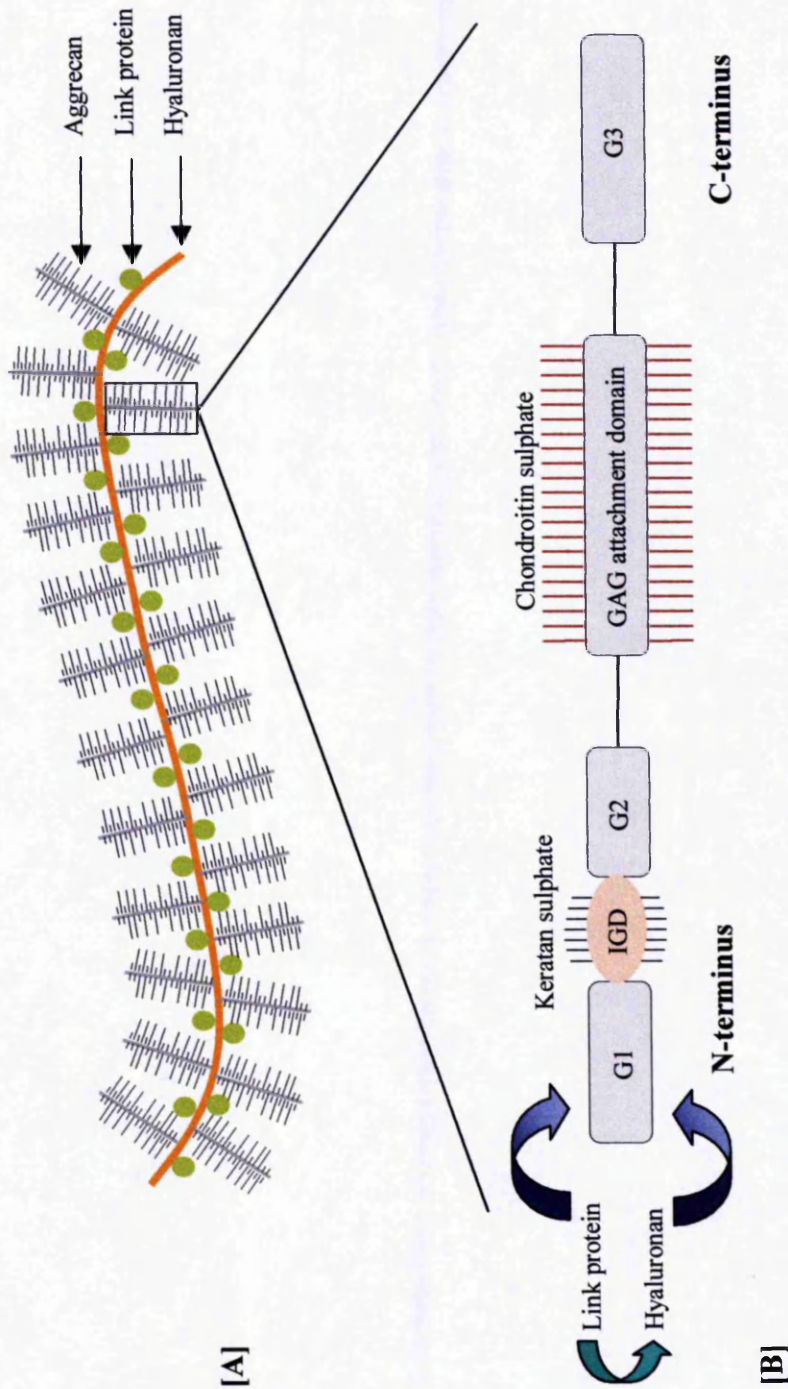


Figure 1.5. Aggrecan and its interaction with hyaluronan in cartilage.

- A. Aggrecan exists in cartilage as a component of large multimolecular aggregates composed of aggrecan monomers bound non-covalently to hyaluronan (about ten to hundred proteoglycan monomers per hyaluronan molecule). This interaction is stabilised by link protein. The high concentration of negative charges in aggrecan, coupled with the large size and polyanionic nature of hyaluronan, endows the aggregates with strong water-binding properties. Together with hyaluronan, aggrecan contributes to swell and hydrate the collagen network in cartilage.
- B. Aggrecan has a multidomain structure with three globular domains (G1-3), a glycosaminoglycan (GAG) attachment domain, and an extended interglobular (IGD) domain. Interaction with hyaluronan and link protein occurs via the G1 domain, which contains distinct binding sites to these two molecules. Link protein also contains a binding site specific to hyaluronan. The function of the G2 domain is unknown. It is possible that the G2 domain is involved in the intracellular synthesis and maturation of aggrecan. There is evidence that the IGD domain contains site(s) for the proteolytic cleavage of aggrecan.

possible involvement of a member of the ADAM (a disintegrin and metalloproteinase) family in the proteolytic cleavage of aggrecan in cartilage (Tortorella *et al.*, 1998). There is evidence that the C-terminal G3 domain may be involved in the intracellular synthesis and maturation of aggrecan. A mutation in the GAG region results in the synthesis of truncated aggrecan lacking G3 (Li *et al.*, 1993b), which fails to be translocated from the endoplasmic reticulum to the Golgi (Vertel *et al.*, 1994).

1.3.2.2. HYALURONAN

Unlike the other GAGs, hyaluronan (HA) is not sulphated and is not attached covalently to any peptide *in vivo*. It is a large linear molecule with an exceptionally high molecular weight of millions (Fraser and Laurent, 1996). HA assumes an open, random coil configuration that occupies large solvent volumes, thus giving rise to solutions with high viscosity. HA molecules are able to self-associate and form extensive meshworks with considerable elasticity. Since HA bears a high negative charge, it can adsorb large amounts of water. Together with aggrecan, HA contributes to swell and hydrate the collagen network in cartilage in order to minimise mechanical deformation of the tissue. In addition, it was found that hypertrophic chondrocytes in the growth plate secrete large amounts of HA, which led to the proposal that HA may play a role in the expansion of hypertrophic lacunae (Pavasant *et al.*, 1996a, b).

1.3.2.3. DECORIN AND BIGLYCAN

Decorin and biglycan are members of the family of leucine-rich proteoglycans called small chondroitin sulphate/dermatan sulphate (CS/DS) proteoglycans (Fisher *et al.*, 1989). Their main characteristic is their ability to control matrix assembly and cell growth by interacting with extracellular matrix and cell surface proteins (Iozzo and Murdoch, 1996). For example, decorin has been shown to bind collagen types II and VI (Bidanset *et al.*, 1992; Vogel *et al.*, 1984). Despite their structural homologies and expression in the same tissues, decorin is found predominantly in the extracellular matrix while biglycan is localised mainly in the pericellular environment. Decorin is thought to play a key role in the regulation of collagen fibrillogenesis (Brown and

Vogel, 1989; Vogel *et al.*, 1984). There is strong evidence that decorin is essential for the orderly assembly of collagen fibrils during development as shown by the abnormal development of fibril structures and skin fragility in transgenic mice harbouring a targeted disruption of decorin (Danielson *et al.*, 1997). Little is known about the roles of biglycan *in vivo*. A low expression of biglycan was found in Turner's syndrome characterised by a short stature while its overexpression was detected in Klinefelter syndrome characterised by an excessive growth and a tall stature (Geerkens *et al.*, 1995). In addition, studies *in vitro* suggested that biglycan may be involved in the regulation of mineralisation (Boskey *et al.*, 1997). This notion is supported by the reduced cartilage mineralisation obtained in biglycan knock-out mice (Boskey *et al.*, 1998).

1.3.3. OTHER COMPONENTS OF THE EXTRACELLULAR MATRIX

1.3.3.1. CARTILAGE MATRIX PROTEIN

Cartilage matrix protein (CMP) is a non-collagenous protein found mostly in the extracellular matrix of cartilage. The precise function of CMP is still unknown. There is some indication that it may interact and bridge type II collagen fibrils (Winterbottom *et al.*, 1992). In addition, CMP has been localised in the maturation zone of the growth plate, which led to the suggestion that it may be considered as a specific marker of the maturation stage of differentiation of chondrocytes during endochondral ossification (Chen *et al.*, 1995). CMP is discussed further in Section 1.4.5.1.

1.3.3.2. CARTILAGE OLIGOMERIC MATRIX PROTEIN

A novel protein has been identified recently in bovine cartilage where it occupies preferentially the territorial matrix surrounding the chondrocytes and has been called cartilage oligomeric matrix protein (COMP) (Ekman *et al.*, 1997; Hedbom *et al.*, 1992). COMP is a member of the thrombospondin family of extracellular calcium-binding proteins. Although the role of COMP is still unknown, several lines of

evidence show its importance in skeletal growth and development. Mutations in the *COMP* gene give rise to human skeletal abnormalities, including pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) (Loughlin *et al.*, 1998; Susic *et al.*, 1997; Briggs *et al.*, 1995). All mutations in the *COMP* gene have been found within the calmodulin-like domain of COMP, thereby possibly altering the protein conformation and calcium-binding property of COMP (Ballo *et al.*, 1997).

1.3.3.3. MATRIX GLA PROTEIN

Matrix gla protein is a member of the Gla protein family characterised by their ability to bind mineral and mineral ions such as calcium, phosphate, and hydroxyapatite crystals through γ -carboxyglutamic acid residues (Gla) (Dowd *et al.*, 1995). Other members of this family include osteocalcin, coagulation factors VII and IX, and anti-clotting factors protein S and C (Ducy *et al.*, 1996; Furie *et al.*, 1988). Studies have suggested a possible function of matrix gla protein as a regulator of calcification of extracellular matrix (Luo *et al.*, 1995). Mice deficient in matrix gla protein developed to term but died within two months as a result of blood vessel rupture caused by arterial calcification (Luo *et al.*, 1997). It was found that these mice also exhibited an inappropriate calcification pattern in the growth plate (from the proliferative zone to the lower hypertrophic zone) leading to a disorganised chondrocyte column structure and short stature. These results strongly suggest that matrix gla protein is responsible for inhibiting calcification of soft tissues such as arteries and cartilage, probably through its mineral ion-binding affinity. More recently, it was found that mutations in human matrix gla protein leading to an absent or non-functional protein, caused Keutel syndrome, a disorder characterised by abnormal cartilage calcification, peripheral pulmonary stenosis, and midfacial hypoplasia (Munroe *et al.*, 1999). This finding further supports the importance of matrix gla protein in matrix calcification.

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1.4. THE CHONDROCYTE, THE GROWTH PLATE, & THE DIFFERENTIATION PATHWAY

1.4.1. THE CHONDROCYTE

As mentioned previously, the differentiation of mesenchymal cells in the limb bud during endochondral ossification (EO) gives rise to a set of differentiated cells known as chondrocytes. The simplest definition of a chondrocyte is that of a cell which is embedded in a matrix of cartilage (Cancedda *et al.*, 1995). The chondrocyte is a highly specialised cell and plays a pivotal role, being the “sole architect of cartilage” (Muir, 1995). Not only is it the cell that becomes committed to the differentiation pathway in the growth regions of the bone to eventually allow longitudinal bone growth but it is also responsible for producing, maintaining, and influencing the extracellular matrix of the cartilage tissue. Chondrocytes can be distinguished from each other on the sole basis of their differential morphology and biochemistry at different stages of their development during EO.

The chondrocyte is a highly differentiated cell with remarkable properties and capabilities. It can survive under extremely low oxygen tensions. Even under aerobic conditions, it undergoes preferentially anaerobic respiration, metabolising glucose by glycolysis to produce lactate (Muir, 1995). The major function of the chondrocyte is to produce extracellular matrix of cartilage. Aggregates of aggrecan and hyaluronan, stabilised by link protein, inflate the collagen fibrillar network, thereby enabling cartilage to resist compressive forces. In contrast, the collagen fibrils provide the tissue with strength to resist tensile forces. In adult articular cartilage, the cells are sparsely distributed and hence, become isolated from their neighbours. This cell distribution is in contrast to the majority of cells found in other tissues where cell-to-cell contact is essential for cell survival.

Under normal conditions, chondrocytes cease to undergo cell division at sexual maturity. However, cell replication and increased matrix synthesis resume when the collagenous network of the cartilage matrix is damaged as in the early stages of

osteoarthritis, a major disease of diarthrodial joints. Studies of the cellular basis of EO, including the development of the chondrocyte, are therefore important for our better understanding of joint diseases such as osteoarthritis.

1.4.2. THE GROWTH PLATE & THE DIFFERENTIATION PATHWAY

The cartilaginous epiphysis is separated from the bony diaphysis by the growth plate, which is responsible for longitudinal bone growth (Figure 1.2). Within the growth plate, chondrocytes are organised into axial columns, which are separated from one another by longitudinal septa. In contrast, horizontal septa separate individual chondrocytes within a column. The chondrocytes progress sequentially through a series of well-defined differentiation stages, thus dividing the growth plate into distinct cellular zones, each corresponding to their respective stage of chondrocyte development: (i) the reserve or resting zone, (ii) the proliferative zone, (iii) the maturation zone, and (iv) the hypertrophic zone (Figure 1.6). Each of these differentiation stages is characterised by cell shape, cell size, the proliferation status of the chondrocyte, the range of biosynthetic products made, and by quantitative and qualitative changes in the composition and structure of the extracellular matrix produced by the chondrocytes. The height of the growth plate is maintained by cellular proliferation on the epiphyseal margin, which is balanced by cellular loss on the diaphyseal margin. Therefore, it is evident that EO is a process that is under strict spatial and temporal regulation because it determines both the rate and extent of growth of long bones.

1.4.2.1. THE RESTING STAGE

Chondrocytes in the resting zone of the growth plate undergo little or no cell division (Kember, 1960). Nearly spherical in shape, the resting chondrocytes are sparsely situated being interspersed by an extracellular matrix rich in type II collagen and aggrecan. They occur either singly or in pairs. There has been some evidence suggesting that the role of resting chondrocytes could be to act as a lipid store for use

THE MAMMALIAN
GROWTH PLATE

Stage of differentiation	Cell shape / cell size	Expression of specific Markers of differentiation	Composition of the extracellular matrix
Resting	Spherical / small (sparsely distributed)	<input type="checkbox"/> Decorin <input type="checkbox"/> Fibroblast growth factor receptor-3	<input type="checkbox"/> Collagen types II, IX, XI <input type="checkbox"/> Aggrecan-hyaluronan complexes
Proliferation	Flattened (high proliferation rate)	<input type="checkbox"/> <i>bcl-2</i> <input type="checkbox"/> <i>c-myc</i>	Same as in the resting stage
Maturation	Rounded morphology	<input type="checkbox"/> <i>bcl-2</i> <input type="checkbox"/> Cartilage matrix protein <input type="checkbox"/> Cartilage-derived morphogenetic protein <input type="checkbox"/> Indian hedgehog <input type="checkbox"/> Parathyroid hormone-related peptide <input type="checkbox"/> PTHrP receptor	Same as in the resting stage
Hypertrophy	Spherical / fully enlarged (5 to 10-fold increase in size)	<input type="checkbox"/> Alkaline phosphatase <input type="checkbox"/> <i>bcl-2</i> (except in terminal hypertrophy) <input type="checkbox"/> Cartilage-associated protein <input type="checkbox"/> Collagenase (elevated expression) <input type="checkbox"/> Galectin <input type="checkbox"/> Stromelysin <input type="checkbox"/> Tissue inhibitor of metalloproteinases (decreased expression) <input type="checkbox"/> Transglutaminase	<input type="checkbox"/> Type X collagen (high expression) <input type="checkbox"/> Extensive cleavage of type II collagen <input type="checkbox"/> Proteoglycan degradation

Figure 1.6. The differentiation pathway of chondrocytes in the mammalian growth plate. The growth plate is divided into distinct cellular zones, each corresponding to their respective stage of chondrocyte development. Chondrocytes at each stage differ in their physical and biosynthetic characteristics as shown in the table above.

at subsequent stages in the differentiation pathway (Brighton, 1978; Brighton *et al.*, 1973).

1.4.2.2. PROLIFERATION

Chondrocytes progress from the resting to the proliferative zone where they undergo very active cell division with a marked increase in their metabolic rate and synthetic function. The cells continue to synthesise an extracellular matrix consisting mainly of aggrecan-hyaluronan complexes stabilised by link protein, and a fibrillar network of collagens, composed of collagen types II, VI, IX, and XI (Mayne, 1989). As a result of an intensive cell division, the chondrocytes are no longer spherical in shape, but acquire a flattened morphology, most probably due to the increased longitudinal compressive forces that result from the stacking of the proliferating cells (Poole, 1991). In the mammalian growth plate, long columns of flattened chondrocytes are formed (Poole *et al.*, 1989). It is known that in any particular organism, all growth plates, including those at different ends of the same long bone, exhibit different rates of elongation at any point in time until the end of EO (Farnum, 1994). It is well established that in mammals, the rate and extent of chondrocytic hypertrophy correlates with the rate of bone elongation (Hunziker, 1994; Breur *et al.*, 1991). It was demonstrated recently that proliferating chondrocytes from growth plates elongating at different rates have different cell cycle times, suggesting strongly that cell proliferation is also a controlling factor in the differential growth of long bones (Wilsman *et al.*, 1996). After a finite number of cell divisions, the chondrocytes pass through the maturation stage.

1.4.2.3. MATURATION

In the maturation zone, the chondrocytes start to enlarge and acquire a more rounded morphology (Poole, 1991). This zone is clearly and easily identifiable as the maturing chondrocytes are sandwiched between the flattened chondrocytes in the proliferative zone and the considerably bigger cells in the hypertrophic region. In addition, the specific expression of three proteins, cartilage matrix protein (CMP), indian hedgehog

(Ihh), and parathyroid hormone-related peptide (PTHrP), distinguish the cells of the maturation zone from those of the proliferative and hypertrophic zones. CMP is discussed below and, PTHrP and Ihh are discussed in Sections 1.6.7 and 1.6.8, respectively.

1.4.2.3.1. Cartilage Matrix Protein

Recent studies carried out by Chen *et al.* (1995a) on chick embryonic tibiotarsal chondrocytes demonstrated the existence of a zone in the epiphyseal growth plate that is very distinct from the proliferative and hypertrophic zones, indicating that the latter two zones are not contiguous with each other. The expression of cartilage matrix protein (CMP) in this particular zone of the growth plate supported the idea that the transition from chondrocyte proliferation to hypertrophy is clearly separated by an additional developmental stage called maturation, as proposed earlier by other groups (Stocum *et al.*, 1979; Kim and Conrad, 1977). This model is in contrast to that proposed by Castagnola *et al.* (1988), who suggested that in the chick system, there is no intermediate stage between proliferation and hypertrophy in the differentiation programme of chondrocytes. According to their model, chondrocytes proceed to hypertrophy immediately after proliferation. The finding of a specific marker for the maturation stage tends to confirm the validity of the "Chen model". However, other studies on chick embryos showed the existence of CMP in the resting as well as in the proliferative zones (Murotaglu *et al.*, 1995). These observations suggest that CMP may not be a marker molecule that is strictly specific to the maturation zone in the growth plate, although it still remains that the maturation stage separates chondrocyte proliferation from hypertrophy (Poole, 1991). In addition, a possibly new marker for the zone of maturation, histidine phosphatase of the endoplasmic reticulum-1 (HiPER1) has been identified recently in the zone of transition between proliferation and hypertrophy (Romano *et al.*, 1998; Reynolds *et al.*, 1996), but its function is still unknown and remains to be characterised.

1.4.2.4. HYPERTROPHY

In the hypertrophic stage of differentiation, chondrocytes continue to increase in size and become more spherical in shape than the cells in the other zones of the growth plate. All hypertrophic cells, including the very terminal ones, are rounded and fully hydrated (Farnum *et al.*, 1990). A positive linear relationship has been found between the rate of bone elongation in growing mammals and the hypertrophic chondrocyte volume, which suggests strongly that chondrocyte hypertrophy is a major determinant in the control of the longitudinal bone growth in mammals (Barreto and Wilsman, 1994). The hypertrophic region of the growth plate can be divided into two zones: the upper hypertrophic zone and the lower hypertrophic zone.

In the upper hypertrophic zone, chondrocytes enlarge five- to ten-fold in size but the mechanisms involved are unclear. Buckwalter *et al.* (1986) found that the principal mechanism of cell enlargement is swelling and fluid accumulation in the cytoplasm and nucleus although an increase in the synthesis of organelles also contributes to cellular enlargement. It has been reported recently that hypertrophic chondrocytes also secrete large amounts of hyaluronan which may contribute to the enlargement of hypertrophic lacunae (Pavasant *et al.*, 1996a). Due to its large size and high negative charge, hyaluronan can adsorb large amounts of water, thereby exerting a hydrostatic pressure on the surrounding matrix, and consequently causing the hypertrophic lacunae to expand (Laurent and Fraser, 1992). It has been shown previously that the volume change of the hypertrophic lacunae is a major factor controlling the rate of longitudinal bone growth (Breur *et al.*, 1991).

In addition, hypertrophic chondrocytes produce an extracellular matrix that differs markedly from that found in other regions of the growth plate. There is an extensive cleavage of type II collagen (Buckwalter *et al.*, 1986), possibly as a result of an elevated concentration of collagenase coupled with a low expression of collagenase inhibitor, tissue inhibitor of metalloproteinases (TIMP) in the hypertrophic zone (Dean *et al.*, 1989; Dean *et al.*, 1985). It has been suggested that type II collagen degradation may help to favour cell enlargement during hypertrophy by reducing

physical constraints on cell swelling (Alini *et al.*, 1992). High expression of metalloproteinases and stromelysin leads to an increased degradation of proteoglycans (Mikuni-Takagaki and Cheng, 1987; Galloway *et al.*, 1983), which are known to inhibit calcification (Chen and Boskey, 1985; Dziewiatkowski and Majznerski, 1985). Proteoglycan breakdown is essential for cartilage mineralisation to occur subsequently (Kawabe *et al.*, 1986). Nurminskaya and Linsenmayer (1996) have identified recently an increased expression of transglutaminase and galectin in the hypertrophic zone. Transglutaminase is thought to participate in modulating the cytoskeleton of chondrocytes as they change shape and enlarge and to alter the extracellular matrix by cross-linking matrix components for subsequent calcification (Nurminskaya and Linsenmayer, 1996). Galectin belongs to a class of lectins involved in crosslinking carbohydrate chains on cell surfaces in the extracellular matrix (Barondes *et al.*, 1994). Another novel protein, cartilage-associated protein (CASP), has been reported to be highly expressed exclusively in the hypertrophic zone although the significance of its accumulation is still unknown (Castagnola *et al.*, 1997).

1.4.2.4.1. Type X Collagen

The most dramatic change in the matrix secreted during hypertrophy is possibly the synthesis of type X collagen. As described in Section 1.3.1.2.4, type X collagen is restricted to the hypertrophic zone of the growth plate (Kirsch and von der Mark, 1991; Capasso *et al.*, 1982; Gibson *et al.*, 1982). Type X collagen has also been found recently in mineralising adult thyroid cartilage (Claassen and Kirsch, 1994) and in regenerating deer antler (Price *et al.*, 1996).

Various studies have shown that type X collagen is localised pericellularly in a capsule-like structure (Lu Valle *et al.*, 1992; Schmid and Linsenmayer, 1990) as well as in the extracellular matrix where it is associated primarily with type II collagen (Schmid and Linsenmayer, 1990; Poole and Pidoux, 1989). The latter observation suggests a possible mechanical function for type X collagen whereby it supplements the properties of type II collagen fibrils and provides mechanical support to hypertrophic chondrocytes. The role of type X collagen in strengthening the cartilage

is further supported by the findings of Rucklidge *et al.* (1996) who reported recently the non-uniform distribution of type X collagen in articular cartilage. It is therefore possible that type X collagen is associated with load-bearing properties in different regions of the articular surface. However, the precise role of type X collagen in EO still remains to be elucidated. It has been implicated in the process of calcification since its synthesis always precedes cartilage mineralisation (Kirsch and von der Mark, 1992). Type X collagen has been reported to be associated with matrix vesicles (Habuchi *et al.*, 1985), which are the primary nucleation sites in cartilage calcification (Anderson, 1969). In addition, the vitamin D-deficient condition of rickets, characterised by the absence of cartilage mineralisation and skeletal growth impairment, is accompanied by a marked decrease in type X collagen synthesis (Kwan *et al.*, 1989). However, conflicting evidence has been presented showing that type X collagen was neither concentrated in focal calcification sites nor was it associated with matrix vesicles (Poole and Pidoux, 1989).

Type X collagen has been implicated in pathological conditions such as metaphyseal chondrodysplasia type Schmid (SMCD) (Wallis *et al.*, 1994; Warman *et al.*, 1993) and osteoarthritis (von der Mark *et al.*, 1992; Hoyland *et al.*, 1991). SMCD is a skeletal disorder characterised by a short stature and abnormal limb development (Wallis *et al.*, 1994; Warman *et al.*, 1993). All the mutations identified in SMCD patients are within the carboxy-terminal NC1 domain (Wallis *et al.*, 1996; 1994). It is thought that these mutations may prevent the correct trimerisation of the molecule since trimer formation is initiated at the carboxy-terminal end of the protein (Warman *et al.*, 1993). Surprisingly, it was found that type X collagen-knock-out mice showed no abnormality in long bone formation and development, which suggests that type X collagen is not required for EO (Rosati *et al.*, 1994). However, a recent study using type X collagen-deficient mice revealed the development of phenotypic changes similar to SMCD (Kwan *et al.*, 1997). Interestingly, the hypertrophic zone of the growth plate remained intact while the other zones were affected, as indicated by a reduction in thickness of the resting zone and an abnormal distribution of proteoglycans and matrix vesicles in the growth plate. These findings led to the

proposal that type X collagen is responsible for the proper compartmentalisation of matrix components to the hypertrophic zone of the growth plate by trapping the components in its pericellular network (Kwan *et al.*, 1997). An abnormal distribution of these components in type X collagen deficiency alters the supporting properties of the growth plate as well as the process of cartilage mineralisation.

1.4.2.5. CARTILAGE MINERALISATION AND VASCULARISATION

Chondrocyte hypertrophy is also marked by the onset of cartilage mineralisation, which occurs in the lower hypertrophic zone of the growth plate. Calcification of the extracellular matrix occurs only in the longitudinal septa, which separate chondrocyte columns from one another (Hunziker, 1988). Various studies have shown a high calcium content in upper hypertrophic chondrocytes (Hargest *et al.*, 1985; Arsenis, 1972) whereas there is very little or no calcium in the lower hypertrophic cells due to loss of mitochondrial calcium as the mineral is deposited in the growth plate. The mechanism of calcification is still not fully understood and it is known that abnormal cartilage calcification is associated with many pathological conditions. It is generally well accepted that matrix vesicles serve as the initial site of calcification during EO (Anderson, 1995). These are small vesicular structures that are formed as a result of a budding process from the plasma membrane of chondrocytes (Anderson, 1995). Matrix vesicles are known to accumulate calcium ions (Ca^{2+}) needed for calcification. It has been reported that this Ca^{2+} influx is activated by interactions between the matrix vesicles and collagen types II and X (Kirsch and Wuthier, 1994). Matrix vesicles are also rich in annexin V (Genge *et al.*, 1989). Annexin V is a membrane-bound protein that has been shown to mediate the influx of Ca^{2+} into matrix vesicles (Kirsch and Wuthier, 1994; Kirsch *et al.*, 1994; Rojas *et al.*, 1992). Matrix vesicles also have a high alkaline phosphatase content (de Bernard *et al.*, 1986; Ali *et al.*, 1970), which confirms the major role of matrix vesicles in calcification. Alkaline phosphatase is an enzyme that liberates inorganic phosphate from organic or inorganic substrates. It is thought that alkaline phosphatase releases inorganic phosphate from acidic phospholipids present in the calcifying matrix (Boskey *et al.*, 1980) and also from pyrophosphate, which is an inhibitor of calcium phosphate formation (Anderson

and Reynolds, 1973). Inorganic phosphate reacts with calcium to give a form of calcium phosphate called hydroxyapatite, which is used for subsequent mineral crystal formation. Alkaline phosphatase is expressed very highly in the hypertrophic zone and its concentration in matrix vesicles can be considered as a means of providing high local concentrations of inorganic phosphate needed for mineral formation (Kirsch *et al.*, 1997). A deficiency of alkaline phosphatase has been shown to lead to hypophosphatasia, characterised by a lack of cartilage and bone mineralisation (Ornoy *et al.*, 1985), thus confirming its essential role in mineralisation.

Cartilage mineralisation is accompanied by the formation of blood capillaries (angiogenesis) and subsequent vascular invasion whereby blood vessels enter the uncalcified last transverse septa separating the hypertrophic zone from the metaphysis (Arsenault, 1987; Schenk *et al.*, 1967). The calcified cartilage matrix acts as a scaffold for osteoblasts, which synthesise woven bone. It is believed that angiogenesis is induced by angiogenic molecules, such as basic fibroblast growth factor, transforming growth factor- β (Gelb *et al.*, 1990; Jingushi *et al.*, 1989), and endothelial cell-stimulating factor (Brown *et al.*, 1987), which act as potential chemoattractants for endothelial cells. Vascular invasion only occurs after hypertrophy (Floyd *et al.*, 1987). Although poorly understood, angiogenesis in the cartilage is essential for normal skeletal growth, and an uncontrolled angiogenesis can cause several diseases, including rheumatoid arthritis and osteoarthritis (Colville-Nash and Scott, 1992; Brown and Weiss, 1988).

1.5. THE FATE OF TERMINAL HYPERTROPHIC CHONDROCYTES

The fate of hypertrophic chondrocytes when they reach the terminal layer of the hypertrophic zone in the growth plate is still under intense debate. Numerous investigations have been carried out to determine whether these terminal hypertrophic chondrocytes ultimately die, and if this is the case, to find the mechanism of cell death, or whether they remain viable and eventually become a source of

osteoprogenitor cells. Since terminal hypertrophic chondrocytes are located at the interface between cartilage and bone, it is crucial to study the ultimate fate of these cells to gain a better understanding of the mechanisms controlling the conversion of calcified cartilage to bone at the chondro-osseous junction.

1.5.1. APOPTOSIS

It was previously hypothesised that terminal hypertrophic chondrocytes eventually die as a result of cellular disintegration caused by metabolic stagnation and depletion of nutrients and oxygen availability (Brighton *et al.*, 1973), possibly due to an extensive calcification of the surrounding matrix. This hypothesis seemed plausible due to the presence of a low oxygen tension in the distal hypertrophic zone (Brighton *et al.*, 1982) and decreased diffusion of nutrients through the calcified matrix (Stambaugh and Brighton, 1980). However, with the development of new and improved techniques for tissue and cell fixation and visualisation, it has become clear that hypertrophic chondrocytes, including the very terminal ones at the last transverse septum, are rounded, fully hydrated, and metabolically active (Farnum *et al.*, 1990). It was observed that in addition to being fully hydrated with intact plasma membranes, terminal hypertrophic chondrocytes also occurred as condensed cells with their plasma membranes attached asymmetrically to the last transverse septum. This observation led to the hypothesis that chondrocyte death occurs by the process of apoptosis, prior to cartilage resorption and vascular invasion (Farnum and Wilsman, 1987). In addition to ultrastructural evidence of apoptotic cell death in the terminal hypertrophic zone, morphological and biochemical evidence has also been presented, including retraction from the pericellular matrix, cytoplasmic and nuclear condensation, and DNA fragmentation, all being characteristic of apoptosis (Zenmyo *et al.*, 1996; Wyllie, 1987b).

Although it is widely accepted that the terminal hypertrophic chondrocytes die by apoptosis, it is still unclear how this process is initiated. It has been shown both *in vivo* and *in vitro* that cartilage resorption fails to occur in the absence of metaphyseal

vasculature (Trueta and Amato, 1960) or vascular agents arising from vascular cells (Gibson *et al.*, 1995). These observations led to the suggestion that apoptosis may be influenced by direct contact with invading vascular cells or agents. Changes in cell-matrix interactions have also been implicated as mediators of apoptosis, as terminal hypertrophic cells retract from the pericellular matrix (Frisch and Francis, 1994; Meredith *et al.*, 1993). Gibson *et al.* (1997) showed that hypertrophy itself is the initiator of apoptosis and put forward the following hypothesis: commitment of chondrocytes to hypertrophic differentiation leads to commitment of the cells to apoptosis and conversely, an inhibition of the hypertrophic phenotype results in the suppression of apoptosis. In addition, the cell survival gene, *bcl-2* (Boot-Handford *et al.*, in press; Hillarby *et al.*, 1996), and the cell death gene, *bax* (Amling *et al.*, 1997), have been localised in the growth plate. Their distributions in the growth plate support the mechanism of cell death by apoptosis in the terminal hypertrophic zone. The importance and roles of *bcl-2* and *bax* in the control of apoptosis in the growth plate is discussed further in Chapter five.

1.5.2. OSTEOBLAST DIFFERENTIATION

Other research groups have argued that the terminal hypertrophic chondrocytes do not die but give rise to osteoblasts. It is generally well accepted that most bone-forming cells are derived from marrow stromal cells (Beresford, 1989). However, it has been postulated frequently that hypertrophic chondrocytes may also differentiate ultimately into bone-forming cells (Moskalewski *et al.*, 1989; Shimomura *et al.*, 1975; Crelin and Koch, 1967). Several studies carried out by different groups demonstrated that hypertrophic chondrocytes may also express markers common to bone cells, including osteocalcin (Lian *et al.*, 1993), osteopontin (Castagnola *et al.* 1991), bone sialoprotein (Chen *et al.*, 1991a), osteonectin (Pacifci *et al.*, 1990), and a switch from collagen types X and II to type I (Descalzi-Cancedda *et al.* 1992). However, it would be difficult to extend these observations obtained *in vitro* to the events that occur *in vivo*, although there is some evidence that such a transition may be possible *in vivo* at the chondro-osseous junction of long bones (Galotto *et al.*, 1994).

1.5.3. TRANSDIFFERENTIATION

Another concept was proposed (Roach, 1997; Roach *et al.*, 1995), which seems to reconcile the two opposing views of the fate of terminal hypertrophic chondrocytes. These studies involved the induction of osteogenic differentiation of hypertrophic chondrocytes by cutting through the hypertrophic cartilage of embryonic chick femurs and then culturing the explants. It was proposed that terminal hypertrophic chondrocytes undergo asymmetric division whereby one daughter cell dies by apoptosis while the other daughter cell remains viable and gives rise to progeny that differentiates into osteogenic cells. Furthermore, Roach *et al.* (1995) argued that osteogenic differentiation of hypertrophic chondrocytes occurs by *transdifferentiation*, i.e. the cells suddenly switch from a chondrogenic to an osteogenic phenotype. This mechanism differs from the gradual and continuous differentiation towards the osteogenic phenotype suggested by others (Gentili *et al.*, 1993; Descalzi-Cancedda *et al.*, 1992). However, it still remains to be defined whether cutting through the hypertrophic cartilage actually simulates the same environment surrounding the chondrocytes at the time of cartilage matrix resorption *in vivo*. It is equally essential to determine whether observations of hypertrophic chondrocytes undergoing osteogenic differentiation in long-term chondrocyte cultures are relevant to the situation *in vivo*.

1.6. REGULATION OF CHONDROCYTE DIFFERENTIATION IN THE GROWTH PLATE

The differentiation of chondrocytes within the growth plate is under strict temporal and spatial control and it is clear that such a highly regulated process is under the influence of autocrine, paracrine, and endocrine factors and their complex interactions with one another. Due to the relative avascularity of growth plate cartilage, the maintenance of chondrocyte viability and their differentiation status depends on the local production of growth factors by the cells themselves and also on the distribution of diffusible factors such as systemic hormones from external sources. Little is known about the precise roles of those regulatory factors and their intricate interplay

in the control of the developmental programme of chondrocytes in the growth plate. However, it is undoubtedly clear that an aberrant expression of these molecules leads to abnormal endochondral ossification (EO), which in turn causes severe pathological conditions of the skeleton.

Three main classes of peptide growth factors have been associated with growth and chondrocyte differentiation within the epiphyseal growth plate leading to endochondral bone formation: the transforming growth factor-beta family (TGF- β), including bone morphogenetic proteins (BMPs) and cartilage-derived morphogenetic proteins (CDMPs); the fibroblast growth factor family (FGFs); and the insulin-like growth factors (IGFs). Other regulatory factors that play a role in chondrocyte differentiation include growth hormone (GH), parathyroid hormone (PTH) and its homologue, parathyroid hormone-related peptide (PTHrP); retinoic acid (RA); epidermal growth factor (EGF); and thyroid hormones. The main functions of these regulatory factors are summarised in figure 1.7.

1.6.1. TRANSFORMING GROWTH FACTOR-BETA

Transforming growth factor-beta (TGF- β) is a disulphide-linked dimer that is found in a wide variety of tissues, including platelets and tumour cells. The most common mammalian isoforms are TGF- β_1 , TGF- β_2 and TGF- β_3 . The differential functions of these various isoforms on the skeletal system are still unknown (Centrella *et al.*, 1994). High levels of TGF- β have been found in the growth plate, epiphysis, and metaphysis of human and chick long bones (Thorp *et al.*, 1992; Sandberg *et al.*, 1988). The mechanism of induction of TGF- β production by differentiating chondrocytes is still uncertain, although it has been shown that fibroblast growth factor stimulates TGF- β production *in vitro* (Gelb *et al.*, 1990). TGF- β is known to be involved in cartilage development as shown by its ability to induce chondrogenesis *in vitro* in the embryonic chick system (Kulyk *et al.*, 1989). In addition, there is a low expression and an altered localisation of TGF- β in the chick growth plate in rachitic conditions where chondrocyte differentiation ceases (Thorp and Jakowlew, 1994). It

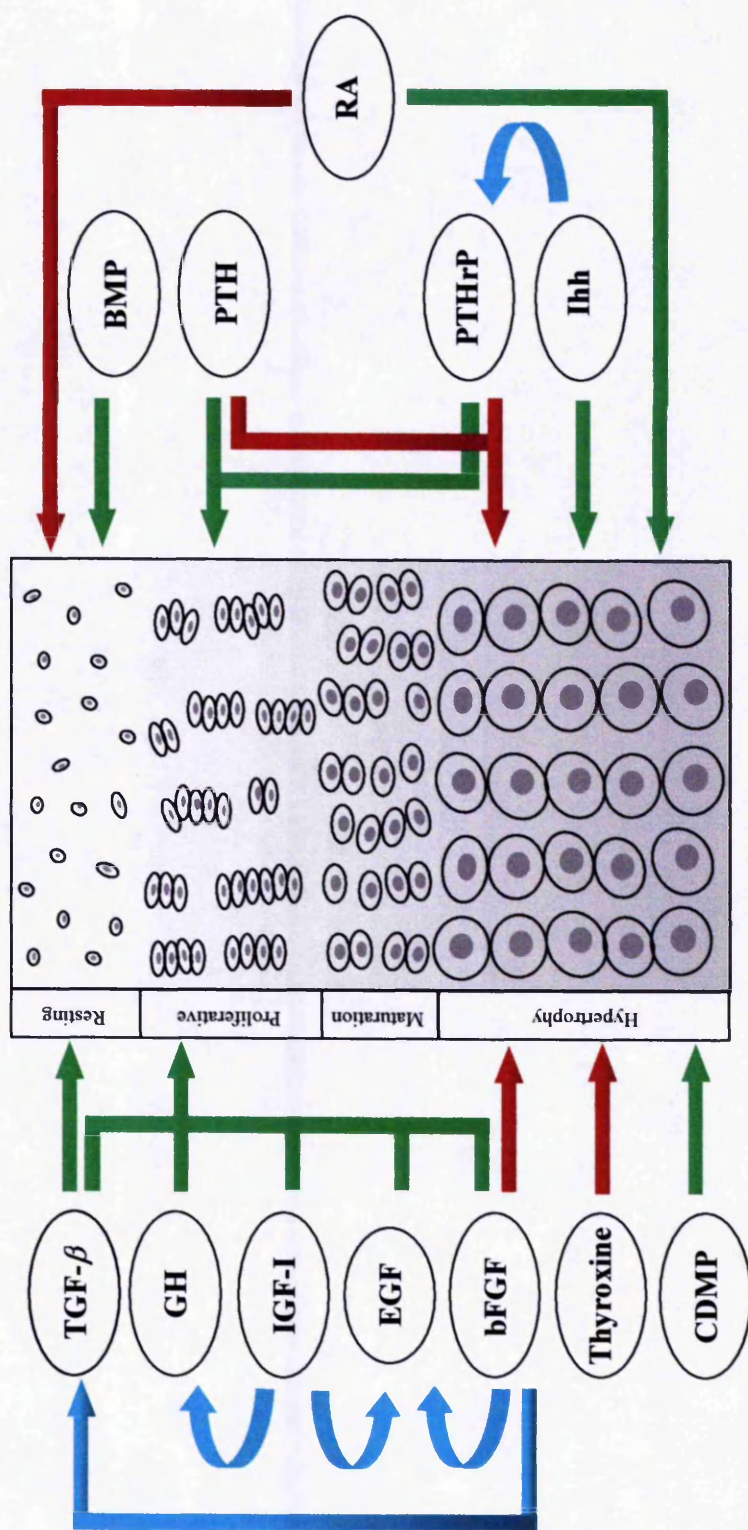


Figure 1.7. Regulation of chondrocyte differentiation in the growth plate. The differentiation of chondrocytes in the growth plate is controlled both spatially and temporally during endochondral ossification. As shown above, the control of chondrocyte differentiation is under the influence of autocrine, paracrine, and endocrine factors, as well as their complex interactions with one another. A green arrow indicates the stimulatory action of a regulatory factor while the inhibitory effect is represented by a red arrow. Any synergistic action between regulatory factors is shown by a blue arrow. It should be noted that most evidence for the effects of these factors has been obtained from experiments *in vitro*.

has been shown to induce chondrocyte proliferation in culture and its effect is enhanced by acting synergistically with basic fibroblast growth factor (Schofield and Wolpert, 1990).

The precise role of TGF- β in chondrocyte differentiation is still unclear. Conflicting findings have been reported on its effect in chondrocyte differentiation. TGF- β has been reported to prevent chondrocytes from reaching terminal hypertrophy by stabilising the prehypertrophic phenotype in three-dimensional pellet cultures of rat epiphyseal chondrocytes (Ballock *et al.*, 1993) and in high-density agarose suspension cultures of chick chondrocytes (Tschan *et al.*, 1993). On the other hand, de Angelo and Pacifici (1997) showed that TGF- β does not seem to be responsible for inhibiting chick chondrocyte maturation to hypertrophy *in vitro* and is more likely to be important in the terminal post-hypertrophic stage of differentiation where it is highly expressed. Such an apparent discrepancy in the effects of TGF- β can be accounted for partly by the critical dependence of the growth factor on a number of parameters including the type of target cell studied, for example rat, human, or chick cell; the stage of differentiation of the target cell; the environmental context of the cell as defined by the cell culture system such as suspension, pellet or monolayer cultures; cell density; growth factor concentration, timing and length of exposure; presence or absence of serum; and presence of other regulatory factors (Flaumenhaft and Rifkin, 1992). The difficulty encountered in attempting to define the functional roles of TGF- β and its mechanisms of actions lies in the choice of *in vitro* model systems (Ballock *et al.*, 1993), especially in the case of chondrocytes which are known to be unstable phenotypically in culture (Benya and Shaffer, 1982).

TGF- β has been implicated in various diseases involving disturbances in EO. A reduced expression of TGF- β has been found in porcine osteochondrosis (Thorp *et al.*, 1995) and in avian dyschondrodysplasia (Law *et al.*, 1997), both characterised by the failure of chondrocytes to reach hypertrophy with little or no matrix calcification

(Thorp *et al.*, 1995). These findings confirm the importance of TGF- β in regulating chondrocyte differentiation *in vivo*.

1.6.2. BONE MORPHOGENETIC PROTEINS

Bone morphogenetic proteins (BMPs) belong to the TGF- β superfamily. They were first identified as molecules derived from bone (Celeste *et al.*, 1990). However, BMP-1 is not involved in bone formation. It has been identified as procollagen C-proteinase (Kessler *et al.*, 1996). BMP-1 processes procollagens to fibrillar collagens, thus playing a role in the regulation of extracellular matrix deposition (Prockop *et al.*, 1998). BMPs are now known to be the most potent inducer of bone formation being the only biological factors capable of inducing osteogenesis at non-skeletal sites such as muscle without depending on the presence of pre-existing cartilage (Sasano *et al.*, 1993). They have been shown to stimulate osteoblastic differentiation of both uncommitted and committed mesenchymal cells *in vitro* (Yamaguchi *et al.*, 1991; Vukicevic *et al.*, 1989) and to induce the expression of osteoblastic markers in cells other than osteoblasts (Katagiri *et al.*, 1994).

BMPs have received considerable attention as potent regulators of mesenchymal differentiation to both chondrogenic and osteoblastic lineages *in vivo* and *in vitro*. They are known to exert their biological function by interacting with their cell surface BMP-receptors (BMPR-I and BMPR-II) (Enomoto-Iwamoto *et al.*, 1998). BMP-3 and BMP-4 promote chondrogenesis in cultures of chick limb bud mesenchymal cells (Carrington *et al.*, 1991; Chen *et al.*, 1991b) while BMP-2, BMP-3, and BMP-4 maintain the cartilage phenotype of already differentiated chondrocytes in culture (Luyten *et al.*, 1992; Hiraki *et al.*, 1991). Carey and Liu (1995) reported the maximal expression of BMP-6 in resting chondrocytes and its minimal expression in hypertrophic cells, which may suggest some possible regulatory role in the terminal differentiation of chondrocytes. Asahina *et al.* (1996) showed that BMP-7 can induce either chondrogenic or osteoblastic differentiation depending on the maturational state of the target cell during endochondral bone formation. In support of the role of BMPs

as inducers of cartilage and bone formation during embryogenesis, a mutation in the gene encoding BMP-5 in *short ear (se)* mice resulted in defects in skeletal structures (Kingsley *et al.*, 1992).

1.6.3. CARTILAGE-DERIVED MORPHOGENETIC PROTEINS

Cartilage-derived morphogenetic proteins (CDMPs) form another set of members of the TGF- β superfamily. The two members so far identified, CDMP-1 and CDMP-2, are expressed primarily in cartilaginous tissues, thus indicating their role in skeletal development and growth (Chang *et al.*, 1994). *In situ* hybridisation and immunostaining studies have shown that CDMP-2 is present in hypertrophic chondrocytes only, suggesting its involvement in the terminal differentiation of chondrocytes and possibly in angiogenesis and osteoblast differentiation. Moreover, its high level of expression in cartilage after birth indicates its possible roles in the postnatal maintenance of cartilage phenotype (Chang *et al.*, 1994).

Of particular interest is CDMP-1, which has been found to be homologous to murine growth differentiation factor-5 (GDF-5). Studies have revealed a possible linkage of *CDMP-1* gene to murine brachypodism (*bp*), a skeletal disorder characterised by limb shortening with no other tissue abnormalities (Storm *et al.*, 1994). Thomas *et al.* (1996) identified a frameshift mutation in *CDMP-1*, which resulted in a form of human chondrodysplasia called Hunter-Thompson type chondrodysplasia. This disorder is characterised by skeletal abnormalities in the limbs and limb joints very similar to those caused by the mouse *bp* mutation (Langer *et al.*, 1989). Another *CDMP-1* mutation was identified which gives rise to another form of human chondrodysplasia, Grebe type (Thomas *et al.*, 1997). This autosomal recessive skeletal disorder is characterised by severe limb shortening and dysmorphogenesis (Langer *et al.*, 1989). Not only is CDMP-1 not secreted but the mutation also prevents the secretion of other BMPs. These findings provide direct evidence for the involvement of CDMP-1 in human skeletal development and also support the

hypothesis that limb and digit morphogenesis is controlled by the composite expression patterns of different BMPs.

1.6.4. BASIC FIBROBLAST GROWTH FACTOR

The fibroblast growth factor (FGF) family groups several members, all bearing the characteristic of interacting with the extracellular matrix (Cancedda *et al.*, 1995). The actions of FGFs are mediated by their binding with varying specificities and affinities to four closely related transmembrane FGF receptors, FGFR-1, -2, -3, and -4 (Givol and Yayon, 1992). FGFs have been implicated in the coordination of skeletal growth and are known to exert major effects on prechondrogenic mesenchymal cells as well as on already differentiated chondrocytes. This section is focused on basic fibroblast growth factor (bFGF), which is involved in chondrocyte differentiation in the growth plate.

bFGF is known to be an important modulator of the maturation cascade of chondrocytes during EO. It is believed to be the most potent mitogen for chondrocytes *in vitro* (Cuevas *et al.*, 1988; Kato and Gospodarowicz, 1984; Kato *et al.*, 1983). It has been localised in proliferating chondrocytes and the extracellular matrix (Luan *et al.*, 1996). Together with one of its high affinity receptors, FGFR-1, bFGF has also been found in the proliferating chondrocytes of human foetal epiphyseal growth plate (Gonzalez *et al.*, 1996). It is hypothesised that bFGF synthesised by proliferating chondrocytes is likely to be deposited into the extracellular matrix until released subsequently from the matrix for its biological activity (Luan *et al.*, 1996). There is evidence for bFGF to interact with heparan sulphate moieties of heparan sulphate proteoglycans present on the cell surface and in the extracellular matrix (Shimazu *et al.*, 1996; Saksela *et al.*, 1988). It is possible that heparan sulphate may act as a storage depot for bFGF during EO (Chintala *et al.*, 1994). It has been shown that bFGF is a potent inhibitor of chondrocyte terminal differentiation by inhibiting type X collagen and alkaline phosphatase expression and calcification (Kato and Iwamoto, 1990). It is interesting to note that bFGF has little

effect on chondrocytes that have already reached hypertrophy (Kato and Iwamoto, 1990). The inhibition of terminal differentiation has been coupled with the loss of bFGF receptors in the hypertrophic zone of the growth plate (Iwamoto *et al.*, 1991). It can be speculated that this stage-specific loss of bFGF receptors is necessary for proliferating chondrocytes to progress to their terminal differentiation.

FGF receptor signalling plays a central role in the regulation of skeletal growth as several human skeletal disorders have been correlated with mutations in FGF receptors (FGFRs). Disruption of the murine *FGFR-3* gene causes severe bone malformation with prolonged bone growth and an expansion of hypertrophic chondrocytes (Deng *et al.*, 1996). This result indicates that FGFR-3 exerts a negative control on EO under normal conditions. Mutations in the *FGFR-3* gene (Rousseau *et al.*, 1994) have also been found responsible for achondroplasia, characterised by short-limbed dwarfism and macrocephaly (Park *et al.*, 1995; Winter, 1995). It appears that achondroplasia may be the result of an activation of the negative effect of FGFR-3 caused by the mutation. Interestingly, FGFR-3 is expressed in resting chondrocytes only and not in the periosteum (Peters *et al.*, 1993), which may explain the occurrence of achondroplasia in bones formed by EO only and not in the intramembranous bones of the skull. Another mutation in FGFR-3 has also been identified in cases of thanatophoric dysplasia (Delezolde *et al.*, 1997), characterised by a disorganised growth plate with markedly reduced proliferative and hypertrophic zones (Park *et al.*, 1995; Winter, 1995). It is clear that further studies are needed to elucidate fully the precise roles of FGFs and their receptors and their implications in skeletal disorders.

1.6.5. INSULIN-LIKE GROWTH FACTORS

Insulin-like growth factors exist in two forms: IGF-I and IGF-II (Humbel *et al.*, 1990). IGF-I is believed to be active mainly in postnatal growth as opposed to IGF-II, which appears to be more effective during foetal life (Vetter *et al.*, 1986). IGFs have been implicated in bone development as both IGF-I and IGF-II are synthesised by chondrocytes (Burch *et al.*, 1986; Nilsson *et al.*, 1986). IGF-II is presumed to be

involved in the regulation of bone formation although its precise role remains to be defined (Gabbittas *et al.*, 1994).

Much work has been focused on IGF-I, which is considered to be the most important anabolic growth factor with respect to cartilage extracellular matrix production (Tyler, 1989). It is known to stimulate proteoglycan synthesis by chondrocytes in serum-free cultures (Bohme *et al.*, 1992), and to stimulate hyaluronan synthesis in epiphyseal growth plate in organ culture (Pavasant *et al.*, 1996b). IGF-I has been shown to stimulate growth plate chondrocytes at all stages of differentiation (Hunziker *et al.*, 1994). It has been localised in proliferative and hypertrophic chondrocytes, suggesting its involvement in EO (Lazowski *et al.*, 1994). Furthermore, IGF-I deficiency can lead to porcine osteochondrosis and avian dyschondroplasia (Thorp *et al.*, 1995). IGF-I is a less potent mitogen than bFGF, but synergistic interactions on chondrocyte growth and differentiation have been demonstrated with bFGF (Trippel *et al.*, 1993) and TGF- β (Tsukazaki *et al.*, 1994). The biological action of IGF-I is mediated by binding to its specific cell-surface receptor, IGF-receptor type 1 (Tollefsen *et al.*, 1991).

1.6.6. GROWTH HORMONE

Growth hormone (GH) is a peptide factor that induces longitudinal bone growth when injected in tibial epiphyseal growth plate of hypophysectomised rats (Isaksson *et al.*, 1982). Although such finding shows the growth promoting effect of GH *in vivo*, it has remained undefined whether GH stimulates skeletal growth directly or whether its effects are mediated by other growth factors. It is well known that IGF-I is a GH-dependent (though not solely) growth factor and is considered generally to be the main mediator of the stimulatory effect of GH on growth. Studies have shown that IGF-I synthesis in proliferative chondrocytes in the growth plate is under the direct influence of GH (Izumi *et al.*, 1995; Nilsson *et al.*, 1986). Consequently, the "somatomedin hypothesis" was postulated whereby GH does not act directly on

cartilage but instead stimulates chondrogenesis and subsequent bone growth indirectly through the somatomedins (now called IGFs) (Daughaday *et al.*, 1972).

Although the "somatomedin hypothesis" was supported by numerous studies, it was also found that GH can exert a direct effect on growth plate chondrocytes *in vivo* (Isaksson *et al.*, 1982). GH receptors have also been localised in rabbit tibial growth plate (Barnard *et al.*, 1988), in rat epiphyseal chondrocytes (Nilsson *et al.*, 1989), in proliferative and hypertrophic chondrocytes of human infant growth plate (Werther *et al.*, 1990), and in avian growth plate chondrocytes (Monson *et al.*, 1993). There is some indication that GH can regulate the expression of its own receptor in rat epiphyseal chondrocytes (Nilsson *et al.*, 1990). It is therefore likely that GH has direct as well as indirect stimulatory effects on cartilage differentiation and bone growth.

Although it has been shown that GH exerts its influence on chondrocytes at each stage of their differentiation (Hunziker *et al.*, 1994), there is evidence that it acts preferentially on proliferating chondrocytes (Tajima *et al.*, 1996), but how GH induces proliferation is unclear. It has been suggested that GH is involved primarily in stimulating the commitment of resting chondrocytes to the proliferative state, but only in synergy with other growth factors including IGF-I (Gevers *et al.*, 1996; Loveridge *et al.*, 1995). It is therefore hoped that further studies both *in vivo* and *in vitro* will help clarify the precise role and mechanism of action of GH in bone growth.

1.6.7. PARATHYROID HORMONE & PARATHYROID HORMONE-RELATED PEPTIDE

Parathyroid hormone (PTH) has been implicated in chondrocyte growth and maturation. In addition to its well established role as an important regulator of blood calcium level (Potts, 1978), PTH has been shown to exert anabolic effects on chondrocytes in culture by stimulating proteoglycan synthesis (Kato *et al.*, 1988b). Koike *et al.* (1990) demonstrated that PTH induces proliferation of rabbit and avian

chondrocytes in soft agar and monolayer cultures, but only in foetal chondrocytes. This result suggests that PTH is a potent mitogen for embryonic chondrocytes only, and becomes ineffective in postnatal life, although the number of PTH receptors does not decrease after birth. In addition, PTH receptors have been localised and shown to be expressed differentially at each stage of chondrocyte differentiation (Iwamoto *et al.*, 1994a). Although the physiological significance of the *in vitro* actions of PTH on chondrocytes is unknown, it can be speculated that PTH may play a role in supporting the rapid growth of cartilage during embryogenesis.

PTHrP was first discovered in tumours that cause malignancy-associated hypercalcemia (Wysolmerski and Broadus, 1994). It has been shown to be present in a wide variety of foetal tissues, including the epithelia (Moseley *et al.*, 1991) and growth plate cartilage (Amizuka *et al.*, 1994). PTHrP is homologous to PTH only at the amino terminal region, which contains a receptor-binding domain essential for receptor activation upon binding (Strewler and Nissenson, 1996). Consequently, the biological activities of both proteins are mediated by the same receptor, PTHrP receptor (Juppner *et al.*, 1991). PTHrP is expressed highly in the maturation zone of the growth plate (Amizuka *et al.*, 1994) where its receptor has also been localised (Iwamoto *et al.*, 1994a). The crucial role of PTHrP in chondrocyte differentiation is now well established and is discussed in Chapter Four.

1.6.8. INDIAN HEDGEHOG

Indian hedgehog (Ihh) is a signalling molecule that is believed to regulate chondrocyte maturation. As the name suggests, it is a member of the Hedgehog family of highly conserved proteins that are known to be involved in embryonic development (Bitgood and McMahon, 1995; Marigo *et al.*, 1995). Ihh has been localized recently in the cartilage of developing long bones in the limbs, particularly in the zone of transition between proliferation and hypertrophy (Vortkamp *et al.*, 1996). Overexpression of Ihh resulted in the arrest of chondrocyte maturation, suggesting clearly that it plays a role in endochondral bone formation. In addition, misexpression of Ihh was found to

cause an upregulation of PTHrP (Vortkamp *et al.*, 1996). Consequently, a model has been proposed for the regulation of chondrocyte differentiation by PTHrP and Ihh (Lanske *et al.*, 1996; Vortkamp *et al.*, 1996). In this model, chondrocytes in the maturation zone of the growth plate produce Ihh. By acting via a signal transduction pathway, Ihh causes an increased synthesis of PTHrP in the perichondrium. PTHrP in turn acts via the PTH/PTHrP receptor on chondrocytes in the growth plate to maintain cell proliferation (Wallis, 1996). The level of Ihh expression is thought to control the number of cells leaving proliferation. Consequently, Ihh expression is stopped completely to permit chondrocytes to proceed to hypertrophy.

1.6.9. RETINOIC ACID

Retinoic acid (RA) is a naturally occurring vitamin A metabolite and is known to influence the maturation process of chondrocytes during EO. RA is generally believed to inhibit chondrogenesis and to promote chondrocyte dedifferentiation *in vitro* by inducing a fibroblastic morphology (Pacifici *et al.*, 1980) and downregulating the expression of type II collagen (Horton *et al.*, 1987).

RA is a potent inducer of chondrocyte terminal maturation and matrix mineralisation, but its effects appear to depend on the maturation stage of the cells. RA promoted chondrocyte hypertrophy and type X collagen synthesis in chick chondrocyte cultures (Oettinger and Pacifici, 1990). Similarly, in RA-treated cultures of mature chondrocytes derived from the chick sternum, RA induced the expression of alkaline phosphatase, osteonectin, osteopontin, and triggered the onset of mineralisation in the absence of ascorbic acid (Iwamoto *et al.*, 1993a). In contrast, in cultures of immature chick chondrocytes, RA induced cell proliferation only but failed to stimulate the expression of markers specific to hypertrophy and mineralisation (Iwamoto *et al.*, 1994b; 1993a). The mechanisms of action of RA have not yet been defined, although RA receptors have been detected in chondrocytes (Kastner *et al.*, 1990).

It has been shown that certain effects of RA are mediated through alterations in the activity of growth factors such as epidermal growth factor (Kinoshita *et al.*, 1992). In addition, it was found that RA appears to inhibit the production of a novel protein, cartilage-derived retinoic acid-sensitive protein (CD-RAP) in treated chondrocytes (Dietz and Sandell, 1996). *In situ* hybridisation has localised CD-RAP specifically to cartilage and the protein seems to be homologous to a growth inhibitory protein recently isolated in a human melanoma cell line (Blesch *et al.*, 1994). Further investigation would help elucidate the functions of CD-RAP and the roles of RA in cartilage development.

1.6.10. OTHER FACTORS

Other factors have been reported to exert an influence on chondrocyte growth and differentiation. For example, thyroxine has been shown to stimulate chondrocyte hypertrophy characterised by cell enlargement, type X collagen synthesis, and an increase in alkaline phosphatase activity in serum-free suspension cultures without exerting any mitogenic effect (Bohme *et al.*, 1992; Quarto *et al.*, 1992). Ballock and Reddi (1994) demonstrated that in pellet cultures with a chemically defined medium containing insulin and growth hormone, thyroxine reproduces a columnar arrangement of chondrocytes reminiscent of the different zones in the growth plate *in vivo*. These results suggest a possible role of thyroxine in the terminal chondrocyte differentiation, including the morphogenesis of columnar cartilage.

In contrast, the role of epidermal growth factor (EGF) in skeletal growth is not clear. Gospodarowicz and Mescher (1977) showed that physiological concentrations of EGF stimulate chondrocyte proliferation in culture, and EGF interacts synergistically with IGFs and FGF to induce DNA synthesis in rabbit costal chondrocytes in culture (Kato *et al.*, 1983). It appears that EGF may enhance growth plate chondrocyte responsiveness to IGF-I *in vitro* by increasing the synthesis of IGF-I receptors per cell (Bonassar and Trippel, 1997). In addition, the presence of EGF-receptors has been demonstrated on cultured rabbit chondrocytes (Kinoshita *et al.*, 1992). The precise

implication of EGF in the regulation of bone growth *in vivo* still remains to be defined.

1.7. ESTABLISHING A CELL CULTURE MODEL OF ENDOCHONDRAL OSSIFICATION

Over the past three decades, major progress has been made in characterising the morphological and biosynthetic features of each stage of chondrocyte differentiation in the growth plate. However, the physical and chemical regulation of chondrocyte differentiation and expression such as cartilage matrix synthesis, and also the various aspects of the chondrocyte differentiation pathway, including the regulation of cartilage calcification, are not fully understood. In addition, the precise molecular mechanisms that initiate the entry of the chondrocytes into the differentiation pathway still remain obscure. Such studies have been limited in part by the lack of a reliable cell culture model, which can reproduce the same events as they occur *in vivo*.

Several attempts have been made during the past thirty years to culture chondrocytes using a wide variety of cell culture systems, each differing in experimental conditions such as culture medium supplements and growth substratum, and the origin and species from which the chondrocytes are obtained. There are a number of important considerations involved in setting up a chondrocyte culture model such as ensuring a maximum cell viability at the time of cell plating and a normal cell growth profile. These factors will be discussed in Chapter two. In addition, it is crucial that the round chondrocytic phenotype is obtained and stabilised during the culture period, accompanied by the induction and maintenance of the expression of markers specific to cartilage.

1.7.1. CHONDROCYTE PHENOTYPE

Obtaining and maintaining the differentiated chondrocyte phenotype *in vitro* is one of the priorities in setting up a cell culture model. Holtzer *et al.* (1960) were among the

first to report on chondrocyte dedifferentiation in which chondrocytes change morphology and lose their ability to synthesise a cartilaginous extracellular matrix. Several questions were raised: (1) Does chondrocyte dedifferentiation result from cellular senescence or the influence of culture environment? (2) Does dedifferentiation occur gradually or abruptly? (3) Is redifferentiation possible and if so, can the cells differentiate into other cell types or can they only revert back to their chondrocytic properties?

1.7.1.1. CHONDROCYTE DIFFERENTIATION VERSUS CHONDROCYTE DEDIFFERENTIATION

It is now well established that growth substratum and cell plating density play a decisive role in controlling the chondrocytic phenotype. A large body of evidence has accumulated demonstrating that chondrocytes lose their round shape and adopt a fibroblastic morphology when cultured in monolayers (Castagnola *et al.*, 1986; Benya and Shaffer, 1982; Deshmukh and Kline, 1976). In contrast, chondrocytes maintain their chondrocytic phenotype and synthesise cartilage-specific proteoglycans when grown in suspension culture using either agarose (Castagnola *et al.*, 1986; Benya and Shaffer, 1982) or collagen gels (McClure *et al.*, 1988; Gibson *et al.*, 1984; Kimura *et al.*, 1984). In addition to cell shape, the type of collagen expressed by chondrocytes is generally another reliable indicator of the transition from the differentiated state (characterised by the synthesis of type II collagen) to the dedifferentiated state when type I collagen is expressed. It has been proposed that the loss of chondrocyte phenotype should be assessed by the loss of cartilage-specific markers such as type II collagen rather than by the appearance of non-cartilage-specific molecules such as type I collagen (Kolettas *et al.*, 1995). It appears that chondrocyte dedifferentiation is an abrupt process since most chondrocytes expressed either collagen types II or I, while both collagen types were coexpressed by very few cells (von der Mark *et al.*, 1977).

The major role played by culture environment in determining and stabilising the chondrocyte phenotype is demonstrated clearly by cell dedifferentiation under

anchorage-dependent conditions and their redifferentiation to chondrocytic phenotype under anchorage-independent growth conditions (Aulthouse *et al.*, 1989; Castagnola *et al.*, 1986; Benya and Shaffer, 1982). It is interesting to observe that chondrocytes grown in monolayers can assume their round phenotype and express cartilage markers if plated at high densities (Koyano *et al.*, 1996; Ruggiero *et al.*, 1993; Amadio *et al.*, 1983). There is evidence indicating that the actin cytoskeleton is the main regulator of cell shape and chondrocyte differentiation *in vitro* (Zanetti and Solursh, 1984).

1.7.1.2. STABILISING THE CHONDROCYTE PHENOTYPE IN CULTURE: THE CHOICE OF A GROWTH SUBSTRATUM

Maintaining the chondrocyte phenotype *in vitro* has remained a challenge primarily because species response varies with culture conditions. For example, rabbit and human chondrocytes dedifferentiate in monolayer cultures (Benya and Shaffer, 1982; Deshmukh and Kline, 1976; Layman *et al.*, 1972) whereas bovine and porcine chondrocytes retain their differentiated phenotype under the same conditions (Kuettnner *et al.*, 1982a, b). Using three-dimensional cultures appears to be a promising approach for maintaining the differentiated chondrocyte phenotype irrespective of the species of origin (Hauselmann *et al.*, 1994; Thomas and Grant, 1988; Delbruck *et al.*, 1986; Benya and Shaffer, 1982). Similarly, chondrocytes were grown as a cell pellet in plastic centrifuge tubes in an attempt to approximate the three-dimensional environment of developing cartilage *in vivo* (Ballock and Reddi, 1994; Kato *et al.*, 1988a). However, it should be noted that each culture system has its own strengths and limitations.

1.7.1.2.1. Collagen gels

Collagen gels have been a popular growth substratum for chondrocytes (McClure *et al.*, 1988; Bates *et al.*, 1987; Gibson *et al.*, 1982). Since chondrocytes are normally embedded within a matrix primarily composed of type II collagen *in vivo*, it would seem appropriate to culture them within a type II collagen gel. However, technical difficulties often encountered in obtaining such gels (Bates *et al.*, 1987) have favoured

the use of gels made of type I collagen. Kimura *et al.* (1984) suggested that collagen provides chondrocytes with a compact environment very similar to the cartilage tissue organisation *in vivo*, thereby promoting the deposition of an extracellular matrix and the resulting maintenance of phenotypic stability.

1.7.1.2.2. Agarose gels

Agarose is also used widely in chondrocyte cultures (Kirsch *et al.*, 1992; Aulthouse *et al.*, 1989; Castagnola *et al.*, 1987). It is believed to form a diffusion pathway resembling that of cartilage matrix, which allows the circulation of nutrients and metabolites (Delbruck *et al.*, 1986). The particularly attractive feature of agarose is its biochemical neutrality; it provides complete cellular immobilisation in an uncharged and inert environment, which does not interfere with chondrocyte phenotype or physiology. In addition, the agarose gel allows the specific effects of culture supplements such as growth hormones to be studied without any biochemical interference (Benya and Shaffer, 1982).

1.7.1.2.3. Alginate gels

Chondrocytes have also been cultured successfully in alginate gels (Hauselmann *et al.*, 1994), where cells are easily recovered by alginate dissolution with chelating agents. The isolated cells are still surrounded by their matrix and can therefore be used for subsequent cultures as a pure chondrocyte population. However, alginate requires 1-2 mM Ca^{2+} for its gel structure (Hauselmann *et al.*, 1994), which therefore makes it unsuitable for culture conditions with lower Ca^{2+} levels.

1.7.2. CELL CULTURE MODELS IN CURRENT USE

As mentioned previously, although the specific morphological and biosynthetic features associated with chondrocytes at distinct stages of differentiation within the growth plate have been well characterised, the precise physical, hormonal, and molecular control of this developmental cascade still remains to be elucidated. The

availability of a suitable cell culture model system that would mimic this pathway *in vivo* would greatly facilitate such studies.

Various cell culture models have been developed for particular studies of the complex differentiation pathway of chondrocytes *in vitro*. Ruggiero *et al.* (1993) proposed a cell culture system of foetal bovine articular chondrocytes grown in high-density monolayers where a cartilage matrix similar to that formed *in vivo* was deposited. This culture system may be useful to study collagen synthesis and its regulation during chondrogenesis. The serum-free model developed by Bruckner *et al.* (1989) using chick resting chondrocytes appears to provide an excellent opportunity to study the influence of exogenous growth factors on chondrocyte differentiation without any interference by undefined components of culture media. Studying the chemical, physical, and biological events at various stages of chondrocyte differentiation *in vitro* is also possible with the development of mineralising culture systems using embryonic chick vertebral chondrocytes grown in monolayers in the presence of ascorbic acid and β -glycerophosphate (Gerstenfeld and Landis, 1991). Other culture systems include rabbit growth plate chondrocytes cultured on type II collagen-coated dishes (Jikko *et al.*, 1993) and in pellets (Kato *et al.*, 1988a). In order to investigate the properties and developmental pathway of chondrocytes at each differentiation stage, an interesting approach was to fractionate bovine growth plate chondrocytes by density gradient separation and to culture each cell fraction separately (Carey *et al.*, 1993). Recently, Yan *et al.* (1997) proposed a novel culture model for EO in which the lectin, concanavalin A, stimulated resting chondrocytes to differentiate to hypertrophy *in vitro*.

The use of cell lines has also been suggested as an alternative to the use of freshly isolated chondrocytes as they represent a renewable source of material. Atkinson *et al.* (1997) used the mesenchymal cell line, C3H10T1/2, in high density micromass cultures in the presence of ascorbic acid, β -glycerophosphate, insulin, transferrin, and a purified mixture of osteoinductive proteins. This system produced a faithful recapitulation of cartilage differentiation up to the hypertrophic stage and appears

suitable to study the regulation of the commitment of mesenchymal cells to the chondrogenic lineage and the subsequent maturation during chondrogenesis. Collagen biosynthesis by chondrocytes can be studied using an immortalised rat chondrocyte cell line, IRC (Oxford *et al.*, 1994; Horton *et al.*, 1988). In addition, several cell lines derived from human chondrosarcoma, including 105KC and HCS-2/8 cell lines, have been established whereby they maintain a stable chondrocytic phenotype in long-term cultures (Block *et al.*, 1991; Takigawa *et al.*, 1989). These cell lines should prove useful in the studies of chondrocyte differentiation, including its control by regulatory factors (Takigawa *et al.*, 1997). Swarm rat chondrosarcoma cell lines also offer the opportunity to examine the post-translational processing of collagens (Fernandes *et al.*, 1997), as well as the transcriptional regulation of chondrocyte-specific genes (Mukhopadhyay *et al.*, 1995).

1.7.3. USE OF 5-AZACYTIDINE IN THE STUDY OF CHONDROCYTE DIFFERENTIATION *IN VITRO*

Previous studies on collagen gene expression have shown that the compound, 5-azacytidine (aza-C), known to be a potent DNA demethylating agent, appears to be able to induce foetal bovine epiphyseal chondrocytes to enter the differentiation pathway in culture (Manning, 1994). Moreover, aza-C has been reported to induce the differentiation of the murine mesenchymal C3H10T1/2 cells into three distinct lineages of myoblasts, adipocytes, and chondrocytes (Taylor and Jones, 1979). These observations offer the possibility of developing a cell culture model, which may prove useful in the molecular studies of the chondrocyte differentiation pathway. Since aza-C is a DNA demethylating agent, it is necessary to understand the importance of DNA methylation in the cell system.

1.8. DNA METHYLATION

1.8.1. POST-REPLICATIVE MODIFICATION OF DNA

DNA is a very stable molecule and due to its function as a carrier of genetic information, it is essential for its structure to be maintained faithfully. It is well-known that the DNA of most prokaryotic and eukaryotic organisms contain chemically modified bases, which either replace the standard bases completely or replace only a small fraction of the bases. DNA modification usually arises from base methylation whereby a methyl group is transferred to adenine or cytosine after DNA synthesis. This process is carried out by a special class of enzymes known as DNA methyltransferases. The commonest methylated base is 5-methylcytosine (Figure 1.8). In eukaryotic DNA, 5-methylcytosine is found mostly in the palindromic dinucleotide sequence 5'-CpG-3' (Adams, 1990). CpG dinucleotides are distributed unevenly in the eukaryotic genome, with A+T-rich regions containing much fewer CpG pairs than G+C-rich regions (Adams and Eason, 1984). This uneven distribution leads to the formation of local areas called CpG islands in the G+C-rich regions of the genome, which consequently have a greater potential for methylation than other regions. The extent of DNA methylation varies according to tissues and species. It is also thought that genome size may play an important role in determining the degree of cytosine methylation.

1.8.2. MAINTENANCE AND *DE NOVO* DNA METHYLATION

During DNA methylation, methyl groups are transferred from the methyl donor, S-adenosylmethionine (SAM), to specific nucleotides, usually cytosine and adenine, to give rise to methylated DNA bases (Figure 1.9). This reaction occurs shortly after DNA replication and is catalysed by a DNA methyltransferase (DNA-MeT) (Adams and Burdon, 1983). Two types of methylation can be distinguished: methylation *de novo* and maintenance methylation. Methylation *de novo* involves the addition of methyl groups to unmethylated DNA whereas maintenance methylation involves the methylation of hemimethylated DNA, which consists of a methylated parental DNA

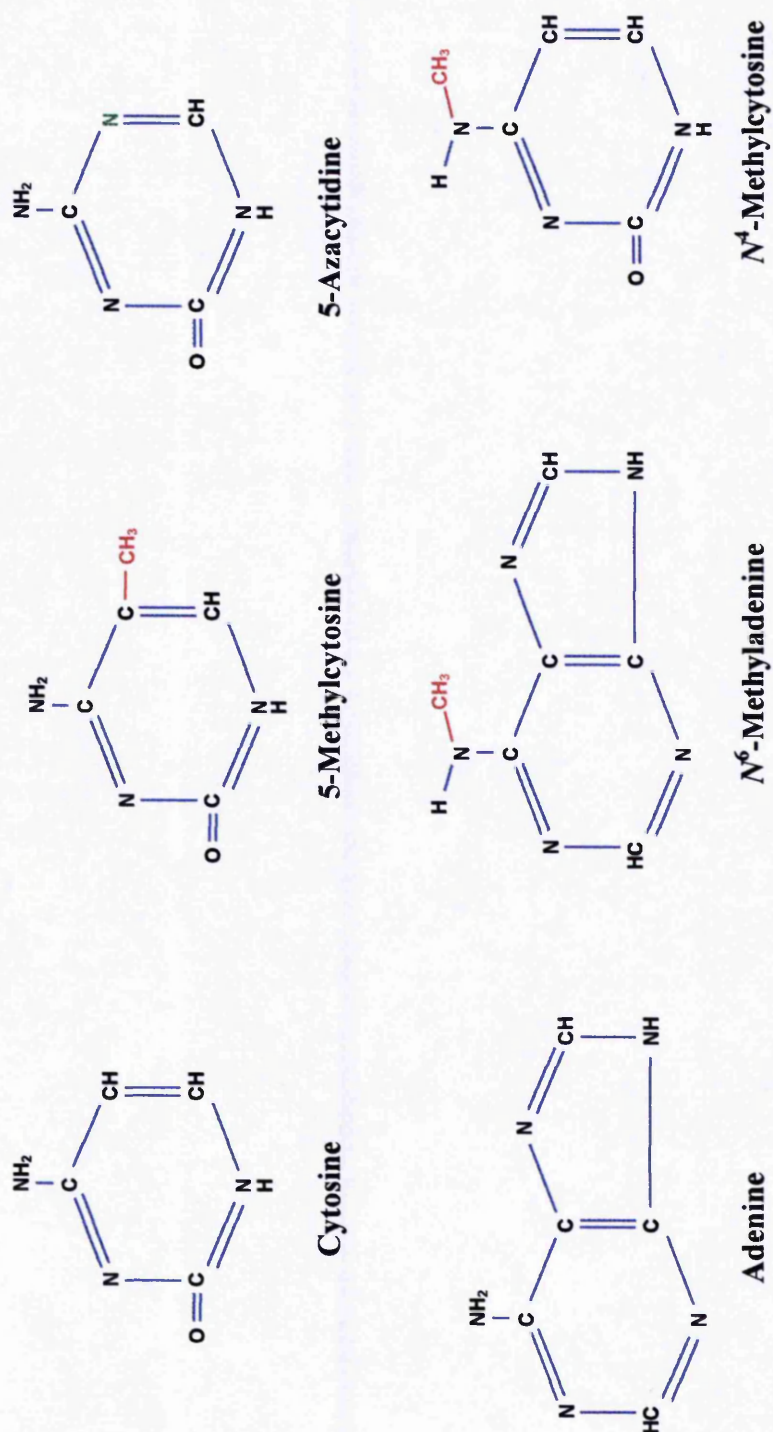


Figure 1.8. Chemical structures of unmethylated bases and their corresponding methylated counterparts. 5-Methylcytosine is the predominant form of methylated base; N⁶-methyladenine and N⁴-methylcytosine are mostly found in prokaryotic DNA. 5-Azacytidine is a cytosine analogue with a nitrogen atom substituting the carbon atom at position 5 in the pyrimidine ring.



Figure 1.9. The DNA methylation reaction. DNA is modified in a post-replicative reaction that is catalysed by the enzyme, DNA methyltransferase (DNA-MeT). During this process, DNA-MeT transfers a methyl group (CH₃) from the ubiquitous methyl donor, S-adenosylmethionine, to the dinucleotide sequence CG which is the potential methylation site in DNA, giving a methylated CG site and S-adenosylhomocysteine.

strand and an unmethylated daughter strand (Bestor and Ingram, 1983). Although only one DNA-MeT has been identified to date as the mediator of DNA methylation, both *de novo* and maintenance (Szyf, 1996), the possibility of the existence of a separate as yet undiscovered *de novo* DNA-MeT cannot be ruled out.

1.8.3. DNA METHYLATION IN PROKARYOTES

The primary function of DNA methylation in prokaryotes is to protect host DNA against cleavage by restriction endonucleases (Arber and Dussoix, 1962). Such DNA modification enables the organism to distinguish between its own DNA and any “foreign” DNA since the latter does not have the same methylation pattern as the host. Any invading “foreign” DNA into the host will be degraded by restriction enzymes at the target sites lacking methyl groups. DNA methylation also plays an important role in the repair of mismatched base pairs to maintain genetic stability (Modrich, 1987). During DNA replication, a mismatch is said to occur when a normal, but incorrect, base is inserted into DNA. Because DNA methylation lags behind DNA synthesis, a newly synthesised daughter strand is always undermethylated in comparison to the parental strand. Due to this lack of methylation on the newly synthesised, but incorrect, daughter strand, this mismatch repair system is able to distinguish the parental DNA from the daughter strand.

1.8.4. DNA METHYLATION IN EUKARYOTES

The exact functional significance of DNA methylation in eukaryotes is yet to be discovered. A major breakthrough came when it was demonstrated that an inactivating mutation of the DNA-MeT gene resulted in abnormal development and embryonic lethality in the mouse (Li *et al.*, 1992). DNA methylation has been proposed to play a major role in the control of eukaryotic gene expression and in carcinogenesis.

1.8.4.1. CONTROL OF GENE EXPRESSION

An inverse correlation between the expression of tissue-specific genes and the level of DNA methylation has been well documented (Ngernprasirtsiri *et al.*, 1989; Mandel and Chambon, 1979). Several *in vitro* methylation studies have provided evidence correlating promoter methylation and transcriptional repression (Guenette *et al.*, 1992; Thompson *et al.*, 1991). It was also observed that DNA methylation is tissue-specific (Waalwijk and Flavell, 1978) and its level differs according to tissue type (Mandel and Chambon, 1979). The interesting question that arises is how DNA methylation influences the expression of specific genes.

Gene activation is a complex multi-step process involving changes in the gene structure and methylation state and the interactions with a large number of *trans*-acting factors with specific *cis*-acting DNA sequences (Cedar, 1988; Mavilio *et al.*, 1983). Transcriptional repression by methylation may occur either by a direct mechanism involving the binding of transcription factors or may be caused indirectly by repressor proteins that bind to methylated DNA, or both (Tate and Bird, 1993). The transition into an active state of tissue-specific genes as a result of their demethylation appears to be due to chromatin becoming less compact (Fajkus *et al.*, 1992; Thompson *et al.*, 1991). Most tissue-specific genes are methylated, probably because the methyl groups generate a "solenoid" chromatin configuration that renders the genes inaccessible, and thus transcriptionally inactive. On the other hand, housekeeping genes, which are expressed constitutively, are demethylated and therefore are maintained in an active "beads-on-string" structure that is accessible to the transcriptional machinery of the cell (Adams, 1990; Cedar, 1988).

1.8.4.2. CARCINOGENESIS

It has been proposed that DNA methylation may be involved in carcinogenesis since cancer cells have been found to exhibit aberrant DNA methylation patterns (Rideout *et al.*, 1994). Any methylated CpG site is a potential mutation hot spot due to the extremely high instability of cytosine compared to the other bases (Jones *et al.*, 1992).

It is speculated that methylcytosine is a powerful endogenous mutagen and its deamination to thymine may contribute to mutations in tumour suppressor genes (Rideout *et al.*, 1990). Due to the inverse correlation observed between DNA methylation levels and gene expression, it has been proposed that hypomethylation of proto-oncogenes (Hanada *et al.*, 1993) and hypermethylation of tumour suppressor genes (Herman *et al.*, 1994) can also lead to cancer induction.

1.8.5. DEMETHYLATION

DNA methylation patterns are established during gametogenesis where tissue-specific genes are methylated by *de novo* methylation. These patterns are maintained throughout early development. Only in the tissue of expression do tissue-specific genes become activated by demethylation and it is this methylation pattern that is inherited clonally (Razin and Riggs, 1980). However, the biochemical and molecular mechanisms responsible for demethylation still remain to be elucidated. In recent years, several experiments have indicated an active process of demethylation that is site-specific and does not involve DNA replication (Paroush *et al.*, 1990; Wilks *et al.*, 1984). Razin *et al.* (1986) suggested that demethylation can result from an enzyme activity that excises methylated cytosine bases and replaces them with non-methylated cytosines without breaking the phosphodiester bond in DNA. It is possible that demethylation can be regulated by the availability of demethylase (Szyf, 1994) and also by *cis*-acting (Paroush *et al.*, 1990) and *trans*-acting factors that determine the accessibility of methylated DNA sequences to demethylase activity. Although no enzyme that specifically removes methyl groups from methylated cytosines has been identified to date, it is clear that demethylation is a central process required by tissue-specific genes for their expression in the differentiated cell type.

1.8.6. 5-AZACYTIDINE, THE DNA DEMETHYLATING AGENT

Since DNA methylation is known to play an important role in the control of eukaryotic gene expression, there has been considerable interest in cytosine analogues such as 5-azacytidine (aza-C) and their effects on the methylation state of genes and

their expression. Aza-C is known to be a powerful inhibitor of DNA methylation (Jones and Taylor, 1980). It differs from cytosine only in that the carbon atom at position 5 in its pyrimidine ring is substituted by a nitrogen atom and consequently, the potential methylation site at this position is removed (Figure 1.8).

Aza-C has provided experimental support to the correlation between DNA methylation and decreased gene expression (Gotzinger *et al.*, 1996). Evidence for methylation-mediated transcriptional repression was provided by treatment of a transformed cell line derived from a human rhabdomyosarcoma with aza-C, which resulted in the transcriptional activation of endogenous $\text{pro}\alpha 1(\text{I})$ collagen gene (Thompson *et al.*, 1991). Aza-C has been shown to induce novel gene expression both *in vitro* and *in vivo*. Taylor and Jones (1979) demonstrated that it converts cultures of the mouse embryo cell line, C3H10T1/2, into three functionally and biochemically differentiated cell types: myoblasts, chondrocytes, and adipocytes. Aza-C also converts teratocarcinoma-derived mesenchymal cells into epithelial cells (Darmon *et al.*, 1984) and induces erythroid differentiation in treated Friend erythroleukemia cells (Creusot *et al.*, 1982).

It is still obscure how aza-C exerts its demethylating effect. The mechanism of drug action appears to be linked to its incorporation into DNA (Creusot *et al.*, 1982; Constantinides *et al.*, 1978). It mediates its effect by inhibiting DNA-MeT rather than by being inherently unable to serve as a methyl acceptor (Creusot *et al.*, 1982). Santi *et al.* (1983) have formulated a proposal for the mechanism of inactivation of DNA-MeT. The interaction between the enzyme and aza-C results in the irreversible formation of a complex in which the enzyme is covalently linked to the cytosine analogue (Christman *et al.*, 1985; Santi *et al.*, 1984). Since this complex is toxic to the cell if unrepaired (Juttermann *et al.*, 1994), the enzyme is degraded, causing its depletion in the cell (Tanaka *et al.*, 1980). Therefore, aza-C indirectly causes genomic hypomethylation.

There still remain some critical questions concerning the correlation between the demethylating action of aza-C and gene activation. It has been demonstrated that aza-C can induce genes in organisms that lack 5-methylcytosine (Tamame *et al.*, 1983), which raises the question of whether the demethylating action of the compound is actually responsible for gene activation. Although aza-C appears to act most specifically on DNA-MeT (Friedman, 1979), it is also known to possess biological effects other than demethylation (Jones, 1985). It can inhibit other DNA metabolising enzymes and can perturb protein-DNA interactions that are essential for the maintenance of gene expression. It has also been reported that aza-C can interfere with chromatin structure when incorporated into DNA (Fajkus *et al.*, 1992; Parrow *et al.*, 1989).

In addition to being used as an experimental tool in DNA methylation research, aza-C has also been utilised in clinical studies as a chemotherapeutic drug due to its anti-tumourigenic potential. The mutagenicity of aza-C (Jackson-Grusby *et al.*, 1997) questions its utility as a chemotherapeutic agent, and novel inhibitors of DNA-MeT that are not incorporated into DNA with no serious side-effects should be developed for the treatment of cancer and other genetic diseases.

1.9. AIMS

Previous work carried out on the role of DNA methylation in the regulation of the expression of the collagen type X gene has demonstrated that aza-C has a remarkable ability to induce foetal bovine epiphyseal chondrocytes to hypertrophy in culture (Manning, 1994). Therefore, this provides an interesting opportunity to develop a cell culture model using aza-C whereby foetal bovine epiphyseal chondrocytes can be induced to enter the chondrocyte differentiation pathway and thus recapitulate the events that occur in the mammalian growth plate.

The work presented here describes the establishment and characterisation of this cell culture model with chondrocytes derived from epiphyseal cartilage of long bones of foetal calves. Epiphyseal chondrocytes do not normally proceed to hypertrophy and do not synthesise type X collagen *in vivo* (Eyre *et al.*, 1987; Svoboda *et al.*, 1989). In this study, aza-C was used to induce these foetal bovine epiphyseal chondrocytes to differentiate into hypertrophic chondrocytes and to recapitulate the differentiation pathway that occurs *in vivo*. The main objectives in this study were: (1) to investigate the suitability of culture conditions for the maintenance of cartilaginous characteristics and viability of the culture system, (2) to characterise the model by examining the expression of a number of markers specific to the various stages of the differentiation pathway, (3) to determine the fate of aza-C treated chondrocytes in this system, and (4) to examine how the aza-C treated chondrocytes respond to the addition of parathyroid hormone-related peptide, which is known to play an important regulatory role in EO.

Once proved reliable, this culture system would provide the opportunity to investigate the molecular mechanisms controlling the chondrocyte differentiation pathway. Several novel cDNA clones coding for genes expressed in specific regions of the growth plate during EO have been isolated with the use of a PCR-based subtractive hybridisation technique (Hillarby *et al.*, 1996). These genes are known to be expressed specifically in distinct regions of the bovine growth plate. The establishment of a cell culture model as described above would enable the characterisation of the novel genes isolated from the specific zones of the growth plate.

Chapter Two

CHAPTER TWO

EFFECTS OF 5-AZACYTIDINE ON FOETAL BOVINE EPIPHYSEAL CHONDROCYTES IN CULTURE

2.1. INTRODUCTION

Long bone formation occurs at the growth plate by the regulated process of endochondral ossification (EO). However, the molecular mechanisms that trigger the various stages in the differentiation pathway of chondrocytes during EO are still unclear. The availability of a cell culture model that can recapitulate the events of EO as they occur *in vivo* would facilitate considerably studies of the molecular mechanisms of EO. Several attempts have been made to set up a cell culture model, each differing in numerous parameters such as the origin of chondrocytes in culture and the culture conditions (see Chapter One, Section 1.7). Various methods have been used to stimulate the differentiation of chondrocytes to hypertrophy in culture, including the use of the lectin, concanavalin A, on rabbit resting chondrocytes (Yan *et al.*, 1997), the supplementation of high calcium levels in bovine chondrocyte cultures (Koyano *et al.*, 1996), the addition of a mixture of ascorbic acid and β -glycerophosphate to chick chondrocyte cultures (Gerstenfeld and Landis, 1991; Thomas *et al.*, 1990), and the use of low serum media supplemented with growth factors (Bohme *et al.*, 1992).

5-Azacytidine (aza-C) is a potential inducer of differentiation *in vitro*. Aza-C is a powerful DNA demethylating agent that has been reported to induce the differentiation of mesenchymal C3H10T1/2 cells into three distinct lineages of myoblasts, adipocytes, and chondrocytes (Taylor and Jones, 1979). Recent studies on the role of methylation in collagen gene expression have also indicated that aza-C appears to be able to induce foetal bovine epiphyseal chondrocytes to differentiate in culture (Manning, 1994). Therefore, the use of aza-C in epiphyseal chondrocyte

cultures provides an interesting opportunity to develop a cell culture system that can mimic the maturation cascade of chondrocytes during EO.

The development of a chondrocyte culture system involves a number of important considerations. Maximum cell viability should be aimed for at the time of cell plating after the isolation of chondrocytes from their source of cartilage. It is also important that a normal cell growth profile is obtained and the cells divide and proliferate. Stabilising the round chondrocytic phenotype, as well as the induction and maintenance of the expression of cartilage markers such as type II collagen, should also be ensured throughout culture (see Chapter One, Section 1.7.1.). The addition of cell culture supplements to the media is also an important factor to consider.

2.1.1. CELL VIABILITY

The isolation procedure chosen to obtain viable chondrocytes from cartilage is of vital importance in the establishment of cell cultures. The tissue needs to be disaggregated mechanically prior to its enzymatic digestion (Bruckner *et al.*, 1989; Thomas and Grant, 1988). Releasing chondrocytes from cartilage is difficult as cartilaginous tissues are hard to digest. Proteolytic enzymes, including trypsin and collagenase, and long incubation periods are required for complete digestion (Ruggiero *et al.*, 1993; McClure *et al.*, 1988; Bates *et al.*, 1987; Castagnola *et al.*, 1986). This procedure leads to an irreversible damage of the chondrocytes, especially the cell surface, which results in an impaired cell viability. It is evident that cartilage digestion conditions should be carefully monitored by the need for maximal cell release and the requirement for maximum cell viability. Cell viability is normally assessed by the trypan blue dye exclusion test. In general, about 85-90% cell viability is achieved at the time of cell plating (Amadio *et al.*, 1983).

2.1.2. CELL PROLIFERATION VERSUS CELL DIFFERENTIATION

Since the availability and amount of tissue samples is often limiting, maximal cell proliferation would be beneficial to permit sample expansion for subsequent experimentation. It has been observed that chondrocytes with a fibroblastic

morphology in monolayer cultures grow faster, while they proliferate poorly in suspension cultures where they express their differentiated phenotype (Aulthouse *et al.*, 1989; Benya and Shaffer, 1982). In order to obtain sufficient material for extensive investigations, it was proposed that chondrocytes are first cultured in monolayers for cell amplification and then they can either be transferred to suspension cultures for the chondrocyte phenotype to be reexpressed or kept frozen for future studies (Aulthouse *et al.*, 1989). A similar approach was taken by Yan *et al.* (1997) who allowed rabbit resting chondrocytes to grow to confluency in 10% foetal calf serum and subsequently treated them with concanavalin A at low serum concentrations for chondrocyte differentiation to proceed.

2.1.3. CULTURE MEDIA SUPPLEMENTS

2.1.3.1. FOETAL CALF SERUM

The addition of supplements to the culture system used is of significant importance. These exogenous factors may have a profound influence on the chondrocyte phenotypic expression and on the pattern of cell differentiation in culture. Foetal calf serum (FCS) is usually included in the culture medium at high concentrations, usually 10%. It has been shown that in embryonic chick chondrocytes, FCS has a potent mitogenic effects and its ability to induce terminal chondrocyte differentiation characterised by the expression of type X collagen (Castagnola *et al.*, 1988; McClure *et al.*, 1988; Tacchetti *et al.*, 1987). In the absence of FCS, neither cell proliferation nor hypertrophic differentiation is obtained and cell viability is maintained only if chondrocytes are seeded at high densities (Bruckner *et al.*, 1989). FCS also protects embryonic chick chondrocytes from toxic compounds derived from molecular oxygen, such as hydroxyl radicals and hydrogen peroxide (Tschan *et al.*, 1990). In addition, rat epiphyseal chondrocytes cultured as an aggregated cell pellet do not terminally differentiate to hypertrophy under serum-free conditions, unless thyroxine is added to a chemically defined medium containing insulin and growth hormone (Ballock and Reddi, 1994). Similarly, serum-free media containing insulin growth factor-I (IGF-1) (Bohme *et al.*, 1992), insulin (Quarto *et al.*, 1992), and thyroid hormone (Bohme *et al.*, 1992) have been reported to induce maturation to hypertrophy in embryonic chick chondrocyte cultures in agarose gels.

2.1.3.2. ASCORBIC ACID

Ascorbic acid is another component that is important in modulating chondrocyte differentiation in culture. Ascorbic acid is known to be essential for extracellular matrix assembly and maximal collagen synthesis *in vitro* (Gerstenfeld and Landis, 1991; Meier *et al.*, 1978), which correlates with its involvement in proline and lysine hydroxylation *in vivo* (Murad *et al.*, 1981; Blanck and Peterkofsky, 1975). Ascorbate supplementation also leads to the hypertrophic differentiation of cultured embryonic chick chondrocytes (Gerstenfeld and Landis, 1991; McClure *et al.*, 1988) and when added with β -glycerophosphate, induces matrix mineralisation (Gerstenfeld and Landis, 1991; Thomas *et al.*, 1990). It should be noted that ascorbic acid is an absolute requirement for matrix assembly *in vitro*, but is not vital for inducing the differentiation pathway of chondrocytes. Leboy *et al.* (1997) demonstrated that ascorbate interacted synergistically with bone morphogenetic protein-2 (BMP-2) to induce a rapid maturation in embryonic chick chondrocyte cultures but alone was not responsible for stimulating type X collagen synthesis. Interestingly, ascorbic acid appears to have differential effects on chondrocytes of different species. For instance, it induces hypertrophic differentiation and matrix mineralisation in embryonic avian chondrocyte cultures (Gerstenfeld and Landis, 1991; Leboy *et al.*, 1989; Tacchetti *et al.*, 1987) but inhibits hypertrophy and type X collagen synthesis in human epiphyseal chondrocytes *in vitro* (Kirsch *et al.*, 1992). The above examples illustrate clearly the varied responses of different species to different culture conditions and stress upon the difficulties involved in setting up a reliable cell culture model.

2.2. AIMS

It is known that chondrocytes derived from the epiphyses of long bones are resting and do not participate in the differentiation pathway that occurs in the growth plate during EO (Svoboda *et al.*, 1988; Eyre *et al.*, 1987). In this study, aza-C was used to treat epiphyseal chondrocytes derived from long bones of foetal calves with a view to inducing their cellular differentiation in culture.

As an initial stage in the construction of this cell culture system, it was necessary to examine the suitability of the culture conditions for the maintenance of phenotypic markers characteristic of cartilage cells. It was also important to assess and ensure that aza-C treatment maintained a normal cell growth cycle as well as favoured the chondrocyte phenotypic characteristics in culture. Several parameters were assessed in cultures maintained in the absence and presence of aza-C, including (i) the variation of cell number with time in culture, (ii) the effect of plating-density on cell morphology, (iii) the expressions of the cartilage marker, type II collagen, and the marker of dedifferentiation, type I collagen, (iv) the ultrastructural features of cultured cells, and (v) the variation of cell morphology and size with and without drug treatment. These strategies of investigation are outlined in figure 2.1.

2.3. MATERIALS

Gibco BRL: Paisley, Scotland.

Dulbecco's phosphate buffered saline (without calcium and magnesium), foetal calf serum, gentamycin, *L*-glutamine, minimal essential medium (with Earle's salts and *L*-glutamine), penicillin-streptomycin solution, sodium bicarbonate (7.5%), trypsin (0.25% w/v in Gibco Solution A).

Sigma Chemicals Co. Ltd: Dorset, U.K.

5-Azacytidine (50X; lyophilised and sterilised), bacterial collagenase type 1A, DAB peroxidase substrate tablets (SIGMA *FAST™*), *L*-ascorbic acid, and trypan blue dye.

Coulter Electronics Ltd: Bedfordshire, U.K.

Isoton

BDH Laboratory Supplies: Dorset, U.K.

Hydrogen peroxide (30%), methanol, sodium chloride, orthoboric acid, and EDTA.

ICN Biochemicals Inc.: Ohio, U.S.A.

Tris (Ultra-Pure).

Biogenesis Ltd: Dorset, U.K.

Bovine type II collagen polyclonal antibody.

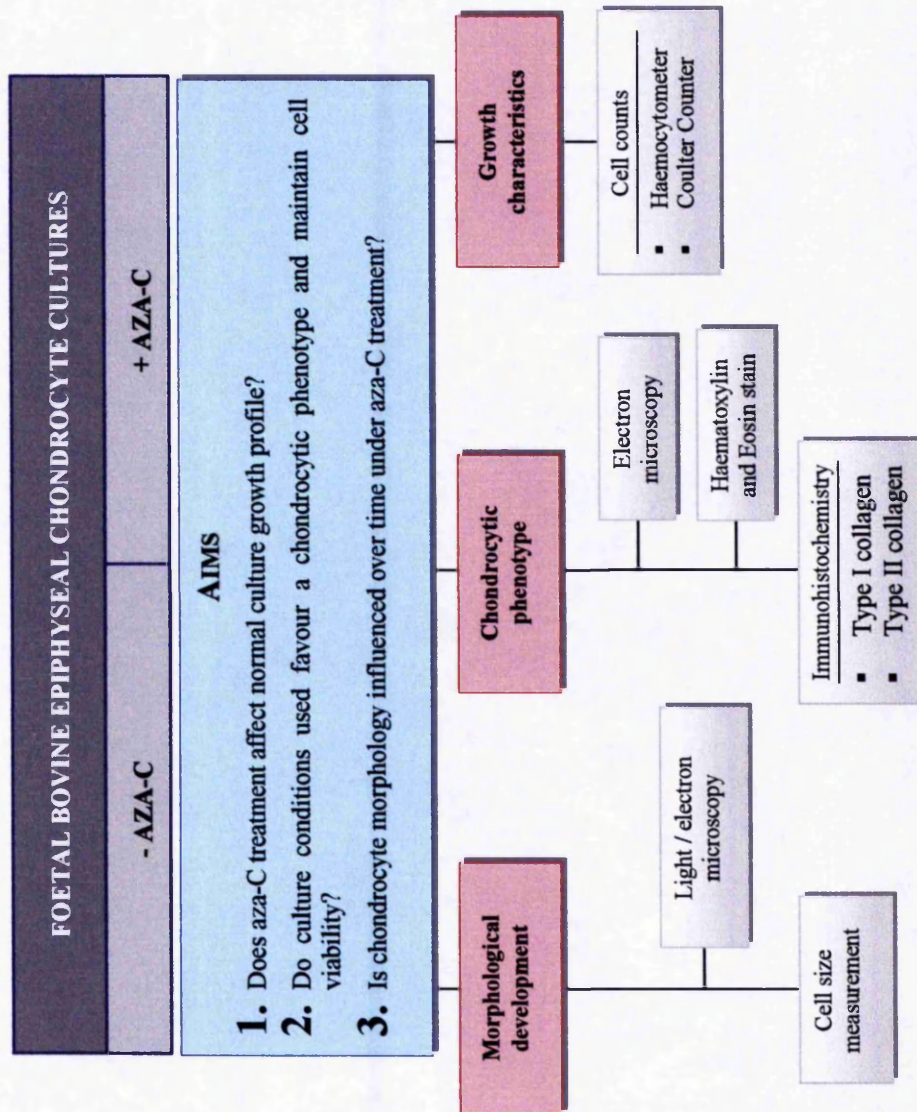


Figure 2.1. Schematic diagram showing the strategies of investigation into the construction of a cell culture system using foetal bovine epiphyseal chondrocytes.

DAKO: Buckinghamshire, U.K.

Biotinylated swine anti-rabbit immunoglobulins, peroxidase-conjugated rabbit anti-guinea pig immunoglobulins, rabbit serum, swine serum, and streptavidin peroxidase.

Loctite: Hertfordshire, U.K.

Loctite® engineering adhesive.

Bovine type I collagen antibody was kindly provided by Dr. Shirley Ayad (University of Manchester).

All reagents were of the highest grade commercially available.

2.4. METHODS

2.4.1. BOVINE CHONDROCYTE CULTURE

Foetal calves of second and third trimester were collected from the local Manchester abattoir and utilised within three hours of death. The long bones were collected and cleaned of adhering tissue. Epiphyseal cartilage was dissected from the long bones well away from the growth plate and from any secondary centres of ossification under sterile conditions. The cartilage was left in organ culture in minimal essential medium (MEM) with Earle's salts and *L*-glutamine, supplemented with 20% (v/v) foetal calf serum (FCS) overnight at 37°C in 5% CO₂ / 95% O₂ atmosphere. The cartilage pieces were washed twice in MEM supplemented with 10% (v/v) FCS and chopped finely in the same medium. In order to release the epiphyseal chondrocytes, the chopped cartilage was digested with bacterial collagenase type IA (10 mg/ml) and trypsin (0.4 mg/ml) in serum-free MEM for 3 hours at 37°C. Following collagenase digestion, the final volume of the cartilage digest was made up to 50 ml with MEM containing 10% FCS. The cell debris was pelleted by centrifugation in an MSE Mistral 2000 bench centrifuge at 100 g for 2-3 seconds and discarded. The resulting supernatant was centrifuged at 250 g for 3 minutes to pellet the chondrocytes. The supernatant was discarded and the cell pellet resuspended in 25 ml MEM containing 10% FCS. The cell suspension was filtered through a 40 µm cell strainer (Becton Dickinson Labware, New Jersey, U.S.A) to further remove any undigested material

and centrifuged at 250 g for 3 minutes. The cell pellet was resuspended in 25 ml MEM containing 10% FCS, filtered, and centrifuged at 250 g for 3 minutes. The final cell pellet was resuspended in 5 ml MEM containing 10% FCS. Cell counts were performed using the haemocytometer to determine the density of the cell suspension. All chondrocytes were plated in 9 cm² NUNCTM plastic tissue culture dishes (Nalge Nunc International, Illinois, U.S.A), unless otherwise stated, at a density of 0.5×10^6 cells per cm². The culture medium used throughout was MEM buffered to pH 7.2 with 10mM HEPES and supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), gentamycin (0.01 mg/ml), *L*-glutamine (2 mM), *L*-ascorbate (25 µg/ml), 0.34% (w/v) sodium bicarbonate, and 10% (v/v) FCS. All cultures were incubated at 37°C in 5% CO₂ / 95% O₂ atmosphere.

2.4.2. 5-AZACYTIDINE TREATMENT OF CHONDROCYTE CULTURES

One hour after plating, cell cultures were treated with 5-azacytidine (15 µg/ml for maximal cell viability) and maintained in this augmented medium for 48 hours. The medium was removed and the cultures were maintained in standard MEM containing 10% FCS, which was replaced every other day throughout the culture period. Control cultures were processed as treated cultures, but without the addition of 5-azacytidine. All cultures were incubated at 37°C in 5% CO₂ / 95% O₂ atmosphere.

2.4.3. DETERMINATION OF CELL NUMBER

Cell counts were carried out using two methods: (1) the haemocytometer together with the trypan blue dye exclusion test at the time of plating, and (2) the Coulter Counter[®] to determine the cell growth profile.

In the "haemocytometer" method, an aliquot of the cell suspension was placed in the counting chamber under the microscope. The cell number within a defined area was counted and the concentration of the cell suspension derived from the count. The trypan blue dye exclusion test was performed to determine cell viability. Non-viable cells take up the dye due to their permeabilised cell membrane, and therefore stain

blue. Conversely, viable cells remain clear (dye-excluding) due to their intact membrane. This test was carried out prior to cell counting.

The Coulter Counter[®] is an electronic particle counter. It consists of two electrodes, one immersed in a liquid electrolyte such as isoton inside a glass counting tube and the other outside the tube, immersed in the cell sample suspended in the same electrolyte. A small orifice in the counting tube ensures a current flow between the two electrodes. As a known volume of the cell sample, usually 500 μ l, is drawn automatically into the glass tube via the orifice, a pulse is registered which appears on an oscilloscope screen and a cell count is recorded on the digital scaler. This counting technique is fast, simple, and accurate. However, care should be taken to avoid cell settling, and the presence of any debris in the cell suspension, which may block the orifice. A good cell suspension was ensured by digesting the cell layers with a mixture of trypsin and collagenase to release the cells from their matrix. The final cell suspension was filtered through a cell strainer to remove any cell debris.

2.4.3.1. METHOD I: THE HAEMOCYTOMETER AND THE TRYPAN BLUE EXCLUSION TEST

Prior to cell plating, an aliquot of the cell suspension was taken and mixed with a 0.4% (w/v) trypan blue solution. Five cell counts were taken microscopically in the haemocytometer after 5-15 minutes.

2.4.3.2. METHOD II: THE COULTER COUNTER[®]

Each well was washed twice with Dulbecco's phosphate buffered saline (PBS). The chondrocytes were released from the plates by incubating in serum-free MEM containing trypsin (0.4 mg/ml) and bacterial collagenase type IA (10 mg/ml) at 37°C for 1 hour. After centrifugation at 250 g in an MSE Mistral 2000 bench centrifuge for 3 minutes, the supernatant was discarded. The cells were resuspended in 5 ml fresh MEM and passed through a 40 μ m cell strainer (Becton Dickinson Labware, New Jersey, U.S.A). An aliquot of 100 μ l of cell suspension was diluted ten times in PBS. An aliquot of 100 μ l of the diluted sample was added to 10 ml of isoton. Five cell

counts were performed using the Coulter Counter®.

2.4.4. TRANSMISSION ELECTRON MICROSCOPY

The ultrastructural characteristics of cultured epiphyseal chondrocytes were studied by transmission electron microscopy (TEM). TEM is used to view ultrathin sections of a tissue sample. In TEM, a beam of electrons is emitted by an electrically heated tungsten filament (cathode). By maintaining the electric potential of the cathode at 50000-100000 V, the beam of electrons is accelerated towards the anode and is focused onto the tissue sample by a magnetic condenser lens. As the electrons pass through the specimen, a series of objective and projector lenses direct them onto a viewing screen or photographic film. Viewing the image depends on the differential scattering of the electron beam by different molecules in the specimen. Prior to tissue processing for TEM, the specimen has to be fixed to preserve the integrity of cellular components. Preservation is achieved by fixing the tissue chemically in glutaraldehyde, which cross-links and immobilises proteins, and osmium tetroxide, which cross-links the lipid components of membranes. Osmium tetroxide also enables the visualisation of different cellular components as it stains preferentially membranes and certain macromolecules, which therefore appear dark in the micrograph. Once fixed, the tissue needs to be embedded in a supporting material before sections are cut for microscopic studies. The tissue is first dehydrated in a series of successively more concentrated solutions of alcohol, followed by a transfer into xylene or propylene oxide and subsequent infiltration with an embedding medium such as waxes or epoxy resins. The tissue is embedded in resin at a high temperature to allow the polymerisation of the embedding material into a hard block. The specimen is ready for sectioning (usually less than 0.1 μm thick) with an ultramicrotome and sections are mounted on fine copper grids for subsequent examination.

2.4.4.1. METHOD

The culture medium was removed and cultures were washed three times with PBS. Cultures were fixed in 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH

7.3), which had been prewarmed to 37°C before use. After two hours, cultures were washed several times in the above buffer with added 0.3 mM calcium chloride, carefully peeled from the culture dishes, cut into small rectangles (approximately 4x5mm) with a sharp blade under a dissecting microscope, and transferred to glass vials for processing. The tissue was post-fixed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.3) for one hour at 4°C, rinsed in buffer and dehydrated in an ascending series of alcohol. After two 15-minute incubations in propylene oxide, the tissue was infiltrated with a 1:1 mixture of propylene oxide and Taab epoxy resin (Taab laboratories Equipment Ltd., Aldermaston, U.K.) for one hour, and left overnight in a 1:3 mixture at 4°C on a rotator. Tissues were subsequently given three 1-hour changes of neat resin at 48°C, placed in flat embedding moulds with three or four rectangles overlying each other, and polymerised at 60°C for 72 hours. Suitable areas were selected for ultrathin sectioning and pale gold sections were mounted on copper grids, contrasted with uranyl acetate and lead citrate. Sections were examined in a Philips EM 301 electron microscope at an accelerating voltage of 60 kV.

2.4.5. PROCESSING, PARAFFIN EMBEDDING AND SECTIONING OF CELL LAYERS

The culture medium was removed from the culture dishes. The cell layers were washed three times with PBS and fixed in 10% buffered formaldehyde at 4°C for 30 minutes. The cell layers were processed using an automatic carousel tissue processor (Shandon Citadel). The samples were put into cassettes, which were in turn placed in a tissue basket and loaded on the processor. The processing programme used was set in the following order: one change of 70% industrial methylated spirit (IMS) (2 hours), one change of 95% IMS (2 hours), six changes of 99% IMS (2 hours each), two changes of 100% xylene (1 hour each), two changes of molten wax at 58-60°C (2 hours each), and two changes of molten wax under vacuum (15 minutes each). The processed cell layers were removed from the processor and embedded in paraffin wax. Paraffin wax sections (5 µm thick) were cut using a sledge-microtome and collected on glass slides previously coated with 3-aminopropyl-triethoxysilane. All slides were left at 50°C to dry overnight.

2.4.6. DEWAXING AND HYDRATING PARAFFIN WAX SECTIONS

Paraffin wax sections were incubated at 80°C for about 30 minutes until the wax has melted. The sections were dewaxed by placing them in three changes of xylene (5 minutes each). The sections were rehydrated in two changes of 100% IMS, one change of 95% IMS, followed by a rinse in cold running tap water, and used for subsequent histological staining as required.

2.4.7. HAEMATOXYLIN AND EOSIN STAINING

The Haematoxylin and Eosin stain is a simple histological technique used to demonstrate the cell organisation and tissue structure in a particular specimen. The method is based on the fact that Haematoxylin binds specifically to the DNA and RNA content in the nucleus such that cell nuclei stain dark blue. In contrast, Eosin stains the cytoplasm and most connective tissues in varying intensities of pink or red.

2.4.7.1. METHOD

Paraffin wax sections were dewaxed and hydrated as described in Section 2.4.6. Sections were stained in Mayer's haematoxylin for 5 minutes, and rinsed in cold running tap water until clear. Sections were placed in hot tap water for 5 minutes, and stained in slightly acidified Eosin for 30 seconds. Sections were rinsed rapidly in tap water and left to air-dry before being mounted with Loctite® adhesive.

2.4.8. IMMUNOHISTOCHEMISTRY FOR THE DETECTION OF TYPE I COLLAGEN

Immunohistochemistry is employed to identify the presence of particular antigens in a tissue. It relies on the specific recognition of the antigen by its antibody, which strongly binds to the antigen. The antibody is usually labelled by conjugating it with either a fluorochrome or an enzyme, such as peroxidase, and the label, either a fluorescence or an enzyme activity given by the production of a coloured reaction, is detected at those sites at which antigen-antibody complexes have been formed. It is therefore possible to localise microscopically the expression of the protein of interest

in the tissue. In this study, the "peroxidase-label" method of detection was used. The principle of the immunohistochemical technique employed is illustrated in figure 2.2. Prior to the application of the primary antibody, it is necessary to block the endogenous peroxidase activity present in the tissue by adding hydrogen peroxide to the sample. It is also essential to block any non-specific binding sites present in the tissue section by incubating the section with serum from the species in which the secondary antibody was made, in this case rabbit. Serum proteins bind to any non-specific sites, thereby blocking any potential non-specific binding with the primary antibody. The section is incubated subsequently with the unlabelled primary antibody at the appropriate concentration. In order to increase the sensitivity of the method of detection, a hydrogen peroxidase-conjugated secondary antibody is added, which binds to the species in which the primary antibody was made, in this case guinea pig. The final step in the procedure is to detect the peroxidase activity by exposing the hydrogen peroxidase conjugated with the secondary antibody to its substrate, hydrogen peroxide (H_2O_2). Oxygen released in this enzymatic reaction reacts with 3, 3'-diaminobenzidine (DAB) to give an insoluble brown precipitate (Trojanowski *et al.*, 1983).

2.4.8.1. METHOD

The culture medium was removed from the tissue culture dishes and the cultures were washed three times with PBS. The cells were fixed in ice-cold methanol for 10 minutes. After rinsing in 0.05 M TBS, the cells were incubated with 3% hydrogen peroxide solution for 5 minutes to block any endogenous peroxidase activity. The cells were rinsed in distilled water followed by 0.05 M TBS prior to incubation with normal rabbit serum (1:5 dilution in 0.05 M TBS) for 20 minutes. The cells were incubated with a 1:100 dilution of the primary antibody against bovine type I collagen for 30 minutes at room temperature. TBS was used instead of the antibody as negative control. After rinsing with 0.05 M TBS, the cells were incubated with a 1:100 dilution of hydrogen peroxidase-conjugated rabbit anti-guinea pig secondary antibody for 1 hour at room temperature. The cells were washed in 0.05 M TBS and incubated with DAB / H_2O_2 solution (one DAB tablet and one Urea Hydrogen

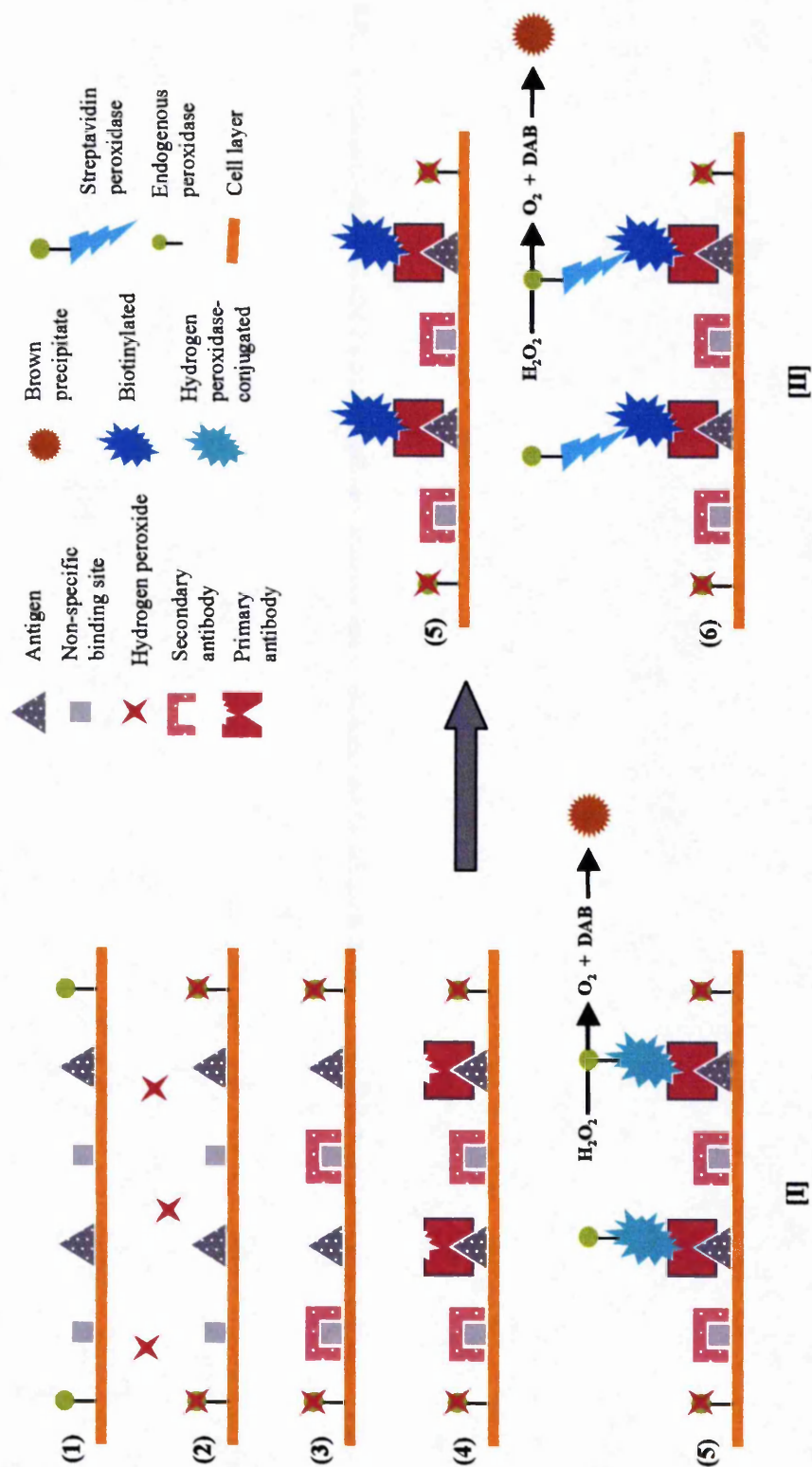


Figure 2.2. Schematic diagram illustrating the principles of immunohistochemistry with peroxidase label. (1-2) Addition of hydrogen peroxide to block any endogenous peroxidase activity. (3) Addition of serum from the species in which the secondary antibody was made to block non-specific binding sites. (4) Addition of primary antibody (5) Addition of the secondary antibody which can be [I] hydrogen peroxidase-conjugated or [II] biotinylated. (6) Addition of streptavidin-peroxidase which binds to the biotinylated secondary antibody. In both [I] and [II], peroxidase reacts with its substrate, hydrogen peroxide (H_2O_2), to release oxygen (O_2) which in turn reacts with DAB to produce an insoluble brown precipitate.

Peroxide tablet in 5 ml of distilled water) for 10 minutes. After rinsing in tap water, the cells were counterstained in Mayer's haematoxylin for 30 seconds followed by 0.05 M TBS for 30 seconds, rinsed in distilled water, air-dried, and finally mounted with Loctite[®] adhesive.

2.4.9. IMMUNOHISTOCHEMISTRY FOR THE DETECTION OF TYPE II COLLAGEN

A slight variation of the immunohistochemical method described in Section 2.4.8. was used to detect the expression of type II collagen in chondrocyte cultures. In this case, the secondary antibody used is biotinylated and as shown in figure 2.2, the cells were incubated with streptavidin peroxidase after exposure to the secondary antibody. Streptavidin has a very high affinity for biotin and therefore binds strongly to the biotin-labelled secondary antibody. The peroxidase activity is detected in the same manner as previously described.

2.4.9.1. METHOD

The same procedures were followed as those described for type I collagen with the following alterations. The cells were incubated with normal swine serum (1:5 dilution in 0.05 M TBS) for 20 minutes to block non-specific binding sites, followed by a polyclonal antibody against bovine type II collagen (1:200 dilution in 0.05 M TBS) for 30 minutes at room temperature. The cells were incubated with biotinylated swine anti-rabbit secondary antibody (1:300 dilution in 0.05 M TBS) for 1 hour at room temperature. After rinsing with 0.05 M TBS, the cells were incubated with streptavidin (1:500 dilution with 0.05 M TBS) for 30 minutes at room temperature. The cells were washed in 0.05 M TBS before incubating with DAB / H₂O₂ solution (one DAB tablet and one Urea Hydrogen Peroxide tablet in 5 ml of distilled water) and processed as previously described.

2.4.10. PHOTOGRAPHY OF CHONDROCYTES

All chondrocytes were photographed using a phase-contrast microscope (Leitz Labovert) fitted with a 35 mm Olympus camera.

2.5. RESULTS

2.5.1. GROWTH CHARACTERISTICS OF FOETAL BOVINE EPIPHYSEAL CHONDROCYTE CULTURES

Bovine epiphyseal cartilage was isolated from long bones of bovine foetuses in regions well away from the growth plate and from secondary centres of ossification. Epiphyseal chondrocytes were released subsequently from the cartilage by collagenase digestion. On average, approximately 8×10^6 cells were obtained per gram of epiphyseal cartilage. The percentage viability of the released cells was at least 95% for each experiment performed. When plated-out on plastic, the cells adhered to the surface within an hour. It was observed that at a density of 0.5×10^6 cells per cm^2 , most of the chondrocytes retained their characteristic round shape, whilst at lower densities, they lost their round phenotype and acquired a fibroblastic morphology (Figure 2.3). It was noted that these above observations were not influenced by the age of the bovine foetuses.

Chondrocytes were released from the culture surface by trypsinisation. It was observed that in general, during the first two days, a small proportion of cells (aza-C treated and untreated) was lost when the culture medium was changed. These cells probably failed to adhere to the plastic surface of the culture dishes after plating. However, after about four to five days in culture, a disc-shaped cell layer was formed, which could be collected with sterile forceps. These cell layers (aza-C treated and untreated) increased in thickness with time in culture as the cells proliferated and produced an extracellular matrix in culture. Consequently, multi-layers of cells were formed in both treated and untreated cultures. The harvested cell layer was digested with collagenase to release the chondrocytes. Cell counts were carried out using the Coulter Counter®.

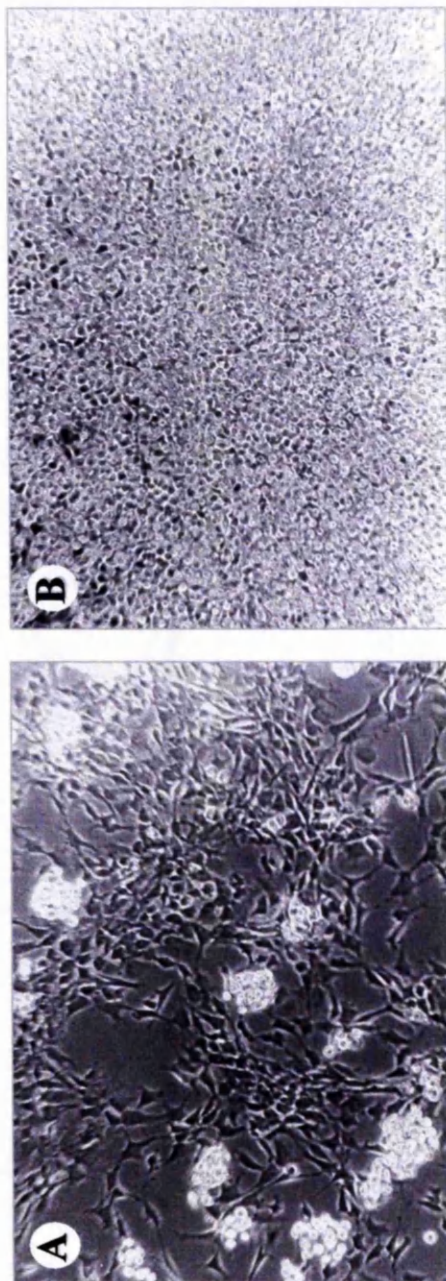


Figure 2.3. Effect of plating-density on the morphology of foetal bovine epiphyseal chondrocytes. Phase contrast micrographs of chondrocytes cultured at low density ($< 0.5 \times 10^6$ cells per cm^2) [A] and high density ($> 0.5 \times 10^6$ cells per cm^2) [B] in medium containing 10% FCS were taken. Note the fibroblastic morphological appearance of chondrocytes at low densities and their round chondrocytic shape at high densities. (Magnification: $\times 188$).

Growth curves were plotted for treated and untreated cultures. Both treated and untreated cultures exhibited similar growth profiles (Figure 2.4). There was an initial decrease in the cell number immediately after plating-out followed by an increase by day 2-3. By the 10-11th day in culture, the cell number reached a constant level and remained unchanged over a ten-day period. These experiments were repeated at least three times and similar growth profiles were obtained for both treated and untreated cultures for each experiment. An initial drop in cell number was obtained followed by a recovery to the starting plating density. However, it should be noted that the apparent protection of aza-C treated cells from cell death between days 1 and 5 was not a consistent finding. As will be discussed in Chapter Five, the occurrence of cell death by apoptosis in culture was investigated in time-course experiments. However, such studies were not carried out on day 0.

2.5.2. EFFECTS OF 5-AZACYTIDINE TREATMENT ON CHONDROCYTE PHENOTYPES IN CULTURE

When foetal bovine epiphyseal chondrocytes were freshly isolated and plated out, they appeared spherical and dispersed rather uniformly on culture dishes. In order to assess the differentiation status of these chondrocytes in culture with and without azacytidine treatment, the expression of collagen types II and I was studied using antibodies specific for types II and I respectively. Type II collagen is normally associated with a differentiated phenotype of chondrocytes while type I collagen is considered to be the marker of dedifferentiation. These immunohistochemical studies showed the presence of type II collagen in the extracellular matrix with and without azacytidine treatment (Figure 2.5). Conversely, type I collagen was absent in all cultures at the time-points studied (Figure 2.5). It should be noted that a positive control should have been included for the immunohistochemical analyses with the type I collagen antibody to further confirm the absence of type I collagen in the sections studied.

Paraffin wax sections of chondrocyte cell layers were cut and used for histological examination under the light microscope. Haematoxylin and Eosin staining of the cut sections revealed a cartilage-like structure where chondrocytes were surrounded by an

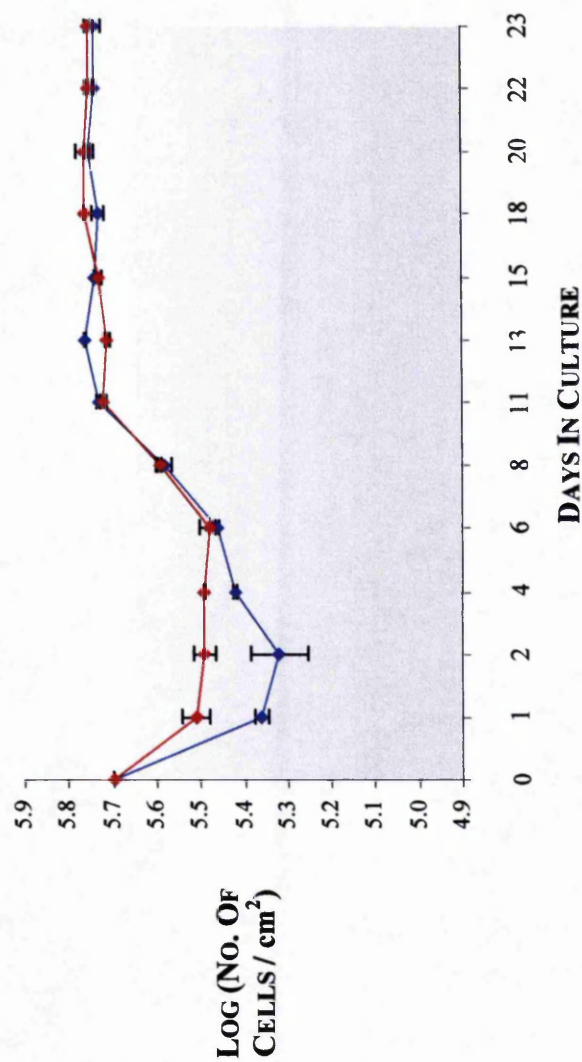


Figure 2.4. Growth profiles of treated and untreated foetal bovine epiphyseal chondrocyte cultures. Chondrocytes were plated in 9 cm²-plastic culture dishes at a density of 0.5×10^6 cells per cm², either in the presence (—◆—) or absence (—◆—) of 15 µg/ml 5-azacytidine. Cell numbers were determined using the Coulter Counter®. Values represent the mean \pm SEM ($3 \leq n \leq 5$, where n is the number of counts performed at each time-point).

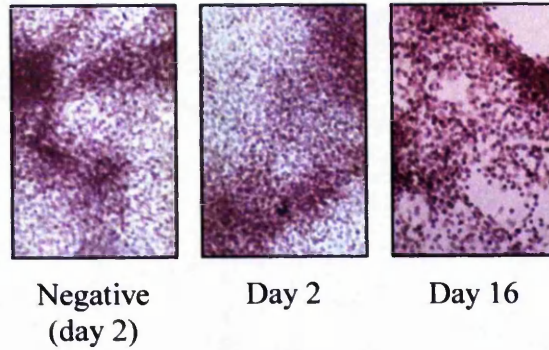
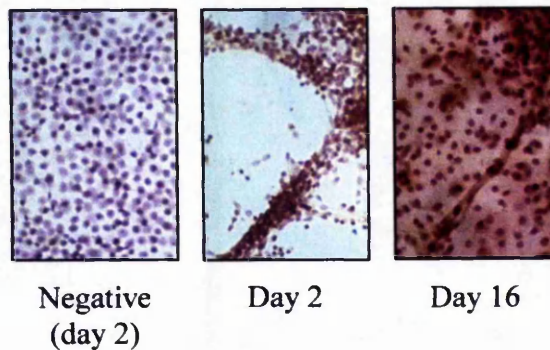
(A) Type I collagen: Aza-C treated cultures**(B) Type II collagen: Aza-C treated cultures**

Figure 2.5. Immunohistochemical analyses of foetal bovine epiphyseal chondrocytes in culture. Cultures of epiphyseal chondrocytes were collected after 2 and 16 days in culture and immunostained using antibodies against collagen types I and II. Negative controls were incubated with TBS buffer. Type I collagen was absent in aza-C treated cultures at all time-points analysed (A). Similar results were obtained in untreated cultures. Type II collagen was expressed both in the presence and absence of aza-C treatment at the time-points studied with an increase in expression with time in culture (B). (Magnification: $\times 240$).

extracellular matrix (Figure 2.6). The cell layers of both untreated and treated cultures could be divided into two populations of cells. The first population consisted of cells found on the surface of the cell layer and therefore, in direct contact with the culture medium. These cells were flattened in shape and were arranged in a one to two cell-thick layer. The second cell population consisted of chondrocytes found in deeper areas of the cell layer. These cells were rounded in shape and were present either singly or in pairs in separate "lacunae".

The ultrastructural characteristics of cultured chondrocytes were analysed by transmission electron microscopy to confirm their chondrocytic phenotype both with and without aza-C treatment. The presence of two distinct cell populations was again evident. The "surface" cells appeared flattened and were surrounded by a relatively sparse extracellular matrix (Figure 2.7). In contrast, cells in the inner areas of the cell layer showed a rounded morphology. These chondrocytes were located in "lacunae" surrounded by a distinct pericellular and finely fibrillar territorial matrix (Figure 2.8). The interterritorial matrix was dense and consisted of randomly arranged collagen fibrils. Proteoglycan granules were found to decorate the collagen fibrils at regular intervals (Figure 2.8). In all cases, cells were healthy and viable as shown by their maintenance of intact plasma membrane and cytoplasmic organelles and a prominent nucleus and nucleoli (Figure 2.9).

2.5.3. MORPHOLOGICAL DEVELOPMENT OF FOETAL BOVINE EPIPHYSEAL CHONDROCYTES UNDER 5-AZACYTIDINE TREATMENT

Photographs of both treated and untreated cells were taken at regular time-points to analyse any possible effect of aza-C on cell morphology. Bovine epiphyseal chondrocytes were released from the cell layers by trypsinisation and collagenase digestion, placed in the haemocytometer and viewed under the light microscope. When epiphyseal chondrocytes were treated with aza-C, they exhibited changes in morphology within a few days in culture. As shown in figure 2.10, by day 6, some of the treated cells had already started to increase in cell size while no such increase was apparent in the untreated cells at the same time-point. At all subsequent time-points

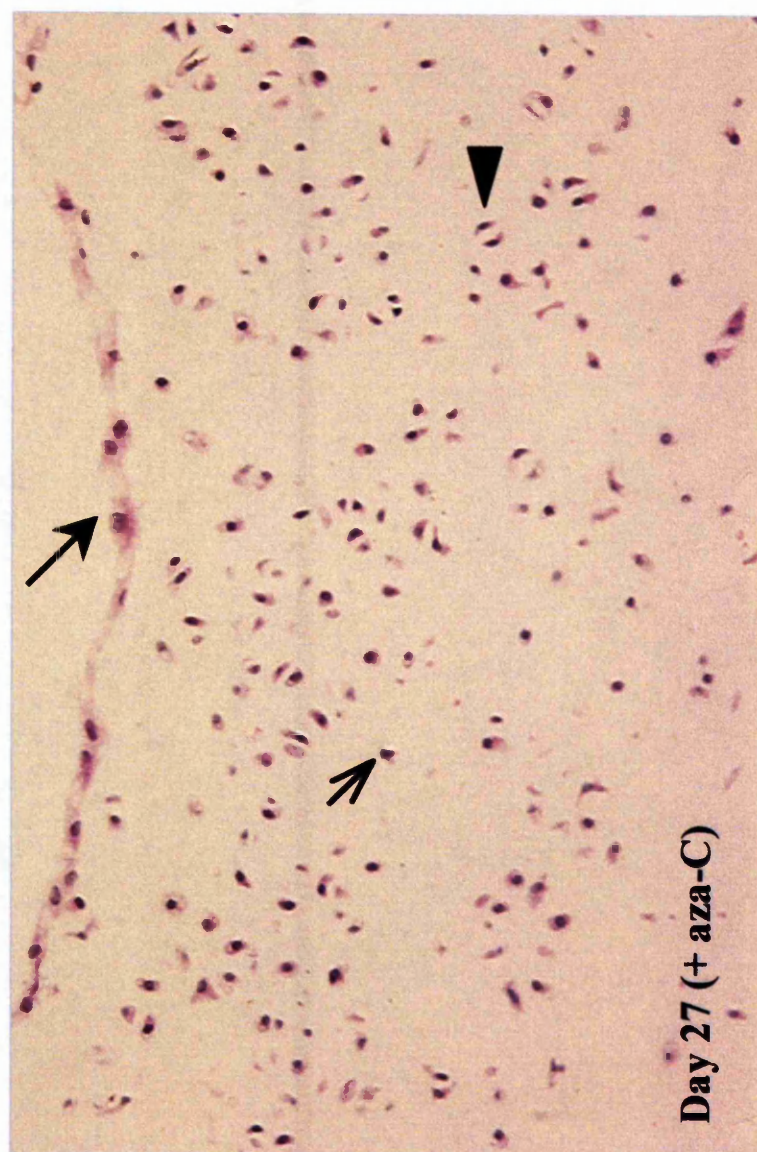


Figure 2.6. Haematoxylin and Eosin stain of foetal bovine epiphyseal chondrocyte cultures. Paraffin wax sections of chondrocyte cultures were stained as described under Methods. Both aza-C treated and untreated cultures showed a structure similar to cartilage *in vivo*. Note the thin layer of flattened cells at the surface of the cell layer (arrow), the presence of round chondrocytes in the inner areas of the cell layer, either singly (open arrow) or in pairs (arrowhead). (Magnification: x242).

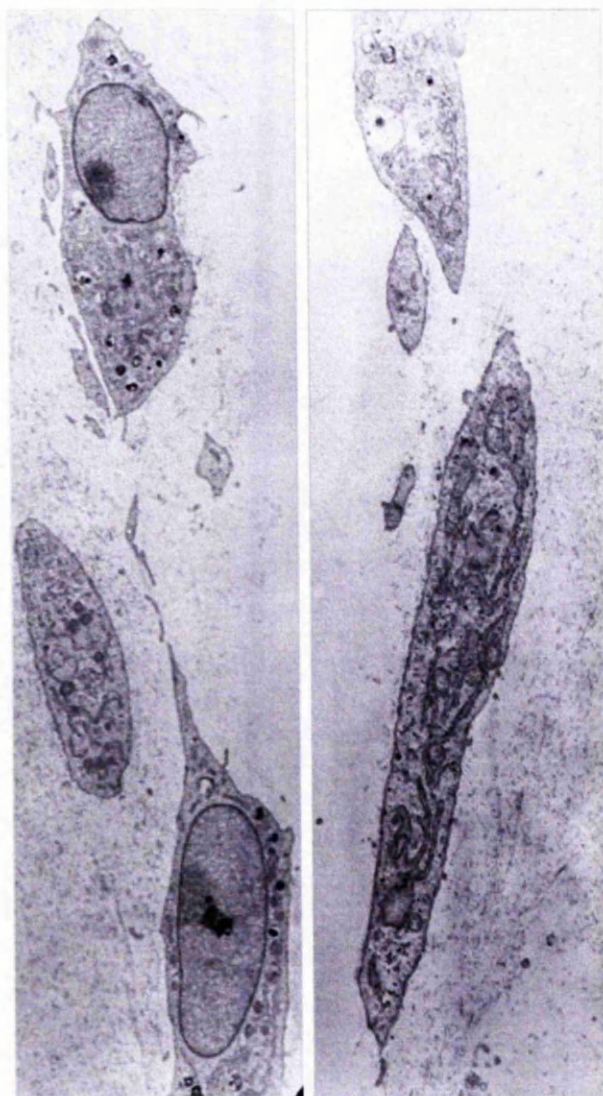


Figure 2.7. Electron micrographs of “surface” cells. Note the sparse extracellular matrix surrounding the cells. Photographs show aza-C treated chondrocytes taken after 20 days in culture and are typical representations of flattened cells lying at the surface of cell layers in both aza-C treated and untreated cultures. (*Magnification: x32000*).

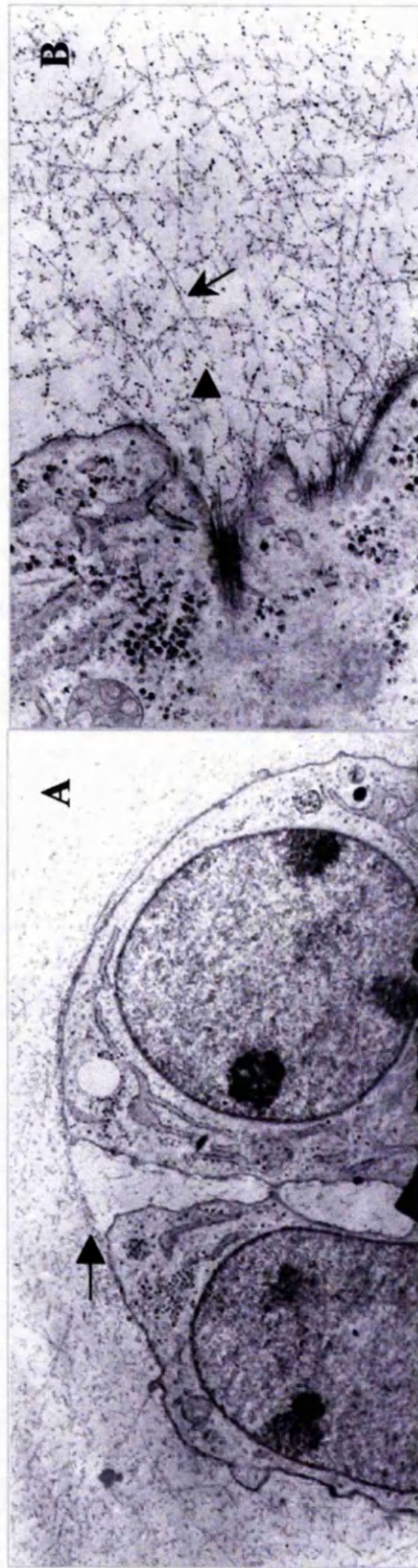


Figure 2.8. (A) Electron micrograph of a pair of "inner" chondrocytes located in lacunae. Chondrocytes found in the deeper areas of the cell layer are round in shape. Note the separation of the territorial extracellular matrix from the interterritorial matrix by a capsular structure consisting of fine fibrils (arrow). (Magnification: x7915). **(B) Electron micrograph showing the extracellular matrix produced by chondrocytes with time in culture.** The extracellular matrix is dense and consists of randomly organised collagen fibrils (arrow) in close association with smaller structures resembling proteoglycans (arrowhead). (Magnification: x11875). All photographs were taken of cells after 20 days in culture and are typical representations of chondrocytes throughout the culture period.

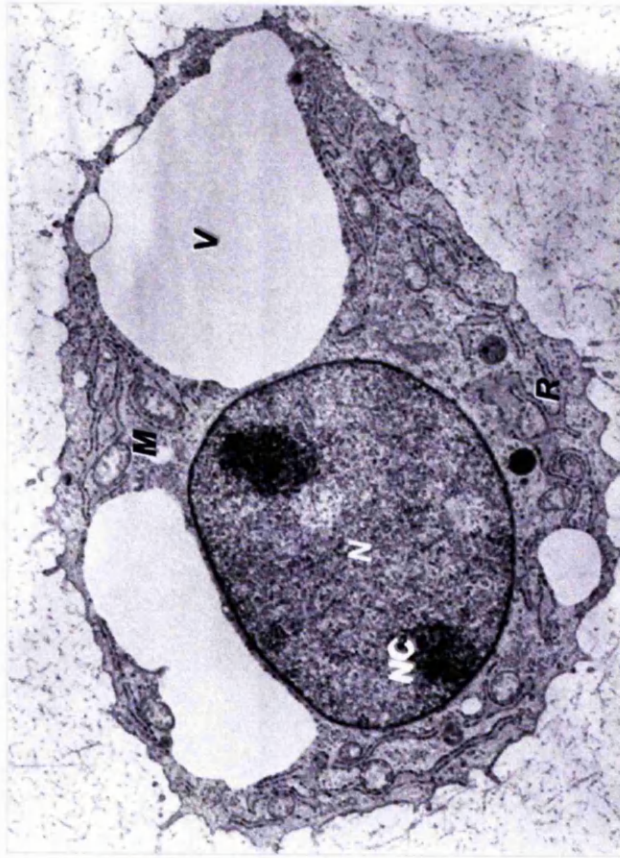


Figure 2.9. Electron micrograph of a typical foetal bovine epiphyseal chondrocyte cultured in monolayers in the presence of 10% FCS. Chondrocytes were viable both with and without azacytidine treatment, as shown by the presence of cytoplasmic organelles including a well-formed nucleus (N) containing one or two nucleoli (NC), rough endoplasmic reticulum (R), mitochondria (M), and vacuoles (V). (*Magnification: x8875*). The photograph shows an aza-C treated chondrocyte taken after 20 days in culture.

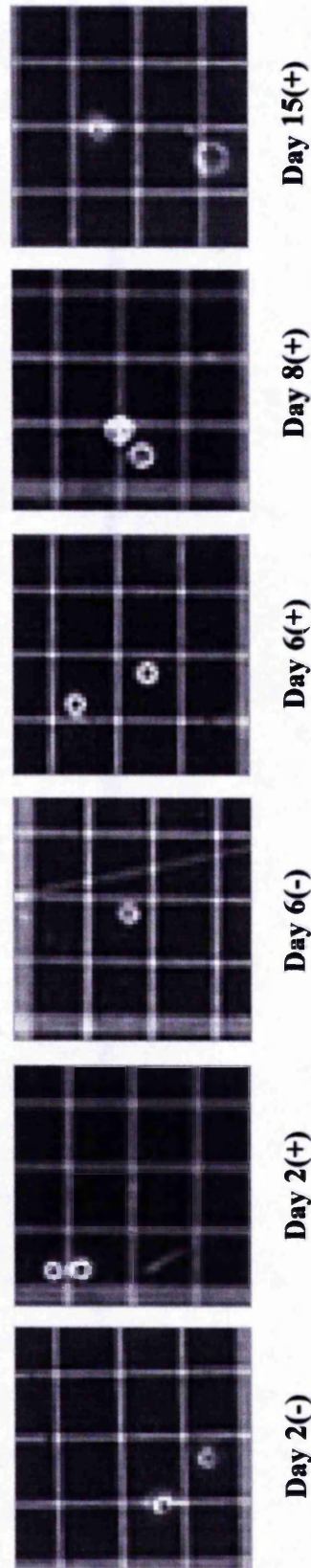


Figure 2.10. Morphological changes of chondrocytes under 5-azacytidine treatment. Foetal bovine epiphyseal chondrocytes were cultured with and without 5-azacytidine (aza-C) treatment at a concentration of 15 $\mu\text{g/ml}$. Cells were released by trypsinisation at regular time-points in culture, placed on the haemocytometer, and viewed under the light microscope. Photomicrographs taken show an increase in cell size with time in culture, starting at around days 6-8 in aza-C treated cultures. No such increase in cell size was apparent in untreated cultures throughout the culture period, as shown by "day 2" photomicrograph which is representative of all time-points studied in untreated cultures. All the photomicrographs shown are representative of photos taken in three separate experiments (Magnification: $\times 500$).

studied, treated cells were larger in size than their untreated counterparts. Cell photographs and measurements of cell size were taken on at least three separate experiments and a similar pattern of increase in cell size was observed on each occasion.

In order to quantify the relative increase in cell volume during the culture period, the diameters of both untreated and treated cells were measured directly from the photographs taken at regular time-points. Since the chondrocytes were released in suspension, it was assumed that they were spherical in shape. The cell volumes were calculated from their corresponding cell diameters measured. Using a ruler, the cell diameters as well as the width of one of the smallest squares of the haemocytometer were measured directly from enlarged photographs of the cells placed on the haemocytometer. Since the actual width of the smallest square of the haemocytometer is known, the actual cell diameter and subsequently, on the assumption that the cell is spherical in shape, the cell volume can be calculated as follows:

Cell diameter (photo)	=	a
Width of smallest square (photo)	=	b
Actual width of smallest square	=	0.05 mm
Actual cell diameter	=	$(a/b) \times 0.05$
	=	d
Actual cell radius, r	=	$d/2$
Actual cell volume	=	$4/3\pi r^3$

A graph of cell volume (μm^3) against days in culture was plotted (Figure 2.11). A steady increase in the cell volume of treated chondrocytes was observed compared to the controls, reaching a 14-fold increase by day 15 in culture. This increase in cell size appears similar to the marked cell enlargement that occurs during hypertrophy in the growth plate *in vivo* (Breur *et al.*, 1991).

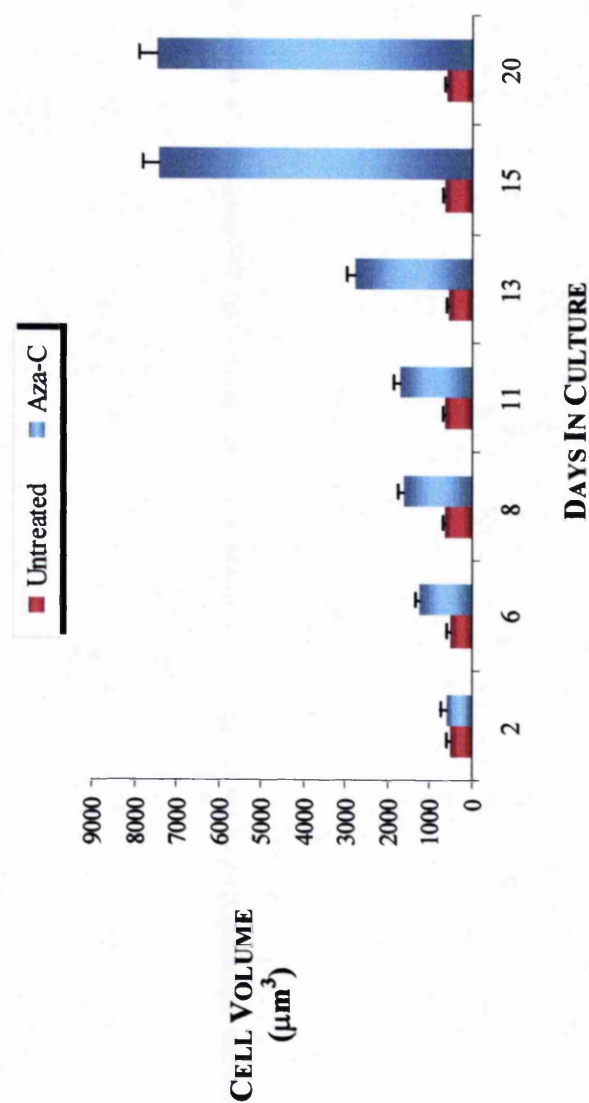


Figure 2.11. Effect of 5-azacytidine on cell size with time in culture. The diameters of azacytidine-treated and untreated chondrocytes were measured at each indicated time-point. Their cell volumes were calculated on the assumption that chondrocytes were spherical in shape. Values represent mean \pm SEM ($n=10$, where n is the number of measurements taken at each time-point). Results shown in this graph are representative of those obtained from three separate experiments.

The ultrastructure of treated and untreated chondrocytes was examined by transmission electron microscopy and compared with a view to assess the expression of cellular characteristics of hypertrophy. It was observed that epiphyseal chondrocytes treated with aza-C showed several characteristics indicative of hypertrophy (Figure 2.12). Their nuclei were generally euchromatic suggesting that the cell transcriptional machinery is active. The cells also contained a large number of mitochondria, dilated rough endoplasmic reticulum, and prominent Golgi apparatuses and vesicles, all indicating a high level of protein synthesis and secretion. In contrast, untreated chondrocytes showed features similar to those of resting cells (Figure 2.12). They contained only moderate amounts of cellular organelles with few rough endoplasmic reticulum and Golgi vesicles and their nuclei contained a condensed chromatin. The increase in cell size of treated chondrocytes was also apparent.

2.6. DISCUSSION

Many different culture systems have been used to grow chondrocytes. It is well established that the initial cell-plating density as well as the growth substrata used play a crucial role in the determination and maintenance of the morphological status of the cell. In this study, foetal bovine epiphyseal chondrocytes were grown on plastic in high-density monolayer cultures. The current view is that the chondrocytic phenotype is stabilised by culture in agarose (Benya and Shaffer, 1982), in collagen gels (Gibson *et al.*, 1984) or in suspension (Castagnola *et al.*, 1986). When grown at low density in monolayer culture, chondrocytes dedifferentiate and acquire a fibroblastic phenotype (Castagnola *et al.*, 1988; Holtzer *et al.*, 1960). Results obtained in this study show that increasing the initial cell-plating density in monolayer culture leads to confluency of the cells, which allows them to change to a rounded morphology. It has been shown that when chondrocytes are grown at low density in monolayer culture and subsequently transferred to agarose-coated dishes or collagen gels, they assume their normal rounded morphology (Castagnola *et al.*, 1986; Gibson *et al.*, 1984). It is possible that in monolayer culture, cell-cell contacts are essential in the maintenance of the normal rounded phenotype of chondrocytes, at least during the early stages of the culture until an extracellular matrix is produced by the cells. In

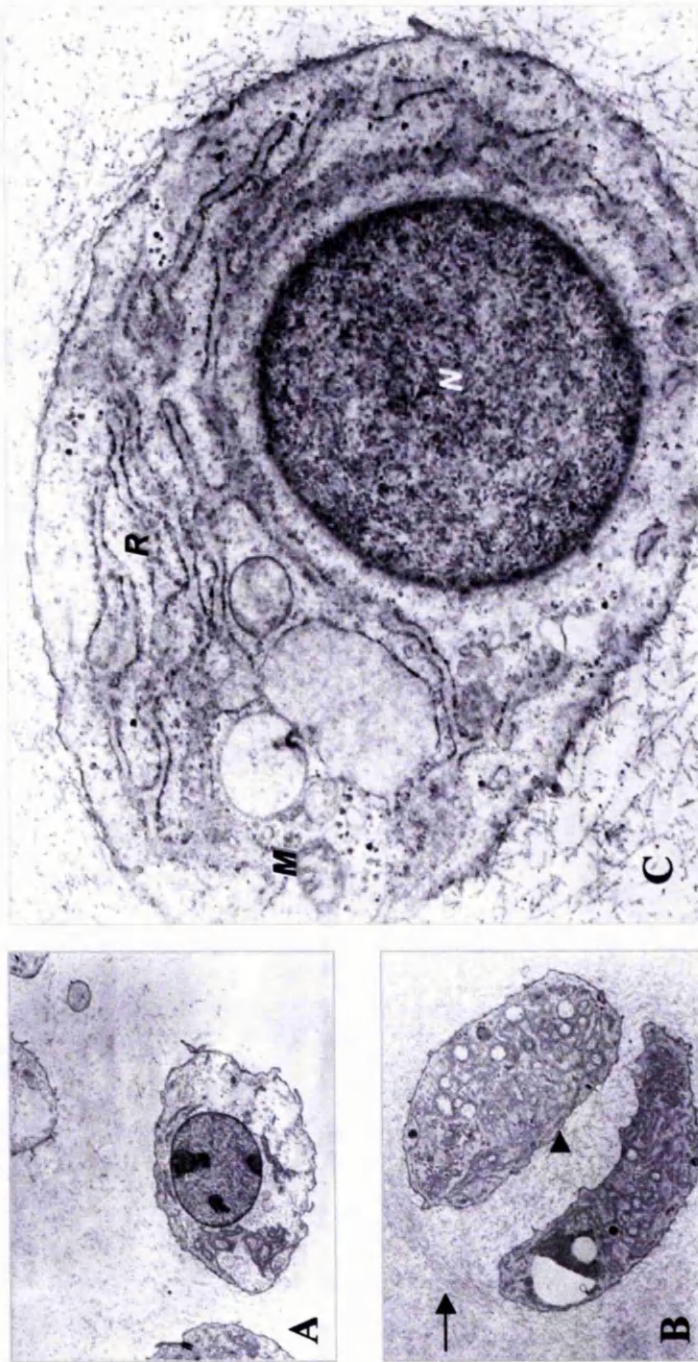


Figure 2.12. (A) Electron micrograph of a foetal bovine epiphyseal chondrocyte grown in the absence of 5-azacytidine treatment. The chondrocyte has relatively few cytoplasmic organelles and its nucleus contains condensed chromatin, all of which indicates that the cell is in a "resting" stage. (Magnification: x3100). **(B) Electron micrograph of a pair of foetal bovine epiphyseal chondrocytes in cultures treated with 5-azacytidine for 48 hours after plating.** The cells contain a large number of cytoplasmic organelles, including a prominent Golgi apparatus (arrowhead). Also note the pericellular concentration of extracellular matrix, which has a finely fibrillar appearance (arrow). (Magnification: x3100). **(C) Electron micrograph showing the ultrastructural characteristics of a hypertrophic chondrocyte after 5-azacytidine treatment.** Note the presence of abundant rough endoplasmic reticulum (R), mitochondria (M) and a euchromatic nucleus with dispersed chromatin (N). (Magnification: x18750). All photographs were taken of cells after 20 days in culture.

contrast, agarose or collagen gel culture systems provide the chondrocytes with a three-dimensional environment that is very similar to the cartilage tissue organisation *in vivo*.

Previous studies on collagen gene expression during chondrocyte differentiation in bovine growth plate cartilage made use of aza-C as an experimental tool to induce the maturation cascade *in vitro* (Manning, 1994). Manning (1994) showed that treatment of bovine epiphyseal chondrocytes with aza-C at concentrations higher than 15 µg/ml for 48 hours proved to be cytotoxic and led to a considerable decrease in cell viability. Several possibilities can explain the cytotoxic effect of aza-C. Aza-C is a potent demethylating agent that can induce the expression of several genes (Darmon *et al.*, 1984; Creusot *et al.*, 1982; Taylor and Jones, 1979). Increased drug concentrations may cause a more global induction of gene expression that in turn can lead to an over-expression of proteins including those responsible for cell lysis. It is known that aza-C inhibits DNA methylation by binding irreversibly to the enzyme, DNA methyltransferase (Christman *et al.*, 1985; Santi *et al.*, 1984). This covalent complex is degraded normally due to its toxicity to the cell (Jutterman *et al.*, 1994). Increased concentrations of aza-C may cause a corresponding increase in the level of the toxic complex that is too high to be cleared by the degradation machinery of the cell. It has been reported that aza-C is capable of causing minor damage in DNA (Stopper *et al.*, 1992; Schmuck *et al.*, 1986). Therefore, it is possible that increased drug concentrations may cause excessive base pair damage that cannot be overcome by the excision repair mechanisms of the cell. Another possible consequence of this considerable base pair damage is the occurrence of double-stranded breaks in DNA that is lethal to the cell (Friedberg *et al.*, 1985).

The present study focused on the isolation of foetal bovine epiphyseal chondrocytes and the subsequent establishment of a culture system with a view to adopt the aza-C-treatment of cells to mimic chondrocyte differentiation that occurs in the growth plate. After animal dissection, epiphyseal cartilage was digested with collagenase and trypsin to release epiphyseal chondrocytes. Both treated and untreated cultures presented similar growth profiles, which indicates that treatment with aza-C does not

affect the normal proliferation status of chondrocytes in culture. The initial decrease in cell number could be due to several reasons. On plating-out, a small number of chondrocytes might fail to attach to the plastic surface and therefore, be lost each time the culture medium was changed during the time-course experiments. Some cells could fail to adjust to the new environmental conditions *in vitro* and therefore, be unable to survive in culture immediately after being removed from the cartilage tissue. Some cell death could occur as a result of the trypsinisation process prior to each cell count. The subsequent increase in cell number could be accounted for by the chondrocytes having adapted to the culture conditions imposed on them and being able to proliferate. It is known that the presence of ascorbic acid in culture promotes the formation and maintenance of an extracellular matrix *in vitro* (Gerstenfeld and Landis, 1991). It is likely that the addition of ascorbic acid to the present culture system also induces extracellular matrix synthesis in culture which might contribute to providing the chondrocytes with an environment that is conducive to cell survival and proliferation. By day 10, the majority of chondrocytes had ceased to proliferate in culture. It is possible that the maximum culture capacity of the culture dishes had been reached, thus possibly explaining the constant cell density obtained at this time-point. It can be suggested tentatively that by day 10, the majority of chondrocytes had possibly reached the hypertrophic stage of differentiation in aza-C treated cultures. This hypothesis was verified and is discussed in later chapters.

It was important to examine the chondrocytic phenotype of untreated cultures under the culture conditions used in this study and also to check if aza-C leads to cell dedifferentiation or maintains the differentiation status of chondrocytes in culture. The expression of type I collagen usually indicates a dedifferentiated state of chondrocytes whereas type II collagen is considered to be the marker of the chondrocyte differentiated phenotype. Immunohistochemical analyses in this study show the presence of type II collagen in both untreated and treated cultures while type I collagen was absent in all cultures. These observations indicate that the culture conditions used here to grow foetal bovine epiphyseal chondrocytes favour a differentiation status of chondrocytes and aza-C does not cause the cells to dedifferentiate and lose their phenotypic characteristics of chondrocytes. These

results also correlate with the round shape of chondrocytes obtained in these cultures, which is another indicator of chondrocyte differentiation.

Histological staining of both treated and untreated cultures revealed the formation of a cartilage-like structure where chondrocytes were present in "lacunae" and surrounded by an extracellular matrix. Similar tissue organisation has been described by others (Gerstenfeld and Landis, 1991; Thomas and Grant, 1988; Kuettner *et al.*, 1982b) and is reminiscent of cartilage *in vivo*. Electron microscopical studies showed viable chondrocytes containing intact cytoplasmic organelles and surrounded by an extensive interterritorial matrix composed of collagen fibrils and proteoglycans, as reported in other studies (Ruggiero *et al.*, 1993; Gerstenfeld and Landis, 1991; Bruckner *et al.*, 1989; Kuettner *et al.*, 1982b). These observations confirm the chondrocytic phenotypes exhibited by cultures both with and without aza-C treatment. Therefore, it appears that culturing foetal bovine epiphyseal chondrocytes under conditions described in this study allows the chondrocytes to grow and establish their own environment by elaborating an extracellular matrix similar to cartilage *in vivo*. It is interesting to note the presence of a small population of flattened cells at the surface of the cell layers while the "inner" chondrocytes were round in shape. The presence of flattened and round cells in the same culture has been observed previously in monolayer cultures of embryonic chick chondrocytes (Gerstenfeld and Landis, 1991) as well as in bovine articular chondrocyte cultures in alginate beads (Hauselmann *et al.*, 1994). It is unknown why cells at the surface of the cultures are flattened in shape, although their appearance resembles that of cells found in the uppermost layer of articular cartilage (Archer *et al.*, 1990; Aydelotte *et al.*, 1988). It is possible that the presence of an extracellular matrix all around the cells is necessary for the maintenance of the typical round chondrocytic morphology. Cells at the surface of the cultures are exposed to the culture medium. Consequently, they lose their round shape and form a palisade of flattened cells at the surface of the cultures. This palisade could in turn create an enclosed environment where chondrocytes in the deeper areas of the cultures are surrounded completely by matrix.

A number of previous studies have shown that aza-C has a remarkable ability to induce phenotypic changes in cells. For instance, it converts cultures of the mouse

embryo cell line, C3H10T1/2, into three distinct lineages of myoblasts, chondrocytes, and adipocytes (Taylor and Jones, 1979), and induces the transformation of teratocarcinoma-derived mesenchymal cells into epithelial cells (Darmon *et al.*, 1984). Manning (1994) showed that aza-C was able to cause cultured foetal bovine epiphyseal chondrocytes to acquire a morphology that is characteristic of growth plate chondrocytes during hypertrophy. Results obtained in the present study confirms this observation. Under normal conditions *in vivo*, epiphyseal chondrocytes do not progress to hypertrophy (Svoboda *et al.*, 1988; Eyre *et al.*, 1987). Photographs of cultured chondrocytes taken during time-course culture experiments indicate that epiphyseal chondrocytes treated with aza-C became much more enlarged than the controls during the culture period. Such an increase in cell size is characteristic of the hypertrophic stage of differentiation (Brighton *et al.*, 1973). The effect of aza-C on the morphology of epiphyseal chondrocytes *in vitro* is also clearly apparent by cell sizing and the subsequent determination of the cell volume with time in culture. Treated chondrocytes increased in volume whereas untreated cells did not. These results are in agreement with those obtained from previous studies, which demonstrated a steady increase in the cell volume as the growth plate chondrocytes progressed down the maturation pathway and reached hypertrophy (Hillarby *et al.*, 1996). In addition, the increase in the cell size of treated chondrocytes was observed by transmission electron microscopy, which also revealed ultrastructural differences between untreated and treated cells. Untreated chondrocytes exhibited characteristics of resting cells with only moderate levels of DNA transcriptional activity, low levels of extracellular protein synthesis and low metabolic rate. In contrast, treated chondrocytes appeared to have high metabolic activities and be engaged in active DNA transcription and protein synthesis, as observed in previous studies (Bruckner *et al.*, 1989). Taken together, these findings suggest that aza-C appears to have pushed the foetal bovine epiphyseal chondrocytes down the maturation pathway and to enter hypertrophy. It also provides another example illustrating the ability of aza-C to induce phenotypic changes in cells *in vitro*.

Chapter Three

CHAPTER THREE

EFFECTS OF 5-AZACYTIDINE ON THE EXPRESSION OF EXTRACELLULAR MATRIX COMPONENTS ON FOETAL BOVINE EPIPHYSEAL CHONDROCYTES

3.1. INTRODUCTION

Endochondral ossification (EO) is a highly regulated process that occurs in the growth plates of developing long bones. The growth plate is divided into well-defined cellular zones, each zone consisting of chondrocytes at a specific stage of their differentiation: (i) resting, (ii) proliferation, (iii) maturation, and (iv) hypertrophy (see Chapter One, Section 1.4.2). As the chondrocytes proceed through the differentiation pathway, the extracellular matrix of the growth plate cartilage undergoes constant degradation and reassembly. The extracellular matrix consists predominantly of proteoglycan aggregates and various cartilage-specific collagen types. Proteoglycan aggregates, which consist mainly of hyaluronan-aggreacan complexes, provide a major resistance to compressive forces as a result of their hydrophilic nature. The primary function of the collagen component of the extracellular matrix is to provide high tensile strength to the cartilage. Type II collagen is the most abundant constituent of the cartilage matrix (see Chapter One, Section 1.3.1.2.1) while collagen types IX and XI are present in smaller amounts (see Chapter One, Sections 1.3.1.2.2 and 1.3.1.2.3, respectively). The extracellular matrix also contains type VI collagen, which is found mainly around chondrocytes (see Chapter One, Section 1.3.1.2.5). On the other hand, type X collagen is expressed exclusively in the hypertrophic zone of the growth plate (see Chapter One, Sections 1.3.1.2.4 and 1.4.2.4.1). Chondrocyte hypertrophy is accompanied by an elevated expression of alkaline phosphatase and the process of mineralisation in the lower hypertrophic zone (see Chapter One, Section 1.4.2.5). Alkaline phosphatase is an enzyme that is expressed highly in the hypertrophic zone of the growth plate. Alkaline phosphatase plays an important role in the process of cartilage mineralisation that occurs in the lower hypertrophic zone where it is

concentrated mainly in matrix vesicles. The role of alkaline phosphatase in the growth plate is discussed in Chapter One, Section 1.4.2.5.

3.2. AIMS

The study discussed in Chapter Two suggests the potential use of 5-azacytidine (aza-C) to induce the differentiation of foetal bovine epiphyseal chondrocytes to hypertrophy in culture. The results obtained were based solely on the morphological examination of the chondrocyte cultures.

In order to validate the cell culture model as a faithful recapitulation of EO, the system was further characterised by examining the expression of extracellular matrix components specific to growth plate cartilage. The pattern of expression of several extracellular matrix markers of chondrocyte differentiation was studied in both aza-C treated and untreated cultures, including (i) type II collagen, (ii) type VI collagen, (iii) type X collagen, (iv) proteoglycans, and (v) the presence of calcification. The expression of alkaline phosphatase was also analysed in culture. Calcification of the extracellular matrix in culture was analysed both in the presence and absence of calcium β -glycerophosphate supplementation since calcium β -glycerophosphate has been shown to be essential for mineralisation in culture (Thomas *et al.*, 1990). Examining the expression of type X collagen in culture would provide the definitive confirmation of the hypertrophic state of cultured chondrocytes as indicated already by their morphological status obtained in Chapter Two. These strategies of investigation are outlined in figure 3.1.

All studies were carried out using time-course experiments in an attempt to construct a differentiation profile of chondrocytes with time in culture. It should be noted that the choice of time-points to be analysed was restricted to a considerable extent by the availability of bovine tissue. The supply of foetal calves from the abattoirs was limited as a result of the problems caused by the occurrence of bovine spongiform encephalopathy (BSE). Consequently, the time-points chosen varied from experiment

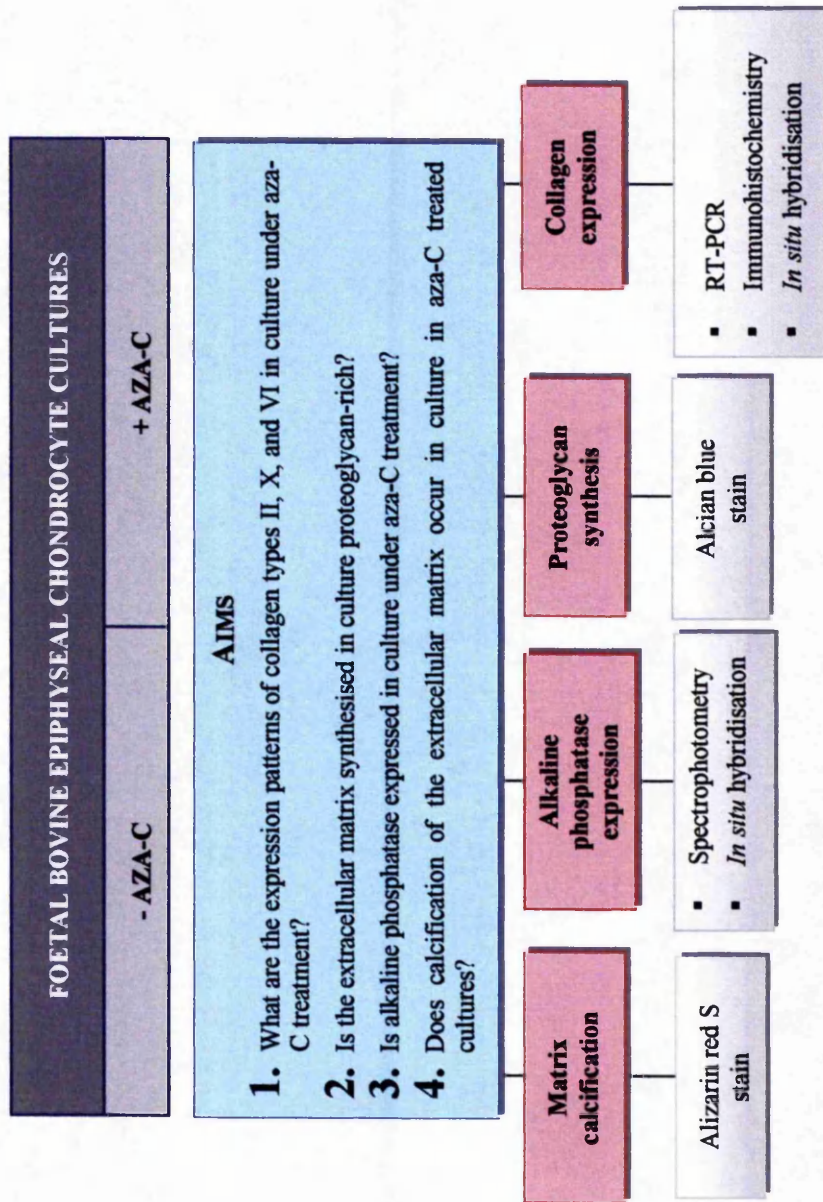


Figure 3.1. Schematic diagram showing the strategies of investigation into the expression of extracellular matrix genes in foetal bovine epiphyseal chondrocyte cultures.

to experiment depending on the marker under investigation. For example, a marker known to be expressed in hypertrophy was analysed predominantly at later time-points in culture. Conversely, a marker specifically expressed at earlier stages of chondrocyte differentiation was tested at earlier time-points in culture.

3.3. MATERIALS

Gibco BRL: Paisley, Scotland.

Dulbecco's phosphate buffered saline (without calcium and magnesium), foetal calf serum, gentamycin, *L*-glutamine, minimal essential medium (with Earle's salts and *L*-glutamine), penicillin-streptomycin solution, sodium bicarbonate (7.5%), trypsin (0.25% w/v in Gibco Solution A), acrylamide/bis solution (35% w/v).

Sigma Chemicals Co. Ltd: Dorset, U.K.

5-Azacytidine (50X; lyophilised and sterilised), bacterial collagenase type 1A, DAB peroxidase substrate tablets (SIGMA *FAST*TM), *L*-ascorbic acid, diethylpyrocarbonate, mineral oil, orange G dye, Ficoll, glycine, hyaluronidase type I-S, paraformaldehyde, polyvinylpyrrolidone, salmon sperm DNA, Sephadex G-50, dextran sulphate, acetic anhydride, dithiothreitol, NP-40, and the Sigma Diagnostics[®] Kit for alkaline phosphatase assay.

Boehringer Mannheim: East Sussex, U.K.

RNase inhibitor, reverse transcriptase (AMV), dATP, dCTP, dGTP, dTTP, Taq DNA polymerase, 1 kb ladder, agarose, RNase A.

Pharmacia Biotech Ltd.: Hertfordshire, U.K.

pd(N)₆ (random hexamer)

Oswel DNA Service: University of Southampton, U.K.

All PCR DNA primers.

BDH Laboratory Supplies: Dorset, U.K.

Hydrogen peroxide (30%), methanol, sodium chloride, chloroform, ammonium acetate, ethanol, magnesium chloride-6-hydrate, potassium chloride, orthoboric acid, EDTA, concentrated hydrochloric acid, sodium citrate, sodium hydroxide,

formamide, ammonium sulphate, acetic acid, sodium acetate, alcian blue, neutral red, calcium chloride, alizarin red S.

Amersham Pharmacia Biotech Ltd: Buckinghamshire, U.K.

³⁵S-dCTP, Amersham Megaprime™ DNA Labelling System

Ilford Imaging U.K. Ltd.: Cheshire, U.K.

K-photographic emulsion, Hypam rapid fixer, Phenisol high contrast film developer, and Ilfostop Pro low-odour stop bath.

ICN Biochemicals Inc.: Ohio, U.S.A.

Tris (Ultra-Pure).

Fluka Chemicals: Dorset, U.K.

Ethidium bromide.

Biogenesis Ltd: Dorset, U.K.

Bovine type II collagen polyclonal antibody, RNazol B.

DAKO: Buckinghamshire, U.K.

Biotinylated swine anti-rabbit immunoglobulins, swine serum, and streptavidin peroxidase.

Loctite: Hertfordshire, U.K.

Loctite® engineering adhesive.

Bovine type VI collagen antibody was kindly provided by Dr. Shirley Ayad (University of Manchester). All cDNA probes were kindly provided by Dr. Judith Hoyland (University of Manchester).

All reagents were of the highest grade commercially available.

3.4. METHODS

3.4.1. BOVINE CHONDROCYTE CULTURE

The procedures are as described in Chapter Two, Section 2.4.1.

3.4.2. 5-AZACYTIDINE TREATMENT OF CHONDROCYTE CULTURES

The procedures are as described in Chapter Two, Section 2.4.2.

3.4.3. TRYPSINISATION OF CHONDROCYTE CULTURES

Each well was washed twice with Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium. The chondrocytes were released from the plates by incubating in serum-free MEM containing trypsin (0.4 mg/ml) and bacterial collagenase type IA (10 mg/ml) at 37°C for 1 hour. After centrifugation at 250 g in an MSE Mistral 2000 bench centrifuge for 3 minutes, the supernatant was discarded. The cells were resuspended in 5 ml fresh MEM and passed through a 40 µm cell strainer (Becton Dickinson Labware, New Jersey, U.S.A). The filtered cell suspension was used as required.

3.4.4. RNA EXTRACTION FROM CHONDROCYTES

During RNA extraction, extreme care has to be taken to protect RNA from being degraded by RNases, which are ubiquitous and very stable enzymes. Treatment of glassware with hydrogen peroxide followed by baking produces RNase-free conditions. Similarly, reagents are made RNase free, using diethylpyrocarbonate (DEPC)-treated water followed by autoclaving. RNase inhibitors are also used wherever appropriate. The method used here is a modification of the Chomzynski and Sacchi method (1987). The first step in RNA extraction involves cell lysis by vortexing the cell suspension in an RNA extraction buffer and chloroform. Chloroform also precipitates DNA and the protein contaminants, leaving RNA in aqueous solution. The cell suspension is treated with ammonium acetate if an extensive extracellular matrix is present. Subsequent centrifugation separates the mixture into distinct layers: an upper aqueous layer containing RNA, a lower organic layer containing the precipitated proteins, and DNA present at the interface of the two phases. The upper aqueous phase is collected and RNA is precipitated with ice-cold isopropanol.

3.4.4.1. METHOD

All standard precautions to prevent RNA degradation were taken. All glassware were treated with hydrogen peroxide solution and baked at 250°C for 3 hours. All solutions were made with DEPC-treated water (0.1% v/v) and autoclaved. Gloves were worn at all times and frequently changed. Chondrocytes released by trypsinisation were collected by centrifugation of the cell suspension in an MSE Mistral 2000 bench centrifuge at 250 g for 3 minutes. The supernatant was discarded and the cells were resuspended in RNazol B and 1/10th volume of chloroform. The cell suspension was transferred to a sterile Eppendorf tube and vortexed for 15 seconds. Half volume of 7.5 M ammonium acetate was added and the mixture was centrifuged in an MSE Micro Centaur bench centrifuge at 10000 g for 5 minutes at 4°C. The supernatant was collected, left at -20°C for 5 minutes, and then centrifuged again at 10000 g for 15 minutes at 4°C. The upper aqueous layer was removed and transferred to a fresh sterile Eppendorf tube. One volume of ice-cold isopropanol was added and the mixture was left at -20°C for at least 15 minutes. After centrifuging the mixture at 10000 g for 15 minutes at 4°C, the supernatant was discarded. The RNA pellet was then washed three times with ice-cold 70% ethanol, dried in a centrifuging evaporator (Heto), and finally resuspended in 0.5% NP-40 containing 1:100 dilution of RNase inhibitor. All RNA samples were kept at -70°C.

3.4.5. CDNA SYNTHESIS

cDNA can be synthesised from RNA by the process of reverse transcription which is catalysed by reverse transcriptase (Maniatis *et al.*, 1989). This process is illustrated in figure 3.2. Reverse transcriptase is found in retroviruses which are RNA-containing eukaryotic viruses such as human immunodeficiency virus (HIV). The unique feature of reverse transcriptase is its ability to synthesise a DNA strand complementary to an RNA template. It acts like DNA polymerase I by synthesising DNA in a 5'→3' direction. A single oligonucleotide primer must be hybridised to the 3' ends of the template before DNA synthesis can proceed. Since eukaryotic mRNAs end in a poly (A) tail consisting of a string of adenylate residues, the primer used is usually a string of thymidylate residues. The RNA sample is first denatured by heating, then cooled

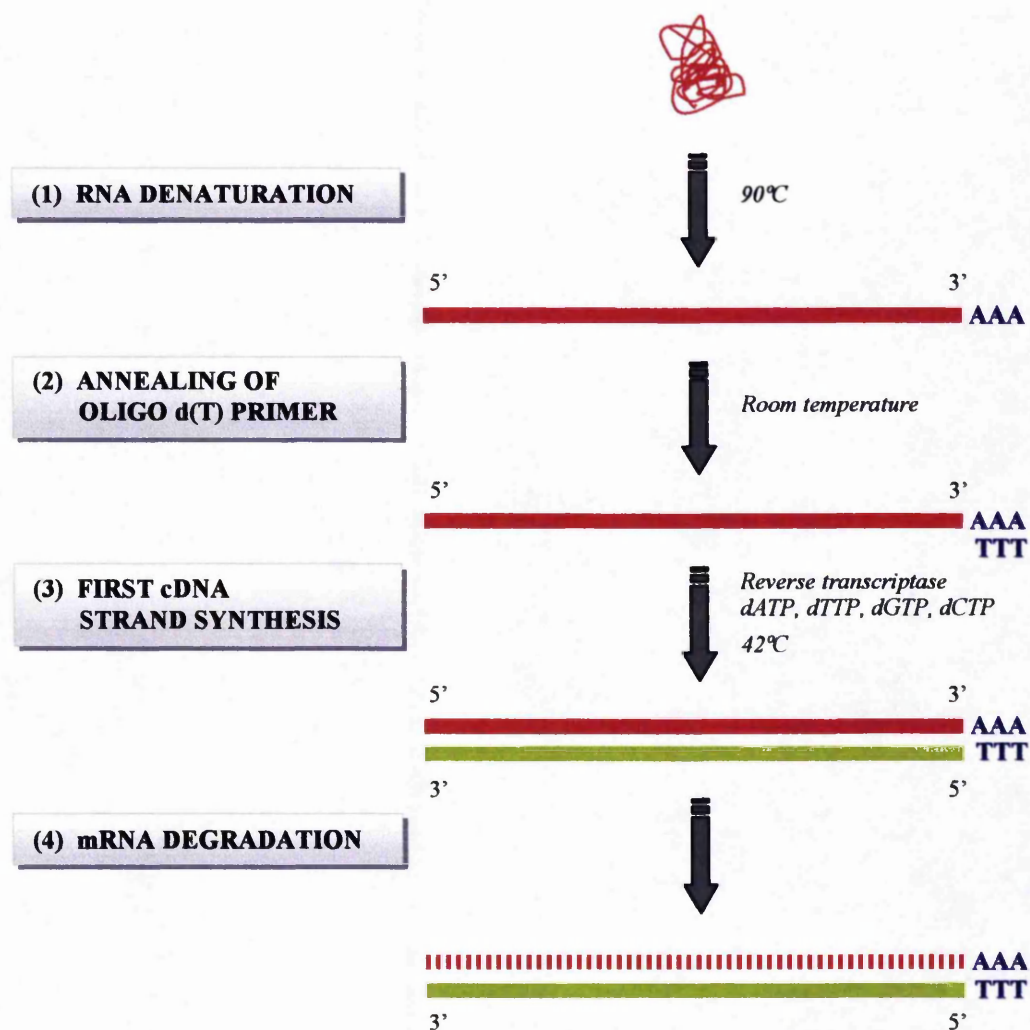


Figure 3.2. The synthesis of cDNA from mRNA by reverse transcription. The first step involves the denaturation of mRNA by boiling. Subsequent cooling to room temperature allows the oligo d(T) primer to anneal to the poly(A) tail at the 3'-end of the mRNA strand. The synthesis of the first cDNA strand is carried by the enzyme, reverse transcriptase, at its optimum working temperature of 42°C, in the presence of the four deoxyribonucleotides according to the complementary rule of base-pairing. The mRNA strand is then degraded by the exonuclease activity of reverse transcriptase.

to room temperature to allow the oligo (dT) primer to anneal to the poly (A) tail of the mRNA template. Subsequent incubation at 42°C allows reverse transcriptase to begin DNA synthesis in the presence of the four deoxynucleotides. The enzyme activity is stopped by heat inactivation. It should be noted that reverse transcriptase is also an exoribonuclease and will therefore degrade specifically RNA of RNA-DNA hybrids soon after the synthesis of the complementary DNA strands. The net result is the production of cDNAs that are representatives of mRNA present in the original preparation.

3.4.5.1. METHOD

RNA (1.0 µg) was denatured by heating at 90°C for 5 minutes, then cooled on ice for 5 minutes. RNA was incubated with freshly prepared cDNA strand buffer (see Appendices for composition) at room temperature for 10 minutes. The mixture was then transferred to 42°C and left for 30-60 minutes. The reaction was stopped by inactivating the enzyme at 95°C for 5 minutes, then cooled on ice.

3.4.6. POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is a powerful technique used to amplify selectively any region of DNA provided the sequences of DNA flanking the DNA region to be amplified are known (Sakai *et al.*, 1988). This amplification allows the generation of large quantities of the DNA region of interest for further analysis. The basic principles of PCR are illustrated in figure 3.3. Oligonucleotide sequences, known as primers, complementary to the 3' ends of the DNA segment to be amplified are prepared. The DNA sample is initially denatured to single strands by heating it to 94°C, then cooled to a specified temperature to allow the oligonucleotides to anneal to their complementary DNA sequences. The annealing temperature depends on the length and base composition of the oligonucleotide sequences (see Appendices). The annealed oligonucleotides then act as primers for the synthesis of DNA which proceeds with a supply of deoxynucleotides. DNA synthesis is catalysed by *Taq* polymerase, which is the DNA polymerase obtained from the thermophilic bacteria *Thermus aquaticus* and is resistant to denaturation by high temperatures. The

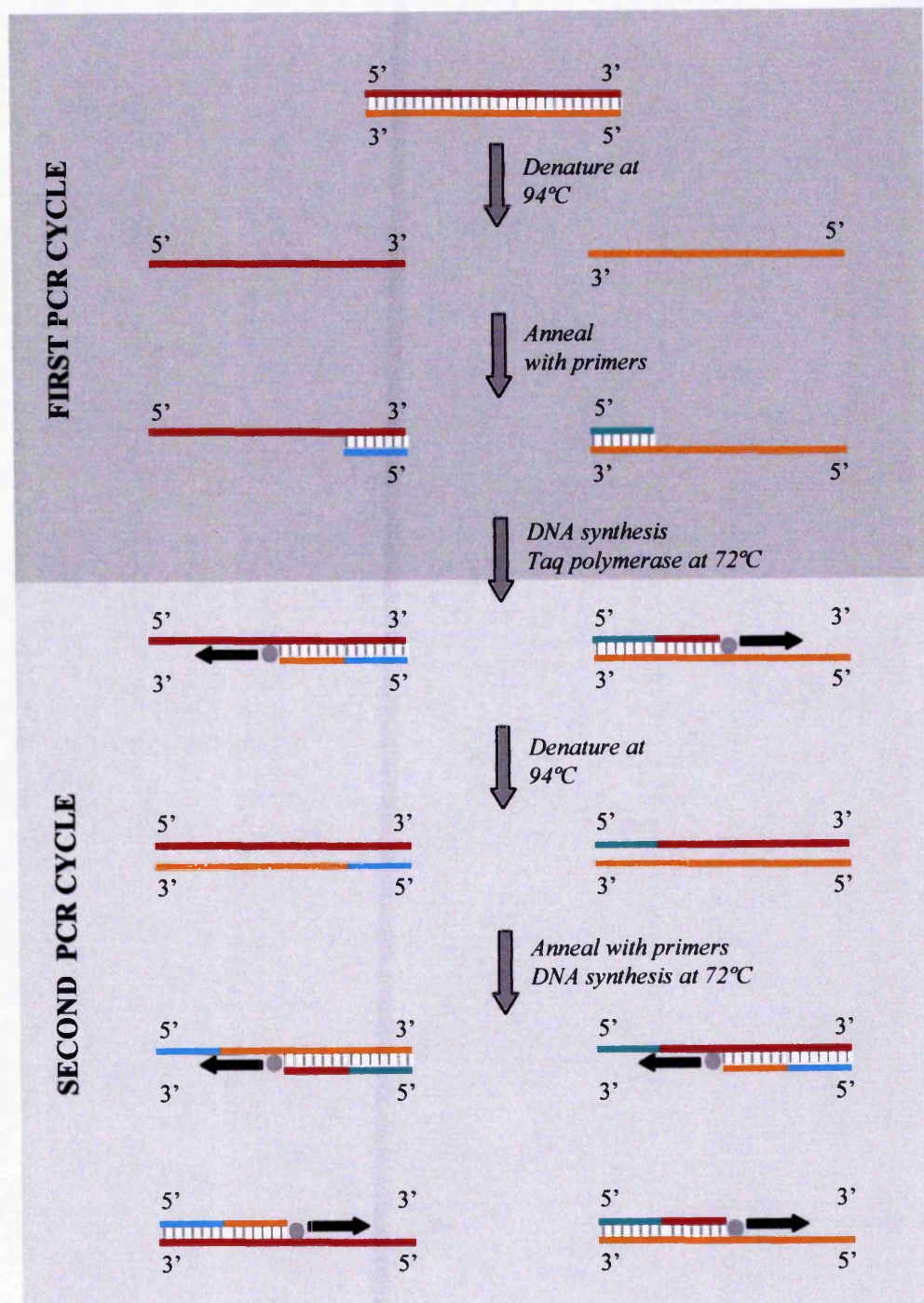


Figure 3.3. Polymerase Chain Reaction (PCR). This technique is used to amplify specific DNA sequences. The DNA sample is first denatured to obtain single stranded molecules. Subsequent cooling to the appropriate temperature allows primers, forward (—) and reverse (—), to anneal to their complementary DNA sequences. *Taq* polymerase (●) is used to extend the DNA sequences flanked by the two primers in a 5'→3' direction at 72°C. Repeated cycles of DNA denaturation, primer annealing and extension result in the exponential amplification of the desired DNA sequence.

extension temperature used is usually 72°C. Once the first round of DNA synthesis is complete, the reaction mixture is heated further to 94°C so that the newly synthesised DNA strands detach from their templates. Cooling to the annealing temperature allows the primers to anneal at their respective complementary DNA sites, including those on the newly synthesised strands. *Taq* polymerase then carries out the second round of DNA synthesis. Provided an excess of primers and deoxynucleotides is present in the reaction mixture, repeated cycles ranging from 30 to 40 can be performed to amplify exponentially the desired DNA sequence. Only traces of DNA are needed initially and the products obtained have precise lengths, delimited exactly by the regions complementary to the primers. The use of a PCR machine takes the reaction mixture automatically through controlled cycles of temperature changes for denaturation, annealing, and synthesis. However, this powerful DNA amplification system is extremely vulnerable to contamination. Even the slightest trace of foreign DNA will be amplified to significant levels, which may produce misleading results. Consequently, cleanliness is of paramount importance when PCR is performed. A negative control is usually included where the DNA sample is replaced with deionised water.

3.4.6.1. METHOD

PCR reactions were carried out in a total volume of 100 µl containing: 1 µl cDNA, 10 µl 10X PCR buffer (see Appendices), 100 pmol forward primer (see Appendices), 100 pmol reverse primer (see Appendices), 10 µl dNTP mix (see Appendices), 2.5 units of *Taq* DNA polymerase, and sterile water to a final volume of 100 µl. Sterile water (1 µl) was used instead of the cDNA sample in the negative control. All PCR reactions were overlaid with 50 µl mineral oil and performed in a Hybaid Omnigene PCR machine. For type II collagen-PCR, reactions were carried out for 35 cycles of 1 minute at 94°C, 1 minute at 57°C, and 1 minute at 72°C. For type X collagen-PCR, reactions were performed for 35 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C.

3.4.7. AGAROSE GEL ELECTROPHORESIS

Agarose was dissolved in 1X TBE buffer (see Appendices) by boiling the mixture (2% w/v). Ethidium bromide was added (0.005% v/v) and the gel mixture was poured onto a plastic plate, surrounded by walls of adhesive tapes to provide a gel of about 3 mm thick. Loading wells were formed by placing a plastic comb in the gel solution, and the gel was left to set. The comb was removed and the gel was placed in the electrophoresis tank and covered with 1X TBE buffer. Each well in the gel was loaded with 13 μ l sample (10 μ l PCR product and 3 μ l Orange G dye). DNA marker molecules of known sizes were also loaded in one well. Electrophoresis was carried out at 100 V for 20-30 minutes using the Bio-Rad power pack. PCR products were viewed subsequently as bands and photographed with ultraviolet transillumination.

3.4.8. IMMUNOHISTOCHEMISTRY FOR THE DETECTION OF COLLAGEN TYPES II AND VI

Cell layers were collected, processed, embedded in paraffin wax, and sectioned as described in Chapter Two, Section 2.4.5. Cut sections were then dewaxed and rehydrated as described in Chapter Two, Section 2.4.6. All sections were placed in 0.05M TBS buffer for 5 minutes, then in 3% (v/v) hydrogen peroxide for 5 minutes. Sections were rinsed in distilled water followed by 0.05M TBS. Some sections were incubated with 1.5 mg/ml hyaluronidase (dissolved in 0.05M acetate / 0.025M saline buffer, pH 5) at 37°C for 1 hour. They were then washed three times in 0.05M TBS (5 minutes each). All sections were incubated with normal swine serum (1:5 dilution in 0.05 M TBS) for 15 minutes at room temperature prior to the addition of the primary antibody for 1 hour. For the detection of type II collagen and type VI collagen, the cells were incubated with a 1:200 dilution of a polyclonal antibody against bovine type II collagen, and a 1:500 dilution of a primary antibody against bovine type VI collagen, respectively. The sections were rinsed three times in 0.05M TBS, then incubated with a 1:300 dilution of biotinylated swine anti-rabbit secondary antibody for 45 minutes at room temperature. After rinsing three times in 0.05 M TBS, the sections were incubated with streptavidin-peroxidase (1:500 dilution with 0.05 M TBS) for 30 minutes at room temperature. They were then washed in 0.05 M TBS before incubating with DAB / H₂O₂ solution (one DAB tablet and one Urea

Hydrogen Peroxide table in 5 ml of distilled water) for 10 minutes. After rinsing in tap water, the cells were counterstained in Mayer's haematoxylin for 1 minute followed by 0.05 M TBS for 1 minute, then rinsed in distilled water, air-dried, and finally mounted with Loctite[®] adhesive.

3.4.9. IN SITU HYBRIDISATION

In situ hybridisation is a technique used to identify the presence and the location of specific gene expression within individual cells. The technique involves the specific annealing of radiolabelled nucleic acid probes to complementary nucleic acid sequences that are retained *in situ* in fixed tissue sections. The location of the probe is visualised by autoradiography. The nucleic acid hybrid molecules may be DNA/DNA, or DNA/RNA, or RNA/RNA between the target nucleic acid and the labelled probe containing the complementary sequence.

In this study, DNA probes were used to identify the presence of their complementary mRNA transcripts. A suspension of chondrocytes obtained by trypsinisation was centrifuged in a cytocentrifuge such that the cells become stuck to glass slides as they are flattened by mechanical forces during the centrifugation process. The tissue was then fixed with a cross-linked fixative, such as paraformaldehyde, to "fix" or retain the cellular RNA within the tissue. This step was carried out in the cold (4°C) to inhibit endogenous ribonuclease activity. In order to facilitate the penetration of the probe into the tissue, sections were treated with hydrochloric acid, which denatures the basic proteins in the cell membrane. At this stage, "RNase-control" sections were subjected to RNase treatment to eliminate any hybridisation signal. All sections were then re-fixed in cold paraformaldehyde to help prevent tissue disintegration. In order to prevent the indiscriminate binding of negatively charged nucleic acid probes to positively charged amino groups present in the tissue, the sections were treated with acetic anhydride, which acetylates the amino groups. Sections were then covered with the radiolabelled cDNA probe for an overnight hybridisation at 37°C.

Following the hybridisation step were a series of post-hybridisation washes aimed specifically at reducing background signal caused by non-specific binding of probe to other cellular components or to related mRNA sequences. Washes were carried out at various stringencies by varying the temperature, salt concentration, and concentration of formamide used. Formamide is a denaturing agent that destabilises double-stranded nucleic acids by disrupting hydrogen bonding. The slides were then coated with photographic emulsion, then processed by autoradiography to reveal the presence of the hybridised probe.

3.4.9.1. METHOD I: TREATMENT OF CHONDROCYTE CULTURES

During the entire experimental procedure, all standard precautions were taken to prevent RNA degradation by RNase contamination. All glassware was treated with 3% (v/v) hydrogen peroxide and baked at 250°C for 3 hours and all solutions were treated with DEPC (0.1% v/v) and autoclaved.

Chondrocyte cultures were trypsinised as described in Section 3.4.3. The filtered cell suspension was centrifuged in an MSE Mistral 2000 bench centrifuge at 250 g for 3 minutes. The supernatant was discarded and the cell pellet was resuspended in sterile PBS to give an approximate cell concentration of 10000 cells per ml. The cell suspension was loaded (100 µl per glass slide) in a cytocentrifuge and centrifuged at 100 g for 30 minutes. The cells were then fixed by placing the slides in 4% (w/v) paraformaldehyde at 4°C for 1 hour. All slides were washed in five changes of DEPC-PBS and stored at 4°C for subsequent hybridisation studies.

3.4.9.2. METHOD II: RADIOACTIVE RANDOM PRIME LABELLING OF CDNA PROBES

Human cDNA probes for type X collagen and alkaline phosphatase were used to identify its mRNA expression in cultured chondrocytes (see Appendices). The cDNA probe was first denatured at 100°C for 5 minutes in a reaction mixture containing: 50 ng cDNA probe, 10 µl primer (Amersham Megaprime™ DNA labelling system), and sterile water to a final volume of 52 µl. The reaction mixture was allowed to cool to

room temperature. The following reagents were then added to the denatured cDNA: 10 µl Reaction Buffer, 8µl of unlabelled dATP, 8 µl of unlabelled dTTP, 8µl of unlabelled dGTP, 10 µl ³⁵S-dCTP, and 4µl Klenow fragment. Note that all reagents added (except ³⁵S-dCTP) were taken from the Amersham Megaprime™ DNA labelling system (see Appendices). The reaction mixture was incubated at 37°C for 1 hour. The labelling reaction was stopped with the addition of 5 µl 0.2 M EDTA, pH 8.0.

3.4.9.3. METHOD III: PURIFICATION OF cDNA PROBES

For each labelling reaction, a 1 ml syringe was plugged with glass wool and filled with sephadex G-50 previously suspended in STE buffer (see Appendices). The syringe was placed in a 15 ml centrifuge tube, centrifuged at 250 g for 1 minute to pack the column, and the centrifuge tube discarded. The unpurified labelled cDNA probe was added to the column that was placed in a fresh 15 ml centrifuge tube, and centrifuged at 250 g for 1 minute. The purified labelled cDNA probe was thus collected in the centrifuge tube ready for use.

3.4.9.4. METHOD IV: PROCESSING OF TISSUE SAMPLES PRIOR TO HYBRIDISATION

All sections were placed in DEPC-water for 10 minutes, then in 0.2 M HCl for 20 minutes, followed by two rinses in 2X SSC (see Appendices) (3 minutes each), one rinse in 0.05 M Tris-HCl, pH 7.4 for 3 minutes, two rinses in 0.2% glycine/PBS (3 minutes each), and then two rinses in DEPC-PBS (3 minutes each). The RNase control slides were covered with 1 mg/ml of boiled pancreatic RNase A previously diluted in 2X SSC (see Appendices) and incubated at 37°C in an Omnislide machine (Hybaid) for 1½ hours. The RNase A was then discarded and replaced with fresh 1 mg/ml RNase A diluted in 2X SSC, and the control slides were incubated for another 1½ hours at 37°C. All test slides were left in 2X SSC during the RNase treatment. All slides were then rinsed in DEPC-PBS for 3 minutes, post-fixed in cold 0.4% paraformaldehyde/PBS for 20 minutes, and rinsed in 0.1 M triethanolamine, pH 8, for 2 minutes. The slides were then placed in freshly prepared acetic

anhydride/triethanolamine (0.25% v/v) for 20-30 minutes, rinsed in DEPC-water for 3 minutes, dehydrated in industrial methylated spirit (IMS) for 3 minutes, and air-dried.

3.4.9.5. METHOD V: HYBRIDISATION

Each purified labelled cDNA probe was added to a hybridisation buffer containing: 0.05 g dextran sulphate, 50 µl of 10X modified Denhardt's, 100 µl of 3 M NaCl, 10 µl of 10 mg/ml salmon sperm DNA, 5 µl of 1 M Tris-HCl, pH 7.4, 0.5 µl of 0.5 M EDTA, 5 µl of 1 M dithiothreitol (DTT), and 250 µl of deionised formamide. The above mixture was then heated to 100°C for 5 minutes and cooled rapidly on ice. Each tissue sample was then covered with approximately 50 µl of the probe mixture, and incubated overnight at 37°C.

3.4.9.6. METHOD VI: POST-HYBRIDISATION WASHES

All slides were rinsed twice (5 minutes each) in 0.5X SSC containing 1mM EDTA and 10 mM DTT, followed by two rinses in 0.5X SSC containing 1 mM EDTA (5 minutes each). The slides were then rinsed in 50% formamide/50% 0.15 M NaCl containing 5mM Tris-HCl (pH 7.4) and 0.5 mM EDTA (pH 8) for 10 minutes, followed by four rinses in 0.5X SSC at 55°C (5 minutes each). The slides were then given a final rinse in 0.5X SSC at room temperature for 5 minutes, dehydrated in IMS, and air-dried.

3.4.9.7. METHOD VII: AUTORADIOGRAPHY

All slides were coated in K5 photographic emulsion, allowed to dry for 3-4 hours away from light, then transferred to a light-proof box and kept at 4°C until ready for developing. In the developing process, the slides were placed in the developer (see Appendices) for 5 minutes, rinsed in stop-bath solution (see Appendices) for 15 seconds, then left in the fixer (see Appendices) for 5 minutes. The slides were washed in cold distilled water for 5 minutes. All the above procedures were carried out in the dark room. Slides were dipped in Mayer's haematoxylin for 1 minute, washed in Scott's tap water substitute (see Appendices) for 5 minutes, placed in Eosin

for 1 minute, and dehydrated in four changes of IMS. All slides were air-dried and mounted with Loctite® adhesive.

3.4.10. ALCIAN BLUE STAINING

The alcian blue staining technique was used for the detection of proteoglycans in chondrocyte cultures. Proteoglycans consist of glycosaminoglycan moieties, which are polysaccharide chains. Each chain is composed of repeating disaccharide units of a nitrogen-containing sugar and a sugar acid. The latter confers affinity for cationic dyes such as alcian blue. Alcian blue binds to carboxyl and sulphate-ester groups at pH 2.5, thus giving a blue colour for positive proteoglycan staining. Alcian blue staining was also carried out on paraffin sections of cell layers using critical electrolyte concentrations (CEC), according to the method by Scott and Dorling (1965). In this method, the procedure uses alcian blue solution containing different concentrations of an electrolyte, such as magnesium chloride (MgCl_2). The CEC is the point at which the electrolyte concentration in the staining solution is sufficient to prevent staining. The electrolyte cations compete with the dye cations for the anionic binding sites on the glycosaminoglycans. Due to their varying glycosaminoglycan composition, different proteoglycans have different CEC points. Therefore, this property is useful in identifying the presence of the various proteoglycans in the tissue under examination as a result of the differential histochemical staining. Three different MgCl_2 concentrations were used here: 0.05 M (all proteoglycans stain blue), 0.5 M (only chondroitin sulphate stains blue), and 0.9 M (only keratan sulphate stains blue).

3.4.10.1. METHOD I: TOTAL PROTEOGLYCAN STAINING

Culture medium was removed from the tissue culture dishes and the cultures were washed three times with PBS. The cells were then fixed in ice-cold methanol for 10 minutes. The cells were rinsed in 0.05 M TBS and then stained with alcian blue (pH 2.5) for 15 minutes. After washing in distilled water followed by running water for 5 minutes, the cells were counterstained with 1% aqueous neutral red for 30 seconds.

The cells were then rinsed in distilled water, air-dried, and mounted with Loctite adhesive.

3.4.10.2. METHOD II: STAINING WITH CRITICAL ELECTROLYTE CONCENTRATIONS

Paraffin sections of cell layers collected at selected time-points were dewaxed and hydrated as described in Chapter Two, Section 2.4.6. Sections were left overnight in the appropriate staining solution (see Appendices) containing 0.1% alcian blue (w/v) in distilled water and MgCl_2 solution at the three different concentrations (0.05 M, 0.5 M, and 0.9 M). All sections were rinsed in their appropriate MgCl_2 solution for 5 minutes, then in distilled water for 5 minutes. Stained sections were dehydrated through one change of 95% IMS followed by two changes of 100% IMS, then cleared in four changes of xylene, and finally mounted in xylene.

3.4.11. SPECTROPHOTOMETRIC ASSAY FOR THE DETECTION OF ALKALINE PHOSPHATASE

Alkaline phosphatase was assayed by using the Sigma Diagnostics[®] kit. When alkaline phosphatase is incubated with *p*-nitrophenyl phosphate at 37°C, the enzyme hydrolyses the phosphate substrate, yielding *p*-nitrophenol and inorganic phosphate (Bessey *et al.*, 1946). The enzyme reaction is stopped by adding sodium hydroxide solution. Under alkaline conditions, *p*-nitrophenol is converted to a yellow complex, and the absorbance is measured spectrophotometrically at 410 nm. The intensity of colour formed is directly proportional to the alkaline phosphatase activity present.

3.4.11.1. METHOD

Bovine epiphyseal chondrocytes were cultured in 96-well plates at a plating density of 0.5×10^6 cells per cm^2 under the same conditions described in Chapter Two, Section 2.4.1. The culture medium was removed and the cells were washed three times with PBS. The alkaline phosphatase assay was performed by incubating the cells in 50 μl alkaline buffer solution (Sigma 221) and 50 μl phosphatase substrate solution (4

mg/ml) (Sigma 104[®]) at 37°C for exactly 15 minutes. A similar reaction was set up using PBS as blank. The reaction was stopped by the addition of 250 µl 0.05 M NaOH. The amount of *p*-nitrophenol released was measured by reading the absorbance at 410 nm using a Titertek® Multiskan plate reader (Flow Laboratories Ltd., Middlesex, U.K.).

3.4.12. ALIZARIN RED S STAINING

The alizarin red S staining method was used to detect the presence of calcification in chondrocyte cultures. Alizarin red S is an anionic dye, which combines with calcium ions to form an insoluble orange chelate. Areas of calcium mineral deposition in tissues are therefore stained orange-red.

3.4.12.1. METHOD

Bovine epiphyseal chondrocytes were grown in glass 4-chamber slides (Gibco, Paisley, Scotland) at a density of 0.5×10^6 cells per cm² under the same culture conditions as described in Chapter Two, Section 2.4.1. Calcium β -glycerophosphate (calcium chloride + sodium β -glycerophosphate) was added to azacytidine-treated and untreated cultures at a concentration of 10 mM and this addition was renewed every 48 hours. Control cultures were maintained without the supplementation of calcium β -glycerophosphate. At selected time-points, slides were collected, the culture medium discarded, and the cells washed three times with PBS. Cells were fixed in ice-cold methanol for 10 minutes and then washed in distilled water. Cells were placed in alizarin red S solution for 2 minutes, rinsed in distilled water for 5 seconds, then placed in a solution containing 0.01% concentrated HCl/99.99% ethanol (v/v) for 15 seconds. Cells were dehydrated in two changes of absolute ethanol, cleared and mounted in xylene.

3.4.13. IMAGE ANALYSIS

In situ hybridisation slides were viewed using a Leica DB research microscope (Leica Ltd., Milton Keynes, U.K.) to which was attached a CF8/1 Kappa monochrome

camera for image capture. All images were fed into a Quantimet 600S image analysis computer (Leica Ltd., U.K.). Hybridisation signals were seen as black grains and the level of mRNA expression was represented by the concentration of black grains obtained. Results were quantified using the computer programme, AGISH, written by John Denton (Department of Rheumatology, University of Manchester). For each time-point studied, ten fields were chosen at random and the number of grains counted in the areas selected. The level of mRNA expression of the protein of interest was expressed as the mean number of grains per unit area of cells.

3.4.14. PHOTOGRAPHY

Cells analysed by immunohistochemistry and histological staining were viewed under the Leica DM microscope (Leica Ltd., Milton Keynes, U.K.) and photographs were taken using a Sony DXC930P colour camera attached to the microscope.

3.5. RESULTS

3.5.1. mRNA EXPRESSION OF COLLAGENS IN FOETAL BOVINE EPIPHYSEAL CHONDROCYTE CULTURES

The messenger ribonucleic acid (mRNA) expression of type II collagen (a marker of chondrocytic phenotype), and type X collagen (a marker of chondrocyte hypertrophy) in foetal bovine epiphyseal chondrocyte cultures was analysed. At selected time-points, total RNA was extracted from azacytidine (aza-C)-treated and untreated cultures and reverse transcribed to give the corresponding total complementary DNA (cDNA). In order to identify the presence of the cDNAs encoding the markers of interest, polymerase chain reactions (PCR) were carried out using primers specific for the cDNA sequences coding for the genes of interest.

Results obtained revealed the constitutive mRNA expression of type II collagen at all time-points studied. Type II collagen was identified by the presence of a 321 bp band after 35 cycles of PCR amplification. Type II collagen was expressed both with and without aza-C treatment (Figure 3.4).

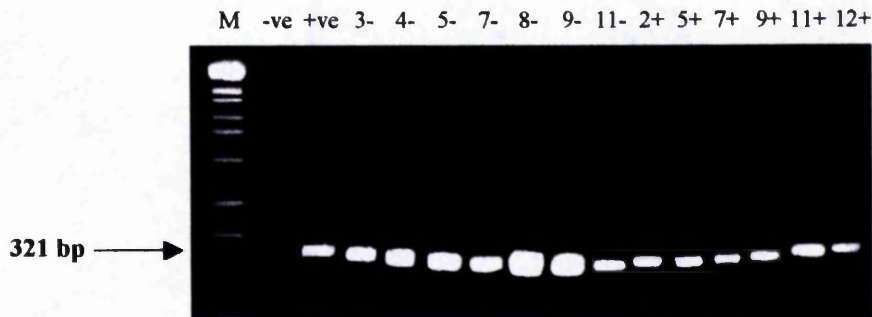


Figure 3.4. mRNA expression of type II collagen in foetal bovine epiphyseal chondrocyte cultures. Total RNA was extracted from both aza-C treated and untreated chondrocyte cultures at selected time-points. RT-PCR was performed to identify the expression of type II collagen, as described under Materials and Methods. Sterile water was used in the place of a cDNA sample as negative control and total cDNA derived from hypertrophic chondrocytes in the bovine growth plate was included as a positive control. The amplified products were analysed by 2% agarose gel electrophoresis. The mRNA expression of type II collagen was detected by the presence of a 321 bp band. (M: 1kb ladder; -ve: negative control; +ve: positive control; -: untreated sample; +: aza-C treated sample).

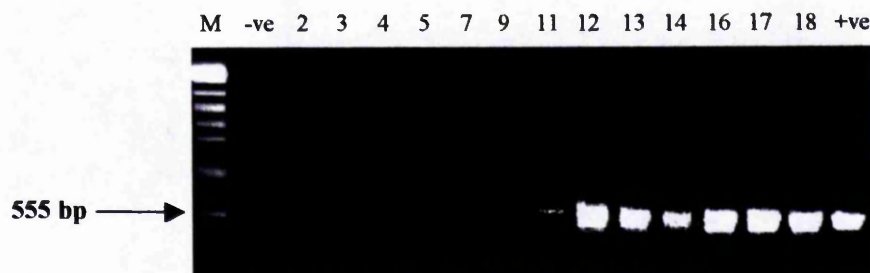


Figure 3.5. mRNA expression of type X collagen in foetal bovine epiphyseal chondrocyte cultures under aza-C treatment. Total RNA was extracted from aza-C treated chondrocyte cultures at selected time-points. RT-PCR was carried out in order to analyse the expression of type X collagen. cDNA was replaced with sterile water as negative control and total cDNA obtained from hypertrophic chondrocytes from the bovine growth plate was used as positive control. The expression of type X collagen was identified as a 555 bp band upon agarose gel electrophoresis of the amplified products. No expression of type X collagen was observed when RT-PCR was performed under the same experimental conditions using total RNA extracted from untreated chondrocyte cultures. (M: 1kb ladder; -ve: negative control; +ve: positive control).

RT-PCR reactions for the detection of type X collagen showed that it was not expressed in aza-C treated chondrocytes during the first 10 days in culture. The mRNA expression of type X collagen was detectable on day 11, given by the presence of a 555 bp band after 35 cycles of PCR amplification. The expression remained switched on at all subsequent time-points analysed (Figure 3.5). No mRNA expression of type X collagen was observed in untreated cultures throughout the culture period under investigation.

With a view to support and confirm the findings obtained from the RT-PCR experiments, the mRNA expression of type X collagen was also analysed by *in situ* hybridisation. *In situ* hybridisation was used in an attempt to examine the synchronisation of the cultures under aza-C treatment. At various time-points, cell layers were harvested and digested with a mixture of trypsin and collagenase to release the chondrocytes. With the use of a cytocentrifuge, the chondrocytes were then fixed onto glass slides and processed for *in situ* hybridisation studies. The slides were viewed using a Leica DB research microscope and results were quantified, as previously published (Mee *et al.*, 1997; Walsh *et al.*, 1993), by the Quantimet image analysis system described under Methods. Hybridisation signals were seen as black grains and the level of mRNA expression was represented by the concentration of black grains obtained. For each time-point, ten fields per slide were chosen randomly and the number of grains counted. Since duplicate slides were analysed for each time-point, the mean grain counts of twenty fields was calculated. The level of mRNA expression of type X collagen was expressed as the grain density per unit area of cells. It was found that type X collagen was not expressed on day 7 under aza-C treatment. Aza-C treated chondrocytes expressed type X collagen on day 13 and this mRNA expression increased steadily at subsequent time-points analysed (days 15 and 20). Type X collagen was consistently absent in aza-C untreated chondrocytes throughout the culture period (Figure 3.6).

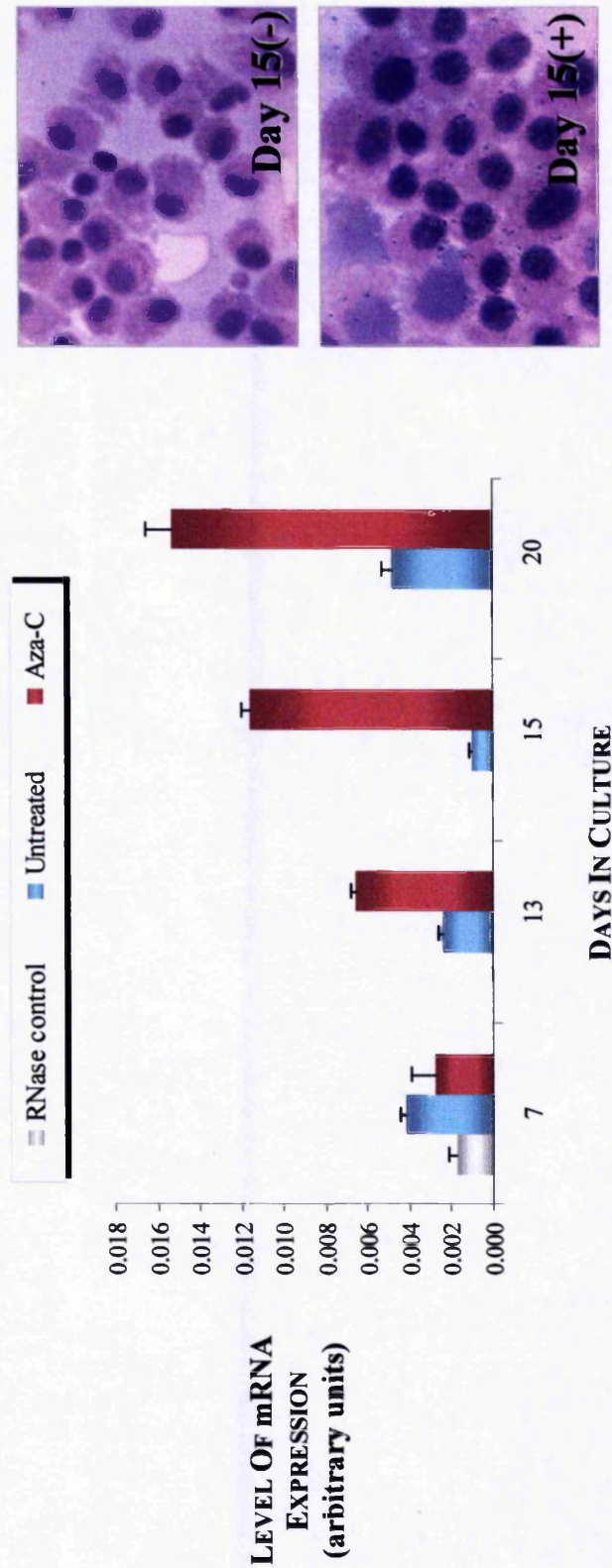


Figure 3.6. mRNA expression of type X collagen in foetal bovine epiphyseal chondrocyte cultures: *In situ* hybridisation. Aza-C treated and untreated chondrocytes were collected at selected time-points and processed for *in situ* hybridisation analyses. A human cDNA probe specific for type X collagen was used to study its mRNA expression in culture. An RNase control was included whereby the chondrocytes were treated with RNase to eliminate any hybridisation signal (+aza-C; day 15). All results were quantified using a Quantimet 600S image analysis system. Values represent mean \pm SEM ($n=20$, where n is the number of fields analysed). Results shown are representative of two experiments performed (Magnification: $\times 582$).

3.5.2. IMMUNOHISTOCHEMICAL ANALYSES OF COLLAGEN EXPRESSION IN CULTURED CHONDROCYTES

The expression of type II and type VI collagen in foetal bovine epiphyseal chondrocyte cultures was assessed immunohistochemically using rabbit anti-bovine type II collagen and type VI collagen antibodies, respectively. All immunohistochemical studies were carried out initially on intact chondrocyte cultures in their culture dishes. It was found that collagen types II (Figure 3.7) and VI (Figure 3.8) were present in both aza-C treated and untreated cultures at the time-points studied. In addition, the pericellular localisation of type VI collagen in chondrocyte cultures was observed (Figure 3.8).

Although the expression of both collagens was detectable, poor immunolocalisation of their presence was obtained with the later time-points. Consequently, the immunostaining procedures were carried out on paraffin wax sections of cell layers collected on days 13, 20, 27, and 35. It was not possible to obtain paraffin wax sections at earlier time-points due to technical problems encountered during the processing, paraffin-embedding, and sectioning of cell layers, which were thin and difficult to manipulate. Paraffin wax sections were either untreated or treated with hyaluronidase prior to the immunostaining procedure in order to remove hyaluronan, which may mask the presence of the collagens in the matrix.

Immunolocalisation of type II collagen in the absence of hyaluronidase treatment of paraffin sections showed a faint expression of type II collagen in the extracellular matrix (Figure 3.9). When the sections were treated with hyaluronidase, type II collagen was found to be present at a high level throughout the interterritorial matrix surrounding the chondrocytes (Figure 3.9). This pattern of distribution in the matrix was observed in sections of both aza-C treated and untreated cultures throughout the culture period. In addition, the presence of chondrocytes in their lacunae was clearly visible as unstained areas.

In the absence of hyaluronidase treatment, a very low expression of type VI collagen was detected in all cultures studied (Figure 3.10). Hyaluronidase treatment of paraffin

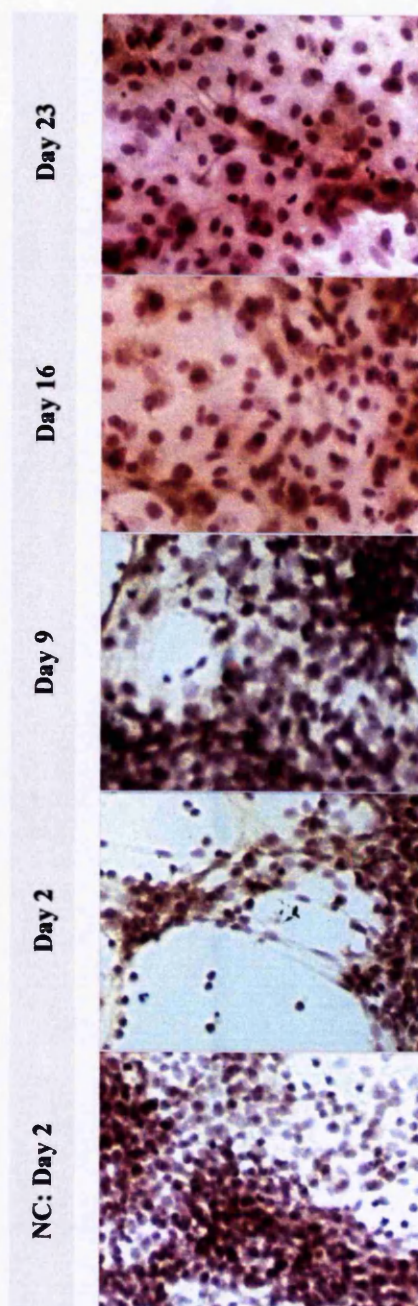


Figure 3.7. Immunohistochemical analyses of type II collagen in foetal bovine epiphyseal chondrocyte cultures. Cultures of epiphyseal chondrocytes were collected at selected time-points and immunostained directly in the culture dishes using a rabbit anti-bovine type II collagen antibody. The antibody was replaced with TBS buffer in the negative control [NC]. The procedures are described under Methods. Photographs show the expression of type II collagen in aza-C treated chondrocyte cultures throughout the culture period. Similar results were obtained in untreated cultures. (Magnification: $\times 1100$).

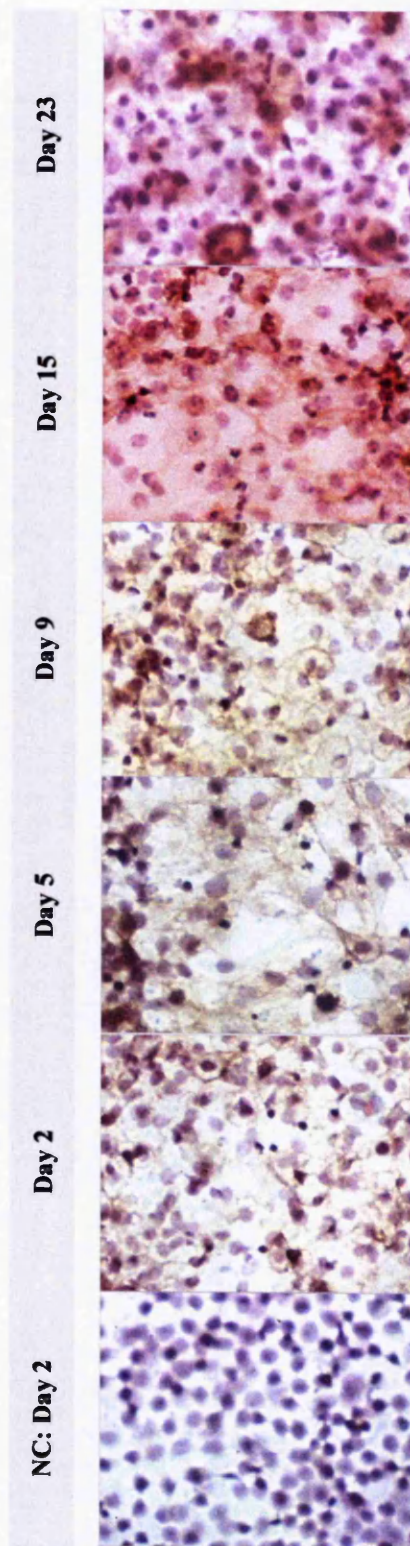


Figure 3.8. Immunohistochemical analyses of type VI collagen in foetal bovine epiphyseal chondrocyte cultures. Cultures of epiphyseal chondrocytes were collected at regular time-points and immunostained directly in the culture dishes using a rabbit anti-bovine type VI collagen antibody. The antibody was replaced with TBS buffer in the negative control [NC]. The procedures are described under Methods. Type VI collagen was present in aza-C treated chondrocyte cultures throughout the culture period. Note the pericellular expression of type VI collagen in the matrix. Similar results were obtained in untreated cultures. (Magnification: $\times 1200$).

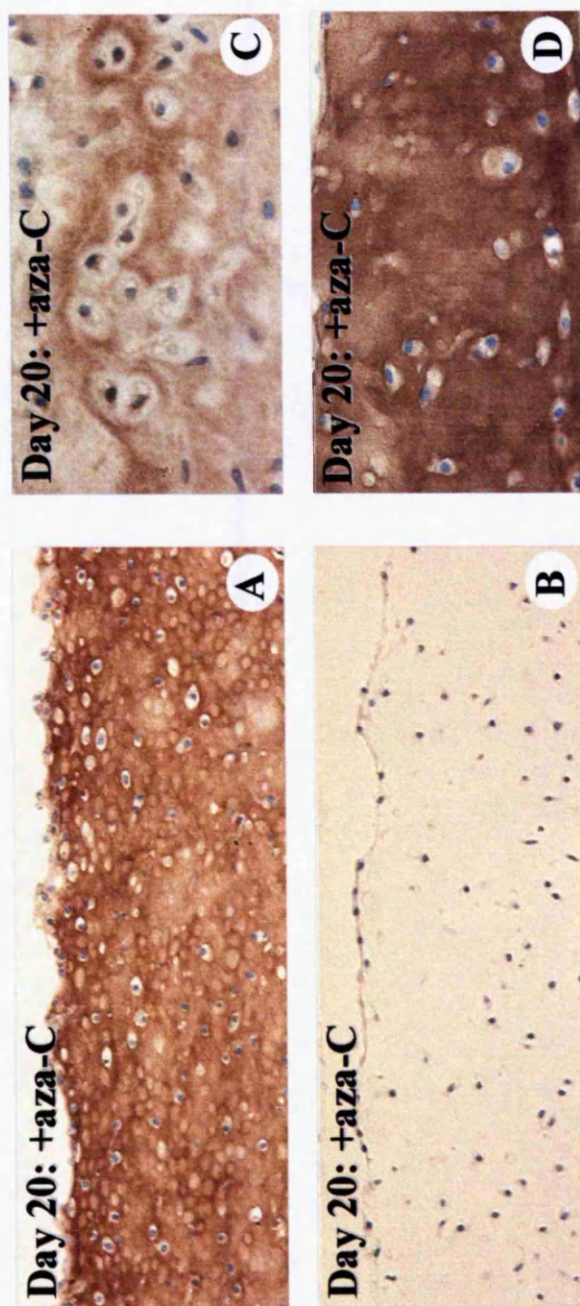


Figure 3.9. Immunohistochemical detection of type II collagen in paraffin wax sections of foetal bovine epiphyseal chondrocyte cultures. At chosen time-points, cell layers of aza-C treated chondrocyte cultures were collected, processed, and embedded in paraffin for subsequent detection of type II collagen by immunohistochemistry. Sections were immunostained using rabbit anti-bovine type II antibody, without [C] and with [A, D] hyaluronidase treatment. TBS buffer was used instead of the antibody in the negative control [B]. All procedures are described under Methods. Results shown are typical representations of type II collagen expression in both aza-C treated and untreated chondrocyte cultures at all time-points throughout the 3-4 week culture period. (Magnifications, A-B: x80; C-D: x170).

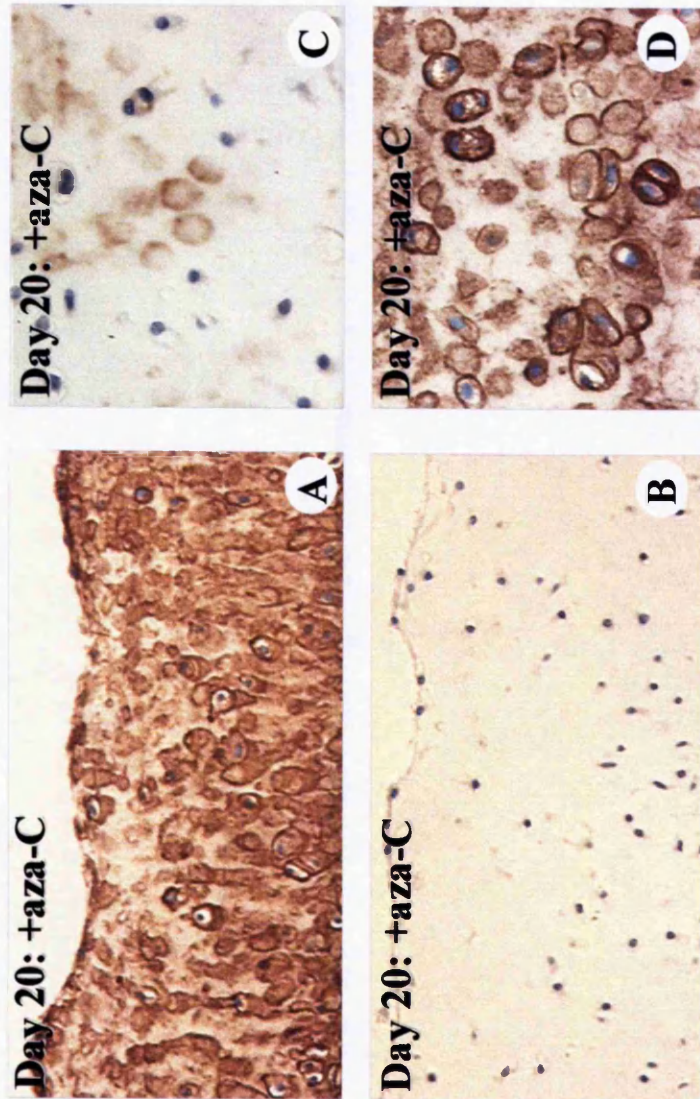


Figure 3.10. Immunohistochemical detection of type VI collagen in paraffin wax sections of foetal bovine epiphyseal chondrocyte cultures. At selected time-points, cell layers of aza-C treated chondrocyte cultures were collected, processed, and embedded in paraffin for subsequent immunohistochemical analyses of type VI collagen. Sections were immunostained using rabbit anti-bovine type VI antibody, without [C] and with [A, D] hyaluronidase treatment. TBS buffer was used instead of the antibody in the negative control [B]. All procedures are described under Methods. Type VI collagen was highly expressed at all time-points and its pericellular localisation in the matrix was clearly apparent [D]. Results shown are typical representations of type VI collagen expression in both aza-C treated and untreated chondrocyte cultures throughout the 3-4 week culture period. (Magnifications, A-B: x103; C-D: x218).

sections prior to immunostaining revealed a high expression of type VI collagen in the cell matrix. Type VI collagen was distinctly concentrated in the pericellular environment of chondrocytes and faintly present in the interterritorial matrix (Figure 3.10). The expression of type VI collagen in the cell matrix was identified in both aza-C treated and untreated cultures throughout the culture period.

3.5.3. PROTEOGLYCAN SYNTHESIS IN CULTURE

When epiphyseal chondrocytes derived from long bones of bovine foetuses were plated at high densities on plastic, they organised progressively an extracellular matrix with time in culture. In order to assess the proteoglycan nature of the cell matrix, alcian blue staining at pH 2.5 was performed on chondrocyte cultures directly in the culture dishes at regular time-points. It was found that the extracellular matrix synthesised in both aza-C treated and untreated cultures was proteoglycan-rich. Matrix synthesis started immediately after cell plating and increased with time in culture (Figure 3.11). In order to identify the type of acid glycosaminoglycans present in the cell matrix, the alcian blue staining was performed on paraffin sections of cell layers at three different concentrations of MgCl_2 (critical electrolyte concentrations). At 0.05 M and 0.5 M MgCl_2 , a positive blue staining was obtained throughout the matrix (Figure 3.11). At 0.9 M MgCl_2 , a much fainter blue staining was observed throughout the matrix (Figure 3.11). These histochemical results were obtained with and without aza-C treatment throughout the culture period.

3.5.4. EXPRESSION OF ALKALINE PHOSPHATASE IN FOETAL BOVINE EPIPHYSEAL CHONDROCYTE CULTURES

The expression of alkaline phosphatase was studied by radioactive *in situ* hybridisation and spectrophotometric assays. In the case of *in situ* hybridisation analyses, ten fields per slide were chosen randomly for each time-point and the number of grains counted. Since duplicate slides were analysed for each time-point, the mean grain counts of twenty fields was calculated. The level of mRNA expression of alkaline phosphatase was expressed as the grain density per unit area of cells. *In situ* hybridisation studies showed that in aza-C treated cultures,

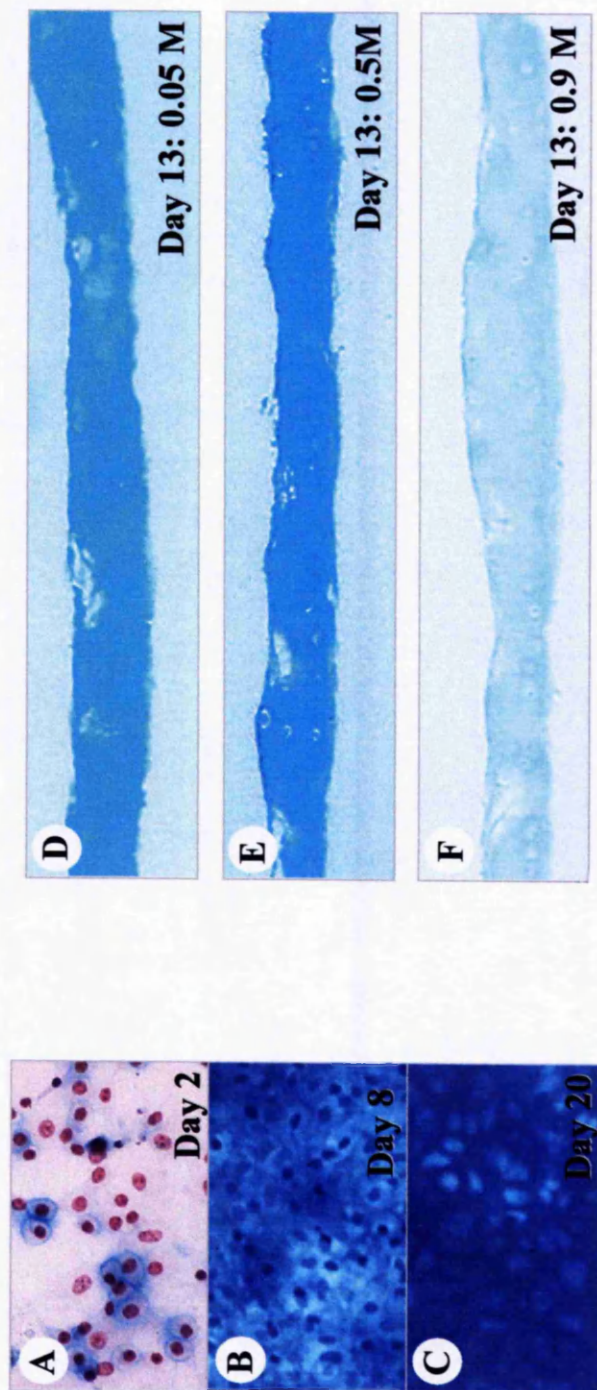


Figure 3.11. Proteoglycan synthesis in foetal bovine epiphyseal chondrocyte cultures. The method of alcian blue staining at pH 2.5 was used to detect the presence of proteoglycans in aza-C treated and untreated chondrocyte cultures at several time-points. The cell nuclei were stained red with aqueous neutral red. The cultures were stained directly in the culture dishes [A, B, C]. Note the increase in staining intensity corresponding to an increase in matrix synthesis as the cells proliferated with time in culture. The staining procedure was repeated using critical electrolyte concentrations of MgCl_2 in order to identify the acid glycosaminoglycans present in the extracellular matrix. Paraffin wax sections of chondrocyte cultures were collected at various time-points and tested histologically [D, E, F]. Note the blue staining throughout the matrix at 0.05 M and 0.5 M [D, E]. Also note the uniform and much fainter staining throughout the matrix at 0.9 M [F]. All results here are typical representations of the presence of proteoglycans in the extracellular matrix in aza-C treated and untreated chondrocyte cultures throughout the culture period. (Magnifications, A-C: $\times 90$; D-F: $\times 344$).

alkaline phosphatase was expressed on days 14 and 20, reaching a maximum expression on day 20 (Figure 3.12). The level of mRNA expression decreased until day 27. These results correlated with those obtained by spectrophotometry, where the expression of alkaline phosphatase in aza-C treated cultures was high on day 14, reached a peak on day 21, and decreased until day 28 (Figure 3.13). Both *in situ* hybridisation studies and spectrophotometric analyses showed a consistently very low level of alkaline phosphatase expression in untreated cultures throughout the culture period.

3.5.5. MINERALISATION IN CULTURE

Foetal bovine epiphyseal chondrocyte cultures were grown in glass chamber-slides and all cultures were either supplemented with or without 10 mM calcium β -glycerophosphate (Ca β GP) throughout the culture period. Cultures were collected at various time-points and stained with alizarin red S in order to detect the presence of mineralisation in culture (Figure 3.14). In aza-C treated cultures, no positive staining for mineralisation was obtained on day 7 with and without the addition of Ca β GP. Some positive reaction, which is identifiable by the presence of orange-red staining in the matrix, was observed on day 14 in aza-C treated cultures supplemented with Ca β GP. More staining was observed on day 25. No orange-red deposit was detected in the absence of Ca β GP supplementation. Likewise, no positive reaction was obtained in aza-C untreated cultures, both with and without the addition of Ca β GP (Figure 3.14).

3.6. DISCUSSION

During the process of endochondral ossification (EO), the differentiation of growth plate chondrocytes is accompanied by the synthesis of several collagen types, which are incorporated into the extracellular matrix of growth plate cartilage. The expression of some of these collagens, namely collagen types II, VI, and X, was examined in foetal bovine epiphyseal chondrocyte cultures, with and without 5-azacytidine (aza-C) treatment.

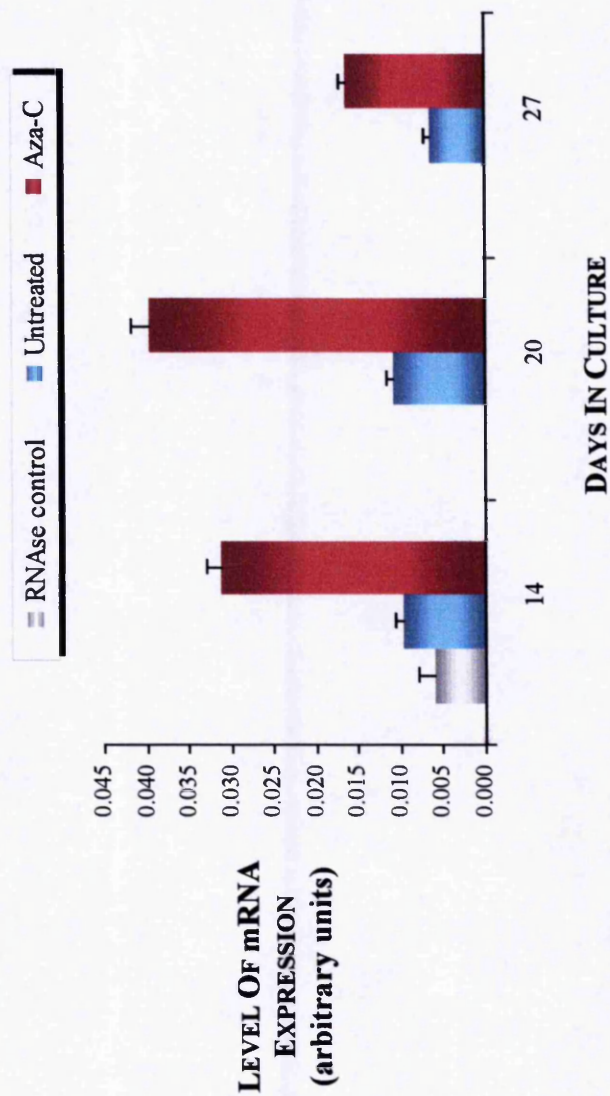


Figure 3.12. mRNA expression of alkaline phosphatase in foetal bovine epiphyseal chondrocyte cultures: *In situ* hybridisation. Aza-C treated and untreated chondrocytes were collected at selected time-points and processed for *in situ* hybridisation analyses. A human cDNA probe specific for alkaline phosphatase was used to study its mRNA expression in culture. An RNase control was included whereby the chondrocytes were treated with RNase to eliminate any hybridisation signal (+aza-C: day 20). All results were quantified using a Quantimet 600S image analysis system. Values represent mean \pm SEM ($n = 20$, where n is the number of fields analysed).

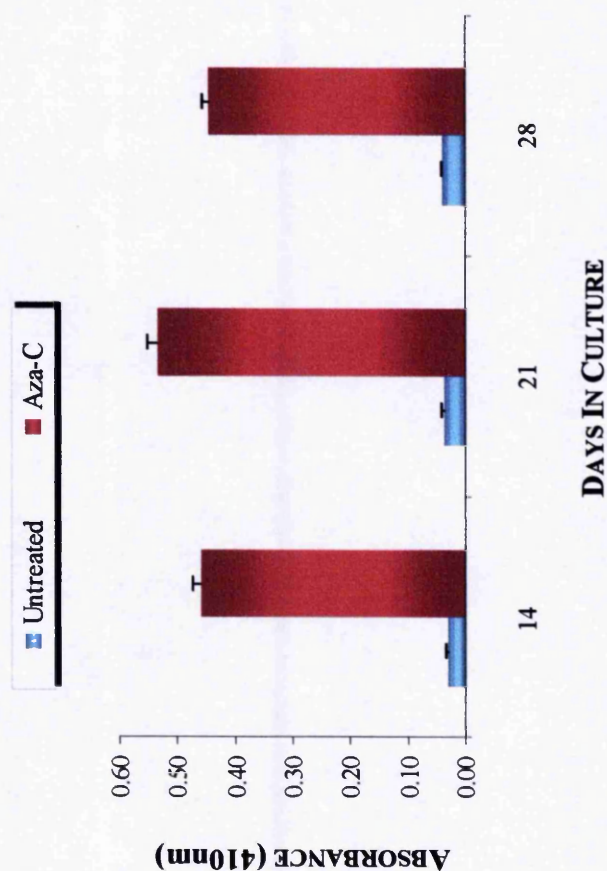


Figure 3.13. Expression of alkaline phosphatase in foetal bovine epiphyseal chondrocyte cultures: Spectrophotometric analyses. Chondrocyte cultures were collected at selected time-points and analysed for alkaline phosphatase using the Sigma Diagnostics® Kit. The assay is based on the hydrolysis of the substrate *p*-nitrophenyl phosphate by alkaline phosphatase to release the yellow complex, *p*-nitrophenol. The absorbance was measured spectrophotometrically at 410 nm. All assays were performed in triplicates. Values represent mean \pm SEM ($n=3$). These results are representative of two experiments performed.

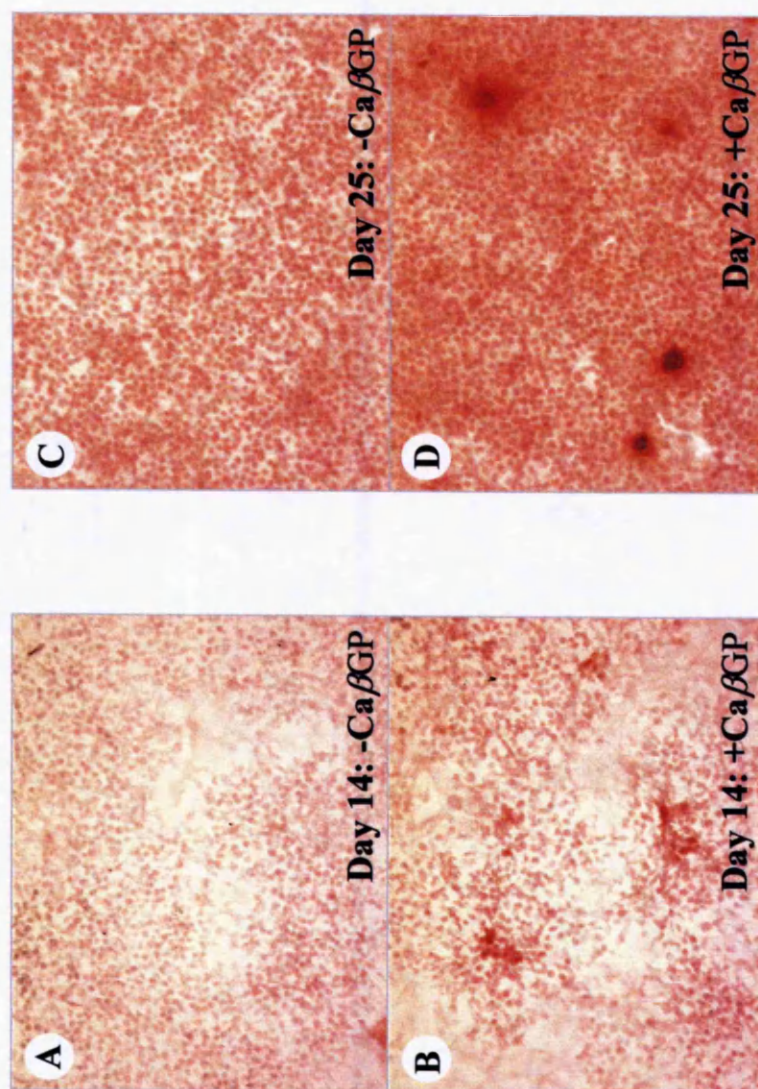


Figure 3.14. Mineralisation in foetal bovine epiphyseal chondrocyte cultures. Foetal bovine epiphyseal chondrocytes were supplemented with 10 mM calcium beta-glycerophosphate (CaβGP) throughout the culture period and stained for the presence of mineralisation using alizarin red S at regular time-points. Photographs above show results obtained in aza-C treated chondrocyte cultures. No calcium mineral deposition was identified in cultures with or without CaβGP [A, B]. Similar results were obtained in cultures on day 7. Some orange-red staining reaction was observed on day 14 only in the presence of CaβGP [C, D]. Note that no positive staining for the presence of mineralisation was detected in aza-C untreated chondrocyte cultures, both with and without the addition of CaβGP. (Magnification: $\times 121$).

Type II collagen was expressed constitutively in both aza-C treated and untreated cultures, as shown by RT-PCR studies, while immunohistochemical analyses revealed the presence of type II collagen throughout the extracellular matrix in all cultures. As discussed in Chapter Two, type II collagen is considered to be a marker of chondrocytic phenotype (Kolettas *et al.*, 1995) and the constitutive expression of type II collagen obtained here confirms further the chondrocytic nature of the cell cultures. The fact that good immunolocalisation of type II collagen was obtained only after treatment of paraffin wax sections of cell layers with hyaluronidase shows a high content of hyaluronan in the extracellular matrix, which masked the presence of the collagen.

Immunohistochemical studies revealed the presence of type VI collagen throughout the culture period in aza-C treated and untreated cultures. Good immunolocalisation of type VI collagen was obtained only when paraffin wax sections of cell layers were treated with hyaluronidase, again indicating the abundance of hyaluronan in the extracellular matrix. Type VI collagen was concentrated mainly in the pericellular environment of chondrocytes with a very low expression in the interterritorial matrix. The pericellular distribution of type VI collagen has been reported previously in articular cartilage (Poole *et al.*, 1988). Type VI collagen is known to function mainly as a structural component of extracellular matrix by maintaining the structural integrity of the tissue and protecting chondrocytes when the cartilage is subjected to compressive forces. There is evidence that type VI collagen links chondrocytes to their surrounding collagen network, thereby stabilising the extracellular matrix components in the pericellular environment of chondrocytes (Poole *et al.*, 1992).

Morphological examination of foetal bovine epiphyseal chondrocytes under the influence of aza-C showed a gradual increase in cell size with time in culture (Chapter Two), thus indicating that aza-C had possibly induced chondrocytes to differentiate to hypertrophy in culture. In order to verify the occurrence of hypertrophy in culture with aza-C treatment, the expression of the specific marker of hypertrophy, type X collagen, was analysed in aza-C treated and untreated cultures. RT-PCR studies showed that type X collagen was expressed from day 11 onwards in aza-C treated cultures only. This observation is supported by *in situ* hybridisation, which also

showed an increase in type X collagen expression with aza-C treatment only. These findings provide the definitive confirmation of the hypertrophic state of aza-C treated chondrocytes, as indicated previously by the morphological status of the treated chondrocytes. Results obtained here also show that the hypertrophic stage of differentiation began on day 11 in aza-C treated chondrocyte cultures. The presence of type X collagen in the hypertrophic zone of the growth plate accompanied by a marked enlargement of hypertrophic chondrocytes has been well documented (Farnum *et al.*, 1990; Capasso *et al.*, 1982).

As reported previously in Chapter Two, long-term monolayer cultures of foetal bovine epiphyseal chondrocytes at high densities produced a thick disc-shaped cell layer with time in culture as a result of a continuous synthesis of extracellular matrix by the cultured chondrocytes. In order to verify the proteoglycan nature of this matrix, alcian blue staining was performed on the cell layers at pH 2.5 at regular intervals in culture. Both aza-C treated and untreated cultures revealed an extracellular matrix rich in proteoglycans. The intensity of the blue reaction increased gradually with time, which indicates an increase in the amount of matrix being produced by the proliferating chondrocytes. It is interesting to observe the chondrocytes being encased individually in alcian-blue positive material at early time-points in culture, which shows that each cell was able to establish actively its own territorial extracellular matrix. The proteoglycan nature of extracellular matrix in culture has also been observed in many other culture systems (Amadio *et al.*, 1983; Capasso *et al.*, 1982). Alcian blue staining using critical electrolyte concentrations allowed the nature of the glycosaminoglycans attached to the proteoglycan molecules to be identified. The uniform positive blue reaction obtained at 0.05 M and 0.5 M of MgCl_2 throughout the extracellular matrix in both aza-C treated and untreated cultures supports the high proteoglycan content of the matrix. It was also observed that the staining reaction at both salt concentrations was more intense around the lacunae enclosing the chondrocytes, which demonstrates a higher accumulation of proteoglycans around the chondrocytes (results not shown). Moreover, the blue reaction obtained at 0.5 M shows the presence of chondroitin sulphate throughout the matrix with a higher concentration around the cell lacunae. Alcian blue staining at 0.9 M revealed a very faint staining reaction throughout the matrix, which indicates a low

content of keratan sulphate in the extracellular matrix. Similar observations have been reported previously by others both *in vivo* and in embryonic chick chondrocyte cultures (McClure *et al.*, 1988). The fact that both aza-C treated and untreated chondrocyte cultures produced similar staining reactions shows that aza-C maintains the chondrocytic nature of the cells in culture by allowing the chondrocytes to elaborate a matrix similar to cartilage matrix *in vivo*.

Numerous studies *in vitro* have reported the expression of type X collagen parallel to the occurrence of calcification, including in cultures of embryonic chick chondrocytes (Thomas *et al.*, 1990), and foetal human growth plate chondrocytes (Kirsch *et al.*, 1992). The expression of type X collagen in aza-C treated cultures obtained in this cell culture model has therefore prompted the investigation into the possible presence of calcification in the model. Both aza-C treated and untreated cultures were supplemented with 10 mM calcium β -glycerophosphate (Ca β GP) throughout the culture period and analysed by alizarin red S staining. Calcium mineral deposition was detected in aza-C treated cultures only during the second week in culture (day 14) with a subsequent increase in positive staining. No calcification was observed in the first week (day 7) in those cultures. The occurrence of calcification during the second week in culture (day 14) coincides with the onset of type X collagen expression in aza-C treated cultures (day 11). However, the occurrence of calcification and type X collagen expression in the hypertrophic zone does not necessarily support an *in vivo* role for type X collagen in calcification (Iyama *et al.*, 1991; Habuchi *et al.*, 1985). In this culture model, only a small minority of aza-C treated chondrocytes were associated with mineral whereas the majority of treated cells were expressing type X collagen. This observation suggests the possibility of non-specific mineralisation taking place in aza-C treated cultures supplemented with Ca β GP and in the presence of alkaline phosphatase expression in culture (see following paragraph). It would be of interest to assess the presence of matrix vesicles in electron micrographs of aza-C treated cultures in order to confirm the occurrence of calcification in culture, although preliminary studies did not reveal the presence of matrix vesicles. A more detailed analysis would therefore be helpful. The process of mineralisation has been obtained in a variety of other cell culture systems whereby chondrocytes were induced to enter hypertrophy (Yan *et al.*, 1997; Kirsch *et al.*, 1992). Interestingly, the induction of the

mesenchymal cell line, C3H/10T1/2, to hypertrophy by aza-C showed no indication of subsequent calcification in the presence of Ca β GP (Atkinson *et al.*, 1997). Several other matrix molecules have also been implicated in mineralisation, including proteoglycans and the C-propeptide of type II collagen (chondrocalcin) (Poole *et al.*, 1984; Suzuki *et al.*, 1981). However, the exact mechanisms of calcification are still not fully understood. It should be noted that the presence of calcification in the cell culture model discussed in the present study was obtained only with the supplementation of Ca β GP. The requirement for Ca β GP in the process of mineralisation *in vitro* has been reported in cultures of embryonic chick chondrocytes (Gerstenfeld and Landis, 1991; Thomas *et al.* 1990). This requirement suggests the importance of phosphate ions in the mineralisation process. Cartilage calcification occurs as phosphate ions react with calcium ions to form hydroxyapatite, which is used in mineral formation. Phosphate ions are made available by the activity of alkaline phosphatase, which is highly expressed in the hypertrophic zone of the growth plate. Several studies have correlated the occurrence of mineralisation *in vitro* with a high activity of alkaline phosphatase (Yan *et al.*, 1997; Kato *et al.*, 1988a).

It was therefore interesting to examine the expression of alkaline phosphatase in the cell culture model. Both *in situ* hybridisation studies and spectrophotometric assays showed that alkaline phosphatase was expressed at the onset of type X collagen expression in aza-C treated cultures. The expression of alkaline phosphatase was highest on days 20-21 and dropped subsequently until days 27-28. The presence of alkaline phosphatase during hypertrophy after aza-C treatment in this cell culture system is consistent with its expression in the hypertrophic zone of the growth plate *in vivo* (de Bernard *et al.*, 1986; Ali *et al.*, 1970). In addition, the expression pattern of alkaline phosphatase observed correlates with the occurrence of calcification in the cell culture system on the assumption that calcification obtained in culture was not non-specific. Some mineralisation was obtained on day 14 when alkaline phosphatase was present. An increase in alkaline phosphatase expression until days 20-21 corresponded with an increase in the amount of calcium mineral deposition obtained in aza-C treated cultures at the same period. This finding suggests that more free phosphate ions were available as a result of an increased enzyme activity, thereby leading to an increased calcification. A decrease in alkaline phosphatase expression

at later time-points could indicate the terminal stage of chondrocyte differentiation *in vitro*. There is considerable evidence that growth plate chondrocytes die by apoptosis at the end of their differentiation *in vivo* (Zenmyo *et al.*, 1996; Farnum and Wilsman 1987). It is possible that chondrocytes also undergo the same fate in this cell culture model. The possibility of apoptotic death in chondrocyte cultures under aza-C treatment was investigated and is discussed in Chapter Five.

Chapter Four

CHAPTER FOUR

EFFECTS OF 5-AZACYTIDINE ON THE EXPRESSION OF FACTORS INVOLVED IN THE REGULATION OF ENDOCHONDRAL OSSIFICATION BY FOETAL BOVINE EPIPHYSEAL CHONDROCYTES

4.1. INTRODUCTION

During the process of endochondral bone formation, growth plate chondrocytes synthesise various different molecules at each stage of their differentiation (Chapter One, Section 1.6). These molecules are not structural constituents of the extracellular matrix of growth plate cartilage, but play a significant part in chondrocyte differentiation. These non-structural components of the extracellular matrix may exert their effect(s) on their own, or more importantly, the coordinated interplay between these molecules plays a critical role in the spatial and temporal regulation of the differentiation programme of growth plate chondrocytes, and ultimately controlling skeletal growth. An aberrant expression of these regulatory factors leads undoubtedly to a disruption of the highly ordered chondrocyte maturation cascade, thereby affecting endochondral ossification (EO), and consequently causing skeletal deformities and often embryonic or postnatal lethality. This chapter focuses on the expression of some of these key differentiation-associated molecules: (i) parathyroid hormone-related peptide (PTHrP), which is expressed in maturing chondrocytes, (ii) PTHrP receptor, also found in the maturation zone of the growth plate, and (iii) α -enolase, a newly identified marker of chondrocyte differentiation.

4.1.1. PARATHYROID HORMONE-RELATED PEPTIDE

As described in Chapter One, Section 1.6.7, PTHrP is expressed specifically in the maturation zone of differentiation in the growth plate cartilage (Amizuka *et al.*, 1994; Lee *et al.*, 1994). PTHrP expression is crucial for the normal and ordered progression

of the chondrocyte differentiation programme. It is known to inhibit the terminal differentiation of growth plate chondrocytes, as demonstrated by the overexpression of PTHrP by proliferating chondrocytes in transgenic mice. These mice were born with cartilaginous endochondral skeletons, suggesting that the process of EO was delayed (Weir *et al.*, 1996). This finding is consistent with previous studies of PTHrP-deficient transgenic mice (Amizuka *et al.*, 1994; Karaplis *et al.*, 1994). These transgenic mice developed skeletal dysplasias as a result of an advanced endochondral bone formation and died at birth. The skeletal defect was characterised by a severe disruption of the growth plate structure, a reduced chondrocyte proliferation, and an accelerated terminal differentiation. There is also evidence that PTHrP plays a role in the apoptotic death of chondrocytes (Amizuka *et al.*, 1996). Mice homozygous for the PTHrP-null mutation showed more apoptotic chondrocytes near the chondro-osseous junction than wild-type littermates (Amizuka *et al.*, 1996; Lee *et al.*, 1996). Moreover, PTHrP overexpression in transgenic mice caused an upregulated expression of the cell death inhibitor, bcl-2, with no apparent change in the expression level of the cell death inducer, bax (Amling *et al.*, 1997). This shift in the bcl-2/bax ratio in favour of bcl-2 delays terminal differentiation and maintains chondrocytes in their prehypertrophic stage. It has been shown recently that PTHrP induces the expression of matrix metalloproteinase-2 (MMP-2) *in vitro* (Kawashima-Ohya *et al.*, 1998), which suggests that PTHrP may be involved in the control of matrix degradation in the growth plate during EO. It has been proposed recently that PTHrP expression in the growth plate may be in part controlled by Indian hedgehog (Ihh), as described in Chapter One, Section 1.6.8.

4.1.2. PARATHYROID HORMONE-RELATED PEPTIDE RECEPTOR

Parathyroid hormone-related peptide receptor (PTH-1 receptor) is a G protein-coupled cell surface receptor that mediates the biological actions of both PTHrP and PTH (Abou-Samra *et al.*, 1992; Juppner *et al.*, 1991). Another member of the G protein-coupled receptors (PTH-2 receptor) has been identified recently. In contrast to PTH-1 receptor, PTH-2 receptor responds only to PTH (Turner *et al.*, 1998; Behar *et al.*, 1996). PTH-1 receptor is expressed abundantly in the maturation zone of the growth plate (Lee *et al.*, 1994). Direct evidence for the importance of PTH-1 receptor in

mediating the effects of PTHrP was provided by the generation of PTH-1 receptor knock-out mice (Lanske *et al.*, 1996). These receptor-negative mice exhibited severe skeletal abnormalities characterised by an accelerated chondrocyte terminal differentiation and an early embryonic death. It has been found that the constitutive activation of PTH-1 receptor is the cause of Jansen-type metaphyseal chondrodysplasia, a skeletal disorder characterised by delayed endochondral ossification (Schipani *et al.*, 1996; 1995). The generation of transgenic mice that express constitutively active PTH-1 receptors has shown a decelerated differentiation of growth plate chondrocytes to hypertrophy, delayed cartilage mineralisation and vascularisation (Schipani *et al.*, 1997). Interestingly, the expression of constitutively active PTH-1 receptors rescued PTHrP-depleted mice by correcting their growth plate abnormalities. More recently, it was reported that the absence of functional PTH-1 receptors as a result of a point mutation may be responsible for Blomstrand chondrodysplasia (Jobert *et al.*, 1998). Blomstrand chondrodysplasia is a skeletal disorder characterised by phenotypes that are the mirror image of those observed in Jansen-type metaphyseal chondrodysplasia, including an accelerated chondrocyte maturation and foetal death (Blomstrand *et al.*, 1985). Taken together, all these findings confirm the crucial role of signalling through the PTH-1 receptor during EO. In this report, PTH-1 receptor is referred to as PTHrP receptor.

4.1.3. α -ENOLASE

α -Enolase is a key enzyme in glycolysis whereby it catalyses the conversion of 2-phospho-D-phosphoglycerate to phosphoenolpyruvate (Wold, 1971). In our laboratory, the cDNA encoding α -enolase has been isolated and identified with the use of an RT-PCR based subtractive hybridisation technique (Chapman, 1998). The expression of α -enolase has been found to be upregulated in the proliferative zone of the bovine growth plate, as demonstrated by *in situ* hybridisation studies and immunohistochemistry (Chapman, 1998). In mammals, α -enolase is present as three isoforms: (1) β -enolase present specifically in muscle, (2) γ -enolase found in neurones, and (3) the ubiquitous α -enolase (Wold, 1971). In addition to its catalytic activity in the glycolytic pathway, α -enolase is thought to have several other functions. There is strong evidence showing that α -enolase displays characteristics of

a plasminogen receptor (Andronicos *et al.*, 1997; Redlitz *et al.*, 1995; Miles *et al.*, 1991). Numerous studies have demonstrated that it binds plasminogen in a variety of systems, including human endothelial cells (Fukao *et al.*, 1992), rat neurones (Nakajima *et al.*, 1994), and on the surface of streptococci (Pancholi and Fischetti, 1998). Plasminogen is a zymogen that is known to activate certain proenzymes (Nagase *et al.*, 1990; Blasi *et al.*, 1987; Liotta *et al.*, 1981), prohormones (Virji *et al.*, 1980), and progrowth factors (Rifkin *et al.*, 1990). It is also thought to be involved in cell migration (Kalderon, 1979) and cell proliferation (Kalderon, 1982). α -Enolase has also been reported to be present in the centrosome of HeLa cells (Johnstone *et al.*, 1992; Rattner *et al.*, 1991), which suggests a possible involvement of α -enolase in the organisation of the cytoskeleton in the cell cycle. In addition, yeast (Chin *et al.*, 1981) and human (Giallongo *et al.*, 1986) enolases show sequence similarity to turtle τ -crystallin, which is a structural component of the lens (Williams *et al.*, 1985). This finding suggests that α -enolase may serve a structural role. The presence of α -enolase in the growth plate is interesting, especially since the chondrocytes are actively proliferating and differentiating in an environment where the extracellular matrix is synthesised, degraded, and reorganised constantly. Furthermore, it has been found recently that human α -enolase is highly homologous with the c-myc binding protein, MBP-1 (Chaudhary *et al.*, 1996). This finding suggests yet another possible function of α -enolase, that of inhibiting c-myc expression in growth plate chondrocytes. The significance of the high level of expression of α -enolase in the proliferating zone of the growth plate remains to be elucidated.

4.2. AIMS

The aims of this study were to analyse the expression of some of the non-structural constituents of the extracellular matrix that are key players in chondrocyte differentiation in the growth plate, namely PTHrP and its receptor. In addition, one of the main purposes of developing a cell culture model of EO is to enable the characterisation of several genes, including a number of novel ones, that have been identified in the growth plate (Hillarby *et al.*, 1996). Among these newly identified genes, α -enolase is a gene that has been reported only recently in EO. It has been

found to be expressed highly in proliferating chondrocytes by molecular biology techniques (Chapman, 1998). In this study, the expression of α -enolase in EO was further defined by using the aza-C cell culture model. The patterns of expression of all three differentiation markers were studied in both aza-C treated and untreated cultures using time-course experiments. As mentioned in Chapter Three, Section 3.2, the choice of time-points was influenced by the availability of tissue materials as well as the pattern of expression of these markers in the growth plate *in vivo*. It was also not possible to repeat all time-course studies described in this chapter due to the limited availability of foetal bovine tissue. These experiments were designed to characterise further the cell culture system and help in the construction of the differentiation profile of chondrocytes (Chapter Three). These strategies of investigation are outlined in figure 4.1.

4.3. MATERIALS

Gibco BRL: Paisley, Scotland

Dulbecco's phosphate buffered saline (without calcium and magnesium), foetal calf serum, gentamycin, *L*-glutamine, minimal essential medium (with Earle's salts and *L*-glutamine), penicillin-streptomycin solution, sodium bicarbonate (7.5%), and trypsin (0.25% w/v in Gibco Solution A).

Sigma Chemicals Co. Ltd: Dorset, U.K.

Acetic anhydride, 5-azacytidine (50X; lyophilised and sterilised), bacterial collagenase type 1A, bovine serum albumin, dextran sulphate, diethylpyrocarbonate, dithiothreitol, Ficoll, glycine, *L*-ascorbic acid, paraformaldehyde, polyvinylpyrrolidone, salmon sperm DNA, Sephadex G50.

BDH Laboratory Supplies: Dorset, U.K.

Ethylenediaminetetraacetic acid, formamide, hydrochloric acid, sodium acetate, sodium citrate, sodium chloride, and tris.

Amersham Pharmacia Biotech Ltd.: Buckinghamshire, U.K.

Megaprime DNA labelling system, and ^{35}S -dCTP.

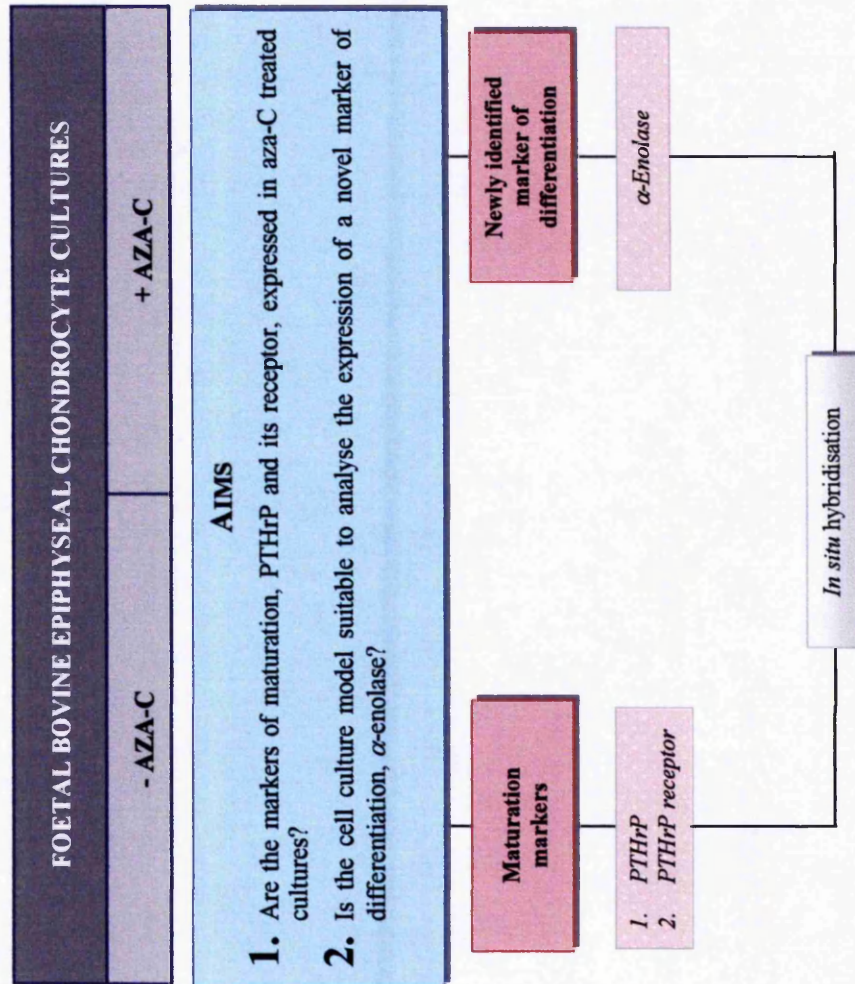


Figure 4.1. Schematic diagram showing the strategies of investigation into the expression of the non-structural components of the extracellular matrix in foetal bovine epiphyseal chondrocyte cultures.

Ilford Imaging U.K. Ltd.: Cheshire, U.K.

K-photographic emulsion, Hypam rapid fixer, Phenisol high contrast film developer, and Ilfostop Pro low-odour stop bath.

cDNA probes for PTHrP and PTHrP receptor were kindly provided by Dr. Judith Hoyland (University of Manchester). The cDNA probe for α -enolase was kindly provided by Kathryn Chapman (University of Manchester).

All reagents were of the highest grade commercially available.

4.4. METHODS

4.4.1. BOVINE CHONDROCYTE CULTURE

The procedures are as described in Chapter Two, Section 2.4.1.

4.4.2. 5-AZACYTIDINE TREATMENT OF CHONDROCYTE CULTURES

The procedures are as described in Chapter Two, Section 2.4.2.

4.4.3. TRYPSINISATION OF CHONDROCYTE CULTURES

The procedures are as described in Chapter Three, Section 3.4.3.

4.4.4. IN SITU HYBRIDISATION

The procedures are as described in Chapter Three, Section 3.4.9. Human cDNA probes for PTHrP, PTHrP receptor, and alkaline phosphatase, and a bovine cDNA probe for α -enolase were used to identify the mRNA expression of these markers in cultured chondrocytes (see Appendices).

4.4.5. IMAGE ANALYSIS

The procedures are as described in Chapter Three, Section 3.4.12.

4.5. RESULTS

4.5.1. mRNA EXPRESSION OF PARATHYROID HORMONE-RELATED PEPTIDE IN FOETAL BOVINE EPIPHYSEAL CHONDROCYTE CULTURES

The mRNA expression of parathyroid hormone-related peptide (PTHrP) in foetal bovine epiphyseal chondrocyte cultures was analysed by *in situ* hybridisation. Chondrocyte cultures were harvested at various time-points and digested with trypsin and collagenase to release the chondrocytes. The cells were fixed on glass slides and processed for *in situ* hybridisation analyses using a ^{35}S -labelled cDNA probe specific for PTHrP. The slides were viewed using a Leica DB research microscope and results were quantified, as previously published (Mee *et al.*, 1997; Walsh *et al.*, 1993) by the Quantimet image analysis system described under Methods. Hybridisation signals were seen as black grains and the level of mRNA expression was represented by the concentration of black grains obtained. For each time-point, ten fields per slide were chosen randomly and the number of grains counted. Since duplicate slides were analysed for each time-point, the mean grain counts of twenty fields was calculated. The level of mRNA expression of PTHrP was expressed as the mean grain density per unit area of cells. Results obtained showed that under aza-C treatment, PTHrP was expressed highly during the first week of the culture period (days 5 and 7) with a peak reached on day 7 (Figure 4.2). PTHrP expression decreased at subsequent time-points tested (days 9, 14, and 20). A much lower level of expression of PTHrP was obtained in untreated cultures at the same time-points studied.

4.5.2. mRNA EXPRESSION OF PARATHYROID HORMONE-RELATED PEPTIDE RECEPTOR IN FOETAL BOVINE EPIPHYSEAL CHONDROCYTE CULTURES

Radioactive *in situ* hybridisation was used to analyse the mRNA expression of parathyroid hormone-related peptide receptor (PTHrP receptor) in foetal bovine epiphyseal chondrocytes in the presence and absence of aza-C treatment. For each time-point, ten fields per slide were chosen randomly and the number of grains counted. Since duplicate slides were analysed for each time-point, the mean

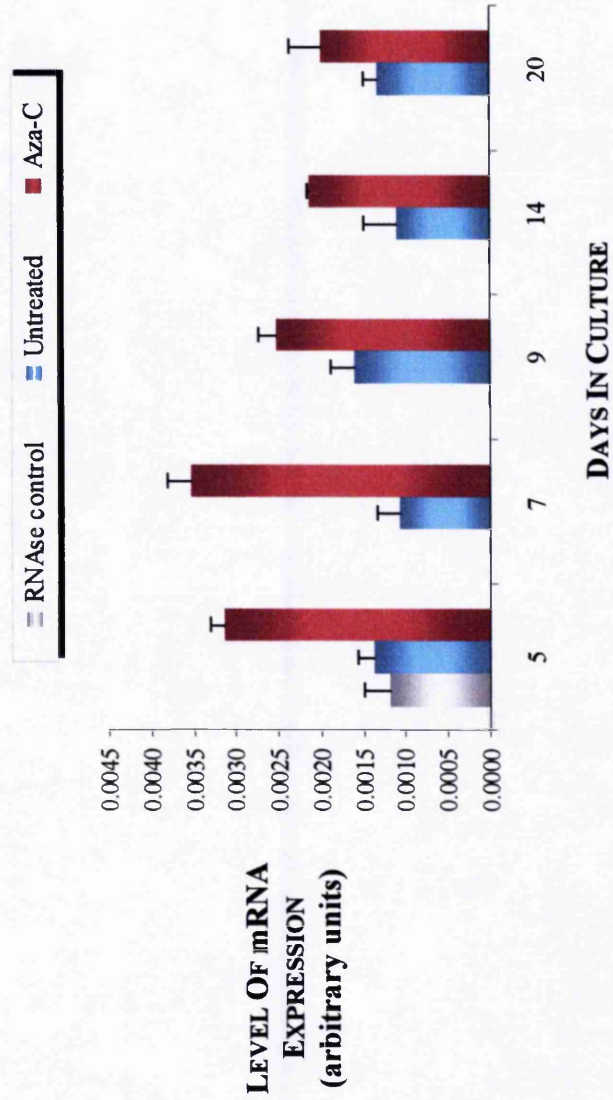


Figure 4.2. mRNA expression of parathyroid hormone-related peptide in foetal bovine epiphyseal chondrocyte cultures. Aza-C treated and untreated chondrocytes were collected at selected time-points and processed for *in situ* hybridisation analyses. A human cDNA probe specific for PTHrP was used to study its mRNA expression in culture. An RNAse control was included whereby the chondrocytes were treated with RNAse to eliminate any hybridisation signal (aza-C; day 7). All results were quantified using a Quantimet 600S image analysis system. Values represent mean \pm SEM ($n=20$, where n represents the number of fields analysed).

grain counts of twenty fields was calculated. The level of mRNA expression of PTHrP was expressed as the mean grain density per unit area of cells. Quantification of results using image analysis system revealed a very low expression of PTHrP receptor in untreated cultures throughout the culture period. In the presence of aza-C treatment, PTHrP receptor was expressed at very low levels during the first week in culture. The level of expression was upregulated markedly on day 9, and decreased considerably until day 23 (Figure 4.3).

4.5.3. mRNA EXPRESSION OF α -ENOLASE IN FOETAL BOVINE EPIPHYSEAL CHONDROCYTE CULTURES

The mRNA expression of α -enolase in aza-C treated and untreated chondrocyte cultures was investigated by radioactive *in situ* hybridisation and the results quantified by image analysis (Mee *et al.*, 1997; Walsh *et al.*, 1993). For each time-point, ten fields per slide were chosen randomly and the number of grains counted. Since duplicate slides were analysed for each time-point, the mean grain counts of twenty fields was calculated. The level of mRNA expression of PTHrP was expressed as the mean grain density per unit area of cells. Very low levels of expression of α -enolase were obtained during the first week in aza-C treated cultures (Figure 4.4). Its level of expression increased markedly on day 9 and decreased until day 12 when it reached only approximately 50% of the maximal expression obtained on day 9. Very low levels of expression were obtained at subsequent time-points analysed (days 14 and 20). Untreated chondrocyte cultures maintained a very low expression level of α -enolase throughout the culture period.

4.6. DISCUSSION

Endochondral ossification (EO) is a process that occurs under strict spatial and temporal control during the embryonic development of the axial and appendicular skeletal system. The control of EO is achieved by a variety of regulatory molecules, which are expressed by chondrocytes at specific stages of cell differentiation in the growth plate. Given the importance of these differentiation-associated molecules in

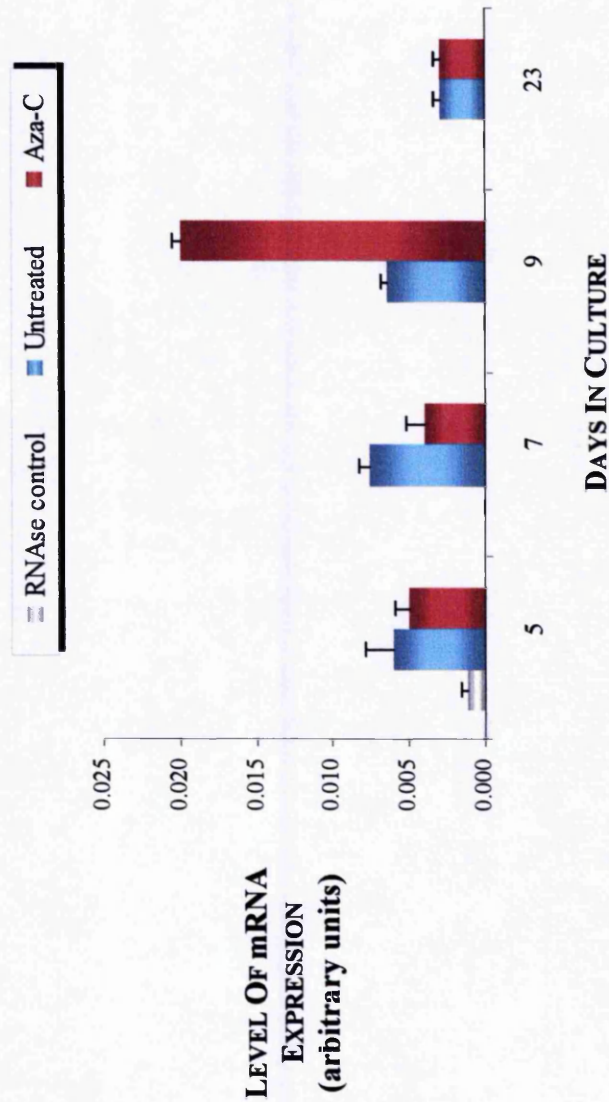


Figure 4.3. mRNA expression of parathyroid hormone-related peptide receptor in foetal bovine epiphyseal chondrocyte cultures. Aza-C treated and untreated chondrocytes were collected at selected time-points and processed for *in situ* hybridisation analyses. A human cDNA probe specific for PTHrP receptor was used to study its mRNA expression in culture. An RNase control was included whereby the chondrocytes were treated with RNase to eliminate any hybridisation signal (+ aza-C; day 7). All results were quantified using a Quantimet 600S image analysis system. Values represent mean \pm SEM ($n = 20$, where n represents the number of fields counted).

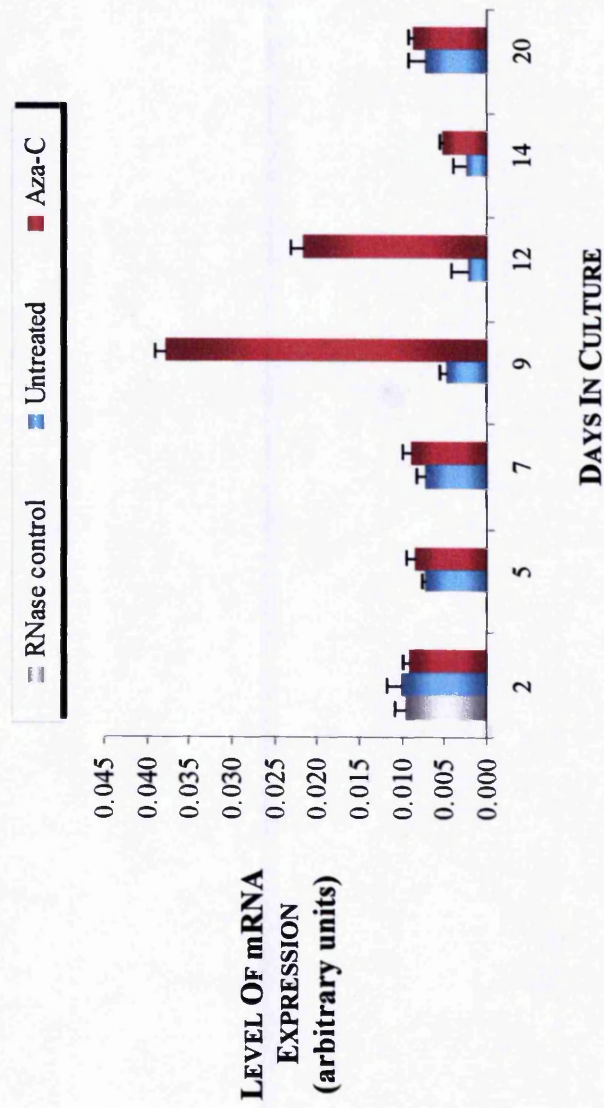


Figure 4.4. mRNA expression of α -enolase in foetal bovine epiphyseal chondrocyte cultures. Aza-C treated and untreated chondrocytes were collected at selected time-points and processed for *in situ* hybridisation analyses. A bovine cDNA probe specific for α -enolase was used to study its mRNA expression in culture. An RNase control was included whereby the chondrocytes were treated with RNase to eliminate any hybridisation signal (+aza-C; day 9). All results were quantified using a Quantimet 600S image analysis system. Values represent mean \pm SEM ($n = 20$, where n represents the number of fields counted).

EO, it was important to analyse the expression of some of these key molecules in the cell culture model described in this study.

Parathyroid hormone-related peptide (PTHrP) is one of the key regulatory factors known to play a crucial role in the chondrocyte differentiation pathway (Weir *et al.*, 1996; Karaplis *et al.*, 1994). PTHrP has been shown to be expressed specifically in the proliferative and maturation zones of the growth plate prior to hypertrophy (Amizuka *et al.*, 1994; Lee *et al.*, 1994). In the present study, results obtained by *in situ* hybridisation revealed a high expression of PTHrP in aza-C treated cultures at early time-points in culture with a maximum level of expression reached on day 7. However, it should be noted that PTHrP was also expressed at subsequent time-points, albeit at lower levels. It is interesting to note that the high expression of PTHrP was obtained prior to the expression of type X collagen, which is a strict marker of hypertrophy. Results described in Chapter Three showed that type X collagen was expressed from day 11 onwards in aza-C treated cultures. The fact that a high expression of PTHrP was obtained before the expression of type X collagen in culture suggests that the chondrocytes were possibly in their maturation stage of differentiation before day 11 under aza-C treatment. Therefore, the expression pattern of PTHrP in this cell culture model appears to be in agreement with that in the growth plate cartilage *in vivo* (Amizuka *et al.*, 1994; Lee *et al.*, 1994). Consequently, it is tentative to define the culture period "days 5-9" as the proliferation/maturation stage of chondrocyte differentiation *in vitro*. The mechanism by which PTHrP influences the differentiation pathway of growth plate chondrocytes is still unclear. While there is evidence that PTHrP is under the control of indian hedgehog (Lanske *et al.*, 1996; Vortkamp *et al.*, 1996), a recent study has provided a strong indication that PTHrP acts on bcl-2, which is an apoptotic suppressor (Amling *et al.*, 1997). It has been suggested that PTHrP promotes the expression of bcl-2, thereby delaying the terminal differentiation of growth plate chondrocytes and their eventual apoptotic death (Amling *et al.*, 1997). The expression of bcl-2 in this cell culture model was investigated and is discussed in Chapter Five.

The biological actions of PTHrP are mediated by its receptor, PTH-1 receptor (referred to as PTHrP receptor in this report) (Abou-Samra *et al.*, 1992; Juppner *et al.*,

1991). There is considerable evidence for the important role of PTHrP receptor in EO, as demonstrated by the occurrence of severe skeletal abnormalities in knock-out and transgenic mice (Schipani *et al.*, 1997; Lanske *et al.*, 1996). Since PTHrP receptor has also been localised in the maturation zone of the growth plate *in vivo* (Iwamoto *et al.*, 1994a; Lee *et al.*, 1994), it was logical to analyse its expression in the cell culture model being characterised in this study. *In situ* hybridisation analyses showed that there were very low levels of expression of PTHrP receptor at early time-points, a maximum expression level on day 9 and very low levels at subsequent time-points in aza-C treated cultures. As in the case of PTHrP, it would appear that PTHrP receptor was expressed highly prior to type X collagen expression upon exposure to aza-C. However, this observation is only highly speculative. Since the expression of the receptor was not measured at any time-point that coincided with type X collagen expression due to a limited availability of bovine materials, it is difficult to assess whether such a high receptor expression occurred only before type X collagen expression or not. However, as will be discussed in Chapter Six, the addition of exogenous PTHrP downregulated the expression of type X collagen on day 14. This result suggests that it is possible that PTHrP receptor is constitutively expressed at a low level after the expression of type X collagen is turned on. If PTHrP receptor is not expressed after the onset of type X collagen expression in aza-C treated cultures or expressed at a low level, these results would then be consistent with previous findings, which showed the expression of PTHrP receptor before the onset of hypertrophy in the growth plate (Iwamoto *et al.*, 1994a; Lee *et al.*, 1994).

In addition to the many well characterised differentiation-associated molecules involved in the chondrocyte differentiation pathway in the growth plate, a number of novel genes expressed in specific regions of the bovine growth plate during EO have been identified (Hillarby *et al.*, 1996). The development of the cell culture model described in this study would enable the characterisation of these novel genes. The cDNA encoding one of these novel genes has been isolated recently by an RT-PCR based subtractive hybridisation technique and identified as α -enolase (Chapman, 1998). α -Enolase is a key enzyme involved in the glycolytic pathway (Wold, 1971). In an attempt to define the expression of α -enolase in EO, the aza-C cell culture model was used. *In situ* hybridisation studies revealed a high expression of α -enolase

on days 9 and 12 in aza-C treated cultures, although the expression level on day 12 was lower than that on day 9. α -Enolase was expressed at very low levels at subsequent time-points in culture. Given that day 9 corresponds to the proliferation/maturation stage of chondrocyte differentiation and day 12 marks the beginning of hypertrophy in the cell culture system, these results seem to be in agreement with the expression of α -enolase in the growth plate *in vivo*. *In situ* hybridisation analyses showed α -enolase to be expressed abundantly in the proliferative zone of the bovine growth plate and to be absent in the terminal hypertrophic zone (Chapman, 1998). It should be noted that the proliferative stage is not clearly distinguishable from the maturation stage in this cell culture system, which explains why these two stages are expressed as one. The expression of α -enolase in the growth plate is interesting, especially since α -enolase appears to display numerous different properties and functions in a variety of systems. It has been reported that α -enolase may be involved in the organisation of the cytoskeleton (Johnstone *et al.*, 1992; Rattner *et al.*, 1991), and it may also play a structural role (Giallongo *et al.*, 1986; Williams *et al.*, 1985). There is increasing evidence that α -enolase acts as a receptor for plasminogen (Andronicos *et al.*, 1997; Redlitz *et al.*, 1995), which is known to be involved in cell migration and proliferation (Kalderon, 1979, 1989), and to activate various proenzymes, including matrix metalloproteinase-3 (Nagase *et al.*, 1990; Liotta *et al.*, 1981) and progrowth factors (Rifkin *et al.*, 1990). These functions of α -enolase may seem plausible in the growth plate since chondrocytes undergo active proliferation and differentiation in an environment where the extracellular matrix is under constant turnover. Interestingly, chemical cross-linking studies revealed the binding of plasminogen to the surface of growth plate chondrocytes, which may suggest that α -enolase could be the receptor (Chapman, 1998). Another interesting possibility is that α -enolase binds the proto-oncogene, c-myc, since there is evidence showing a high homology between α -enolase and the c-myc binding protein, MBP-1 (Chaudhary *et al.*, 1996). c-myc has been shown to be expressed abundantly in the proliferating zones of the growth plate in chick embryo sterna (Iwamoto *et al.*, 1995) and is known to maintain chondrocytes in their proliferative state. The high expression of α -enolase just prior to hypertrophy in this cell culture model suggests that it may bind the constitutive c-myc, thereby inhibiting cell

proliferation and allowing chondrocytes to enter hypertrophy. The exact significance of the abundant presence of α -enolase in the proliferating chondrocytes in the growth plate is yet to be found.

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Chapter Five

CHAPTER FIVE

THE FATE OF HYPERTROPHIC CHONDROCYTES UNDER 5-AZACYTIDINE TREATMENT *IN VITRO*

5.1. INTRODUCTION

Evidence obtained from numerous studies has shown that growth plate chondrocytes undergo apoptotic death when they reach the terminal hypertrophic stage of their differentiation. Apoptosis is a mode of cell death that occurs under normal physiological conditions in a wide variety of biological systems, including normal cell turnover, embryogenesis, morphogenesis, development and maintenance of the immune system, and exposure of cells to toxic agents (Cohen *et al.*, 1992; Arends and Wyllie 1991). The other form of cell death is necrosis, which occurs in response to severe physical injury to the cell, often caused by mechanical damage, hypoxia and exposure to toxic agents (Wyllie, 1987b). Apoptosis and necrosis exhibit differential morphological and biochemical features.

Necrosis is associated with an impaired maintenance of homeostasis by the cell, leading to an influx of water in the cell. Subsequently, cytoplasmic organelles, including mitochondria, and the entire cell swell and lyse, releasing cytoplasmic contents, including lysosomal enzymes, into the extracellular environment (Wyllie, 1987b). Necrosis triggers a strong inflammatory response and the cell debris is phagocytosed by macrophages (van Furth and van Zwet, 1988). Necrosis is accompanied by random DNA degradation, which is seen as a DNA smear after agarose gel electrophoresis (Afanas'ev *et al.*, 1986). Necrotic death is a passive process and has no energy requirement.

One of the early manifestations of apoptosis is the compaction of nuclear chromatin, which aggregates at the nuclear membrane. Chromatin aggregation is accompanied by nuclear and cytoplasmic condensation or "blebbing", which results in nuclear and

cellular fragmentation, and eventually the formation of membrane-bound vesicles called apoptotic bodies. *In vivo*, apoptotic bodies are phagocytosed by neighbouring cells such as macrophages (Savill *et al.*, 1989), and degraded within lysosomes. *In vitro*, apoptotic bodies are not phagocytosed but swell and lyse in a manner similar to necrotic death. This process is termed secondary necrosis. Apoptosis is an ATP-driven process. In contrast to necrosis, the distinctive morphological features of apoptosis are the preservation of the integrity of the membrane and cytoplasmic organelles, and the absence of an inflammatory response. In addition, the biochemical hallmark of apoptosis is the degradation of genomic DNA into mono- or oligonucleosomal fragments of 180-200 base pairs or multiples thereof (Wyllie *et al.*, 1984). DNA fragmentation has been shown to result from the activity of Ca^{2+} and Mg^{2+} -dependent nuclear endonucleases (Shiokawa *et al.*, 1994). Upon agarose gel electrophoresis, these DNA fragments reveal a characteristic ladder pattern (Arends *et al.*, 1990; Wyllie, 1980).

Apoptosis has been shown to be essential for normal development and its dysregulation can lead to a spectrum of disease states (Roy *et al.*, 1995; Donehower *et al.*, 1992). Therefore, there is a need to understand and identify both positive and negative regulators of apoptosis and the molecules that modulate their functions. The regulation of the apoptotic pathway involves a large number of genes that can be classified as: (a) genes that suppress apoptosis, "survival genes", such as members of the *bcl-2* family, (b) genes that promote apoptosis, "death genes", including members of the interleukin-1 β converting enzyme (ICE) family of cysteine proteases, now known as caspases, and (c) transcription factors closely associated with cell proliferation such as *myc*, *fos*, *jun*, and *p53*.

Apoptosis has been shown to be the mode of death undergone by terminal hypertrophic chondrocytes in the growth plate (Farnum and Wilsman 1989, 1987; Zenmyo *et al.*, 1996). In addition, the expression of the cell death inhibitor, *bcl-2*, has been found to be upregulated in the proliferative and maturing chondrocytes in the growth plate, while the expression of the cell death inducer, *bax*, increases progressively towards the hypertrophic zone of the growth plate (Amling *et al.*, 1997).

Bcl-2 is the prototype member of a large gene family encoding proteins that can be divided into two groups: (a) those that inhibit apoptosis, and (b) those that promote apoptosis (Korsmeyer *et al.*, 1992). *Bcl-2* is an inhibitor of apoptosis (Hockenberry *et al.*, 1990), while *bax* is known to induce the apoptotic pathway (Oltvai *et al.*, 1993). The determining factor for cell survival or the occurrence of apoptosis is the ratio of the level of expression of *bcl-2* and *bax*. These two proteins have been shown to interact with each other to form *bcl-2/bax* heterodimers (Oltvai *et al.*, 1993). Apoptosis is suppressed when half or more of the endogenous *bax* is associated with *bcl-2* (Oltvai *et al.*, 1993). These findings are consistent with the level of expression and spatial distribution of *bcl-2* and *bax* in the growth plate. The *bcl-2/bax* ratio is in favour of *bcl-2* in the proliferative and maturing chondrocytes but in favour of *bax* in the hypertrophic zone (Amling *et al.*, 1997). The importance of *bcl-2* as a major player in chondrocyte differentiation during endochondral ossification is demonstrated by *bcl-2* knockout mice studies. These studies showed limb deformation, a marked reduction in growth plate thickness and decreased bone length (Amling *et al.*, 1997). These results suggest strongly that the absence of *bcl-2* caused a shift in the *bcl-2/bax* ratio in favour of *bax* and led to an accelerated terminal chondrocyte differentiation and apoptosis. Therefore, it is evident that apoptosis is a key stage in the differentiation of growth plate chondrocytes during long bone formation. Surprisingly, a recent study showed that the absence of *bcl-2* in knock-out mice had only a mild effect on the growth plate phenotype and produced a marked effect on bone deposition by osteoblasts (Boot-Handford *et al.*, in press).

5.2. AIMS

The purpose of this study was to determine whether foetal bovine epiphyseal chondrocytes (initially treated with 5-azacytidine) died by apoptosis after they had entered the hypertrophic stage of differentiation *in vitro*. Several approaches were taken in order to identify the occurrence of apoptotic death in culture: (i) the gross morphology of the nucleus was examined by fluorescence microscopy, (ii) the ultrastructural features of cell nuclei were analysed by transmission electron microscopy, (iii) the integrity of the plasma membrane of cultured chondrocytes was examined by using annexin V-FITC, a fluorescent probe which binds to the plasma

membrane of apoptotic cells, and (iv) the expression of *bcl-2* and *bax* was studied by *in situ* hybridisation. The *in situ* hybridisation studies were carried out in time-course experiments with a view to determining the ratio of *bcl-2/bax* expression in culture. As mentioned in Chapter Three, Section 3.2, the choice of time-points was influenced by the availability of tissue materials as well as the pattern of expression of these markers in the growth plate *in vivo*. These strategies of investigation are summarised in figure 5.1.

5.3. MATERIALS

Gibco BRL: Paisley, Scotland

Dulbecco's phosphate buffered saline (without calcium and magnesium), foetal calf serum, gentamycin, *L*-glutamine, minimal essential medium (with Earle's salts and *L*-glutamine), penicillin-streptomycin solution, sodium bicarbonate (7.5%), and trypsin (0.25% w/v in Gibco Solution A).

Sigma Chemicals Co. Ltd: Poole, Dorset, U.K.

Acetic anhydride, 5-azacytidine (50X; lyophilised and sterilised), bacterial collagenase type 1A, bovine serum albumin, dextran sulphate, diethylpyrocarbonate, dithiothreitol, Ficoll, glycine, *L*-ascorbic acid, paraformaldehyde, polyvinylpyrrolidone, salmon sperm DNA, Sephadex G50.

BDH Laboratory Supplies: Poole, Dorset, U.K.

Ethylenediaminetetraacetic acid, formamide, hydrochloric acid, sodium acetate, sodium citrate, sodium chloride, sodium hydroxide, and tris.

Amersham Life Science: Buckinghamshire, U.K.

Megaprime DNA labelling system, and ³⁵S-dCTP.

Ilford Imaging U.K. Ltd.: Cheshire, U.K.

K-photographic emulsion, Hypam rapid fixer, Phenisol high contrast film developer, and Ilfostop Pro low-odour stop bath.

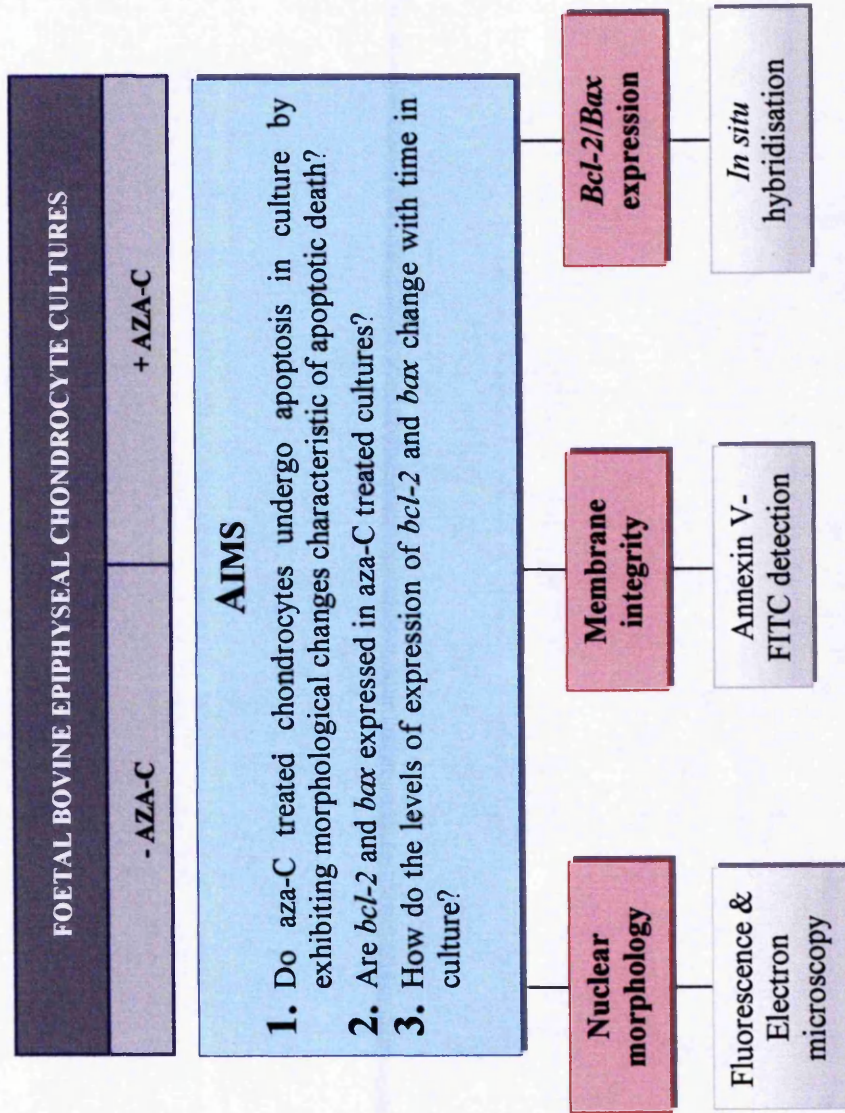


Figure 5.1. Schematic diagram showing the strategies of investigation into the occurrence of death by apoptosis in foetal bovine epiphyseal chondrocyte cultures.

All cDNA probes were kindly provided by Dr. Judith Hoyland (University of Manchester).

All reagents were of the highest grade commercially available.

5.4. METHODS

5.4.1. BOVINE CHONDROCYTE CULTURE

The procedures are as described in Chapter Two, Section 2.4.1.

5.4.2. 5-AZACYTIDINE TREATMENT OF CHONDROCYTE CULTURES

The procedures are as described in Chapter Two, Section 2.4.2.

5.4.3. DAPI FLUORESCENT STAINING

In order to examine the morphological characteristics of the nuclei in cultured chondrocytes, the cells were stained with the fluorescent dye, 4,6-diamidino-2-phenylindole (DAPI). DAPI binds to DNA by intercalating between stacked base-pairs with a particularly strong affinity for A + T-rich DNA sequences (Manzini *et al.*, 1983). The cell nuclei were visualised by fluorescence microscopy.

5.4.3.1. METHOD

Paraffin sections of cell layers were dewaxed and hydrated as described in Chapter Two, Section 2.4.6. Sections were washed three times with PBS, covered with DAPI (1:1000 dilution in PBS) in the dark for 5 minutes, then rinsed with PBS. Sections were mounted with DABCO and viewed under the fluorescence microscope.

5.4.4. TRANSMISSION ELECTRON MICROSCOPY

The procedures are as described in Chapter Two, Section 2.4.4.

5.4.5. ANNEXIN V-FITC DETECTION OF APOPTOSIS

Annexin V is a very sensitive probe for detecting cells that are dying by the process of apoptosis. In viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the plasma membrane. During apoptosis, PS is translocated from the cytoplasmic to the outer surface of the plasma membrane (Martin *et al.*, 1995; Fadok *et al.*, 1992). Annexin V has a very high affinity for PS in the presence of calcium (Raynal and Pollard, 1994). Therefore, cells undergoing apoptosis are marked with annexin V labelled with fluorescein isothiocyanate (FITC) and these cells can be identified by fluorescence microscopy (Vermes *et al.*, 1995). In order to distinguish apoptotic cells from necrotic cells, annexin V is usually used in conjunction with the vital dye, propidium iodide (PI). PI is a fluorescent dye that stains DNA only by crossing a damaged plasma membrane. In contrast to apoptotic cells, which maintain an intact cell membrane during early apoptosis, necrotic cells develop a permeabilised cell membrane and as a result, they take up PI. Under the fluorescence microscope, (i) cells undergoing apoptosis appear green (annexin V positive, PI negative), (ii) necrotic cells are red (annexin V negative, PI positive), (iii) cells that have already died by apoptosis stain both green and red (annexin V positive, PI positive), and (iv) cells that stain negative for both annexin V and PI are viable.

5.4.5.1. METHOD

Bovine epiphyseal chondrocytes were cultured in 0.8 cm² Permanox 8-chamber slides (Gibco BRL, Paisley, Scotland) under the same conditions described in Chapter Two, Section 2.4.1. At selected time-points, the culture medium was removed and replaced with fresh cold MEM. The Annexin V-FITC apoptosis detection kit was used (see Appendices). A volume of 20 µl Media Binding Reagent was added followed by the addition of 2.5 µl Annexin V-FITC. All cultures were kept in the dark and left at room temperature for 15 minutes. The cells were incubated in 300 µl of cold 1X Binding Buffer and 20 µl propidium iodide at 4°C in the dark for 5 minutes. The reagent mixture was removed and replaced with 200µl of cold 1X Binding Buffer. A glass cover-slip was placed on the chamber slides and the cells were viewed under the fluorescence microscope immediately.

5.4.6. TRYPSINISATION OF CHONDROCYTE CULTURES

The procedures are as described in Chapter Three, Section 3.4.3.

5.4.7. *IN SITU* HYBRIDISATION

The procedures are as described in Chapter Three, Section 3.4.9. Human cDNA probes for *bcl-2* and *bax* were used to identify the mRNA expression of these markers in cultured chondrocytes (see Appendices).

5.4.8. IMAGE ANALYSIS

The procedures are as described in Chapter Three, Section 3.4.17.

5.4.9. PHOTOGRAPHY

Cells analysed by immunofluorescence were viewed under the Leica DM microscope (Leica Ltd., Milton Keynes, U.K.), using the dichroic mirror cat. No. 13 513808 (Leica Ltd., U.K.) for FITC and PI staining, and the dichroic mirror cat. No. D 513805 (Leica Ltd., U.K.) for DAPI staining. Photographs were taken using a 35 mm camera and a Leica MPS 48 exposure unit (Leica Ltd., U.K.) attached to the microscope.

5.5. RESULTS

5.5.1. MORPHOLOGICAL CHARACTERISTICS OF FOETAL BOVINE EPIPHYSEAL CHONDROCYTES AFTER THREE WEEKS IN CULTURE

In order to assess the possible occurrence of apoptosis in foetal bovine epiphyseal chondrocyte cultures, the gross morphology of cell nuclei in 5-azacytidine (aza-C) treated and untreated cultures was examined. Paraffin sections of cell layers taken on days 13, 27, and 35 were used in immunofluorescence studies using the fluorescent DNA-binding dye, DAPI. Visualisation of stained sections under the fluorescence

microscope showed the presence of intact cell nuclei in both aza-C treated and untreated chondrocytes on day 13 (Figure 5.2). Examination of sections taken on days 27 and 35 revealed the presence of many condensed cell nuclei with characteristic "blebbing" in aza-C treated chondrocytes. However, in order to obtain a precise evaluation of the extent of apoptosis in culture, quantitation of the proportion of apoptotic cells in the sections examined should be performed. Preliminary quantitation revealed about 10% apoptotic chondrocytes in aza-C treated cultures on day 27. Untreated chondrocytes displayed generally intact nuclei at the same time-points analysed (Figure 5.2).

Further support for the presence of apoptotic death in aza-C treated cultures was obtained by examination of cultured chondrocytes collected on days 27 and 35 using transmission electron microscopy. Chondrocytes that had been treated with aza-C showed clear ultrastructural characteristics of apoptosis. The nuclear chromatin was highly condensed and distributed throughout most of the cross-sectional area of the nucleus. There was also some indication of nuclear fragmentation as shown by the presence of small vesicle-like structures containing condensed chromatin (Figure 5.3). The cytoplasmic organelles generally appeared intact and the integrity of the plasma membrane was well preserved. Untreated chondrocytes appeared healthy and viable at the same time-points studied. No chromatin condensation and nuclear fragmentation was observed in these untreated cells.

Another feature of apoptosis is the translocation of phosphatidylserine (PS) from the cytoplasmic to the outer surface of the plasma membrane. Additional support for the apoptotic death of chondrocytes in aza-C treated cultures was obtained by using fluorescence-tagged annexin V as a specific probe for PS in the presence of calcium. Annexin V was used in conjunction with the vital dye, propidium iodide (PI) with a view to distinguish apoptotic cells from necrotic cells. The tests were carried out using chondrocyte cultures collected on days 20 and 26. Fluorescence microscopy of aza-C treated chondrocyte cultures on day 26 revealed the presence of a large population of apoptotic cells that appeared green (annexin V positive, PI negative). Some chondrocytes also stained red only, indicating necrotic death (annexin V negative, PI positive). Chondrocytes from aza-C treated cultures collected on day 20

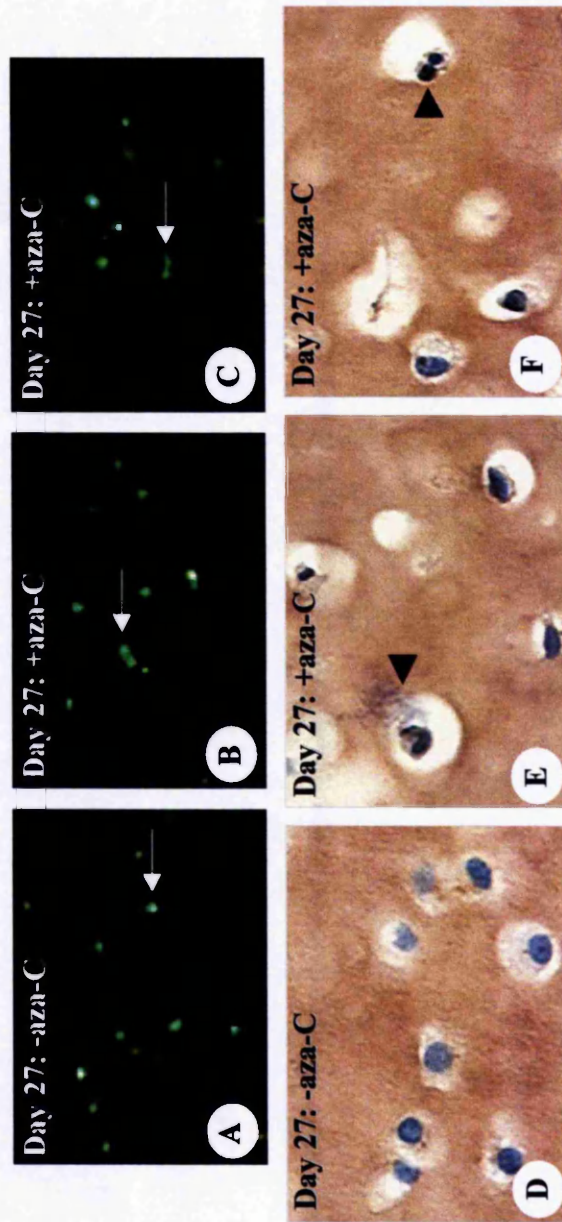


Figure 5.2. Morphology of cell nuclei of foetal bovine epiphyseal chondrocytes after three weeks in culture. Chondrocytes derived from foetal bovine epiphyseal cartilage were cultured with and without 15 $\mu\text{g/ml}$ 5-azacytidine. Untreated and treated cultures were collected at selected time-points and embedded in paraffin wax. Cut paraffin wax sections of cell layers were stained with DAPI and viewed under the fluorescence microscope [A-C]. Note the intact cell nuclei in the untreated cells [arrow: A]. This result is representative of all untreated cultures collected on days 13, 27 and 35. Note the condensed and fragmented appearance of the nuclei in the aza-C treated cultures, indicative of apoptosis [arrows: B-C]. These observations are typical representations of aza-C treated cultures studied on days 27 and 35. Intact nuclei were detected in aza-C treated cultures collected on day 13. Similar observations were obtained when paraffin wax sections of aza-C treated and untreated cultures were analysed immunohistochemically for type II collagen on day 27 [D-F]. Note the nuclear fragmentation and chromatin condensation indicated by the darker nuclear staining in apoptotic chondrocytes [arrowheads: E-F] and the intact nuclei in the untreated cells [D]. Note also the cell shrinkage in the lacunae of apoptotic cells [arrowheads: E-F] (Magnifications, A-C: $\times 80$; D-F: $\times 170$).



Figure 5.3. Electron microscopical examination of apoptotic chondrocytes in culture. Foetal bovine epiphyseal chondrocytes were either treated with 15 $\mu\text{g/ml}$ of azacytidine (aza-C) for 48 hours after plating or left untreated. All cells were cultured for a four-week period in the presence of 10% FCS. Chondrocytes were collected on days 20, 27 and 35 for subsequent examination by transmission electron microscopy in order to detect the occurrence of apoptosis in culture. The photograph shown is a typical representation of apoptotic chondrocytes detected in aza-C treated cultures on days 27 and 35. These chondrocytes exhibit morphological characteristics typical of apoptosis, as shown by the condensation of chromatin [C] and discrete clumps of condensed chromatin, which indicates the occurrence of nuclear fragmentation (arrow). No such observations of apoptosis were observed in aza-C treated cultures collected on day 20 and in untreated cultures at all time-points examined. (Magnification: $\times 8875$).

were viable and generally stained negative for both annexin V and PI. Likewise, untreated chondrocytes taken on days 20 and 26 stained negative for annexin V and PI, although some chondrocytes stained red for PI (Figure 5.4).

5.5.2. mRNA EXPRESSION OF *bcl-2* AND *bax* IN FOETAL BOVINE EPIPHYSEAL CHONDROCYTE CULTURES

Since *bcl-2* and *bax* are involved in the process of apoptosis, their mRNA expression in aza-C treated and untreated chondrocyte cultures was analysed using *in situ* hybridisation. Cells were collected at selected time-points in culture, processed as described under Methods, and hybridised with ³⁵S-labelled cDNA probes for either *bcl-2* or *bax*. The slides were viewed using a Leica DB research microscope and results were quantified, as published previously (Mee *et al.*, 1997; Walsh *et al.*, 1993), by the Quantimet image analysis system described under Methods. For each time-point, ten fields per slide were chosen randomly and the number of grains counted. Since duplicate slides were analysed for each time-point, the mean grain counts of twenty fields was calculated. The level of mRNA expression of either *bcl-2* or *bax* was expressed as the mean grain density per unit area of cells.

Results obtained in the study of *bcl-2* expression showed that in aza-C treated chondrocyte cultures, the level of expression of *bcl-2* was high during the first week in culture (day 7) and increased until day 12 (Figure 5.5). The expression level decreased slightly, at subsequent time-points in culture (days 23 and 33). The expression of *bcl-2* remained low throughout the culture period in untreated cultures. In addition, at all time-points analysed, aza-C treated chondrocytes expressed *bcl-2* at higher levels than untreated cells.

Analyses of the mRNA expression of *bax* indicated a very low level of its expression during the first three weeks in aza-C treated cultures (days 7 and 12). A marked increase in the expression of *bax* was observed on day 23 and its expression increased steadily until day 33 (Figure 5.6). *Bax* was present at very low levels in untreated cultures throughout the culture period.



Figure 5.4. Detection of apoptosis in foetal bovine epiphyseal chondrocyte cultures using annexin V-FITC. The occurrence of apoptosis in chondrocyte cultures was tested by detecting the presence of phosphatidylserine on the outer surface of the plasma membrane with the combined use of annexin V-FITC and the vital dye, propidium iodide (PI). Fluorescence microscopy indicated the abundant presence of apoptotic chondrocytes in aza-C treated cultures collected on day 27, as indicated by the green fluorescence of cells with exposed PS [A]. Some chondrocytes also stained positive with annexin V and PI, showing that the cells had already undergone apoptosis (not shown). No fluorescence was obtained in aza-C treated cultures on day 20. Untreated cultures were viable and stained negative with annexin V and PI on days 20 and 26 [B]. A few chondrocytes stained red in untreated cultures, indicating the occurrence of necrosis [C]. (Magnification: x126).

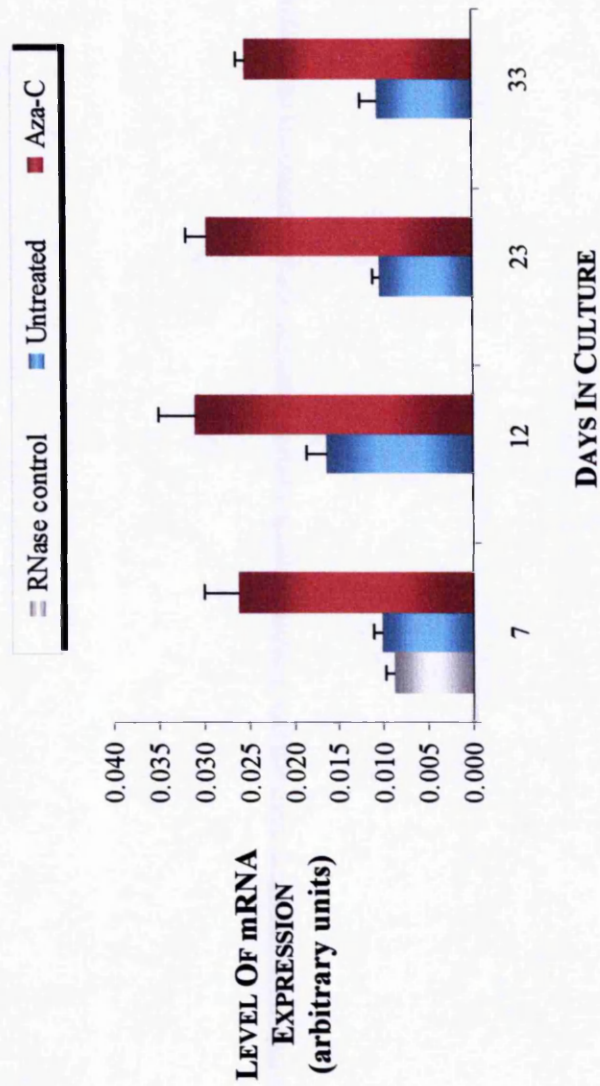


Figure 5.5. mRNA expression of *bcl-2* in foetal bovine epiphyseal chondrocyte cultures. Aza-C treated and untreated chondrocytes were collected at selected time-points and processed for *in situ* hybridisation analyses. A human cDNA probe specific for *bcl-2* was used to study its mRNA expression in culture. An RNase control was included whereby the chondrocytes were treated with RNase to eliminate any hybridisation signal (+aza-C: day 7). All results were quantified using a Quantimet 600S image analysis system. Values represent mean \pm SEM (n=20, where n represents the number of fields counted).

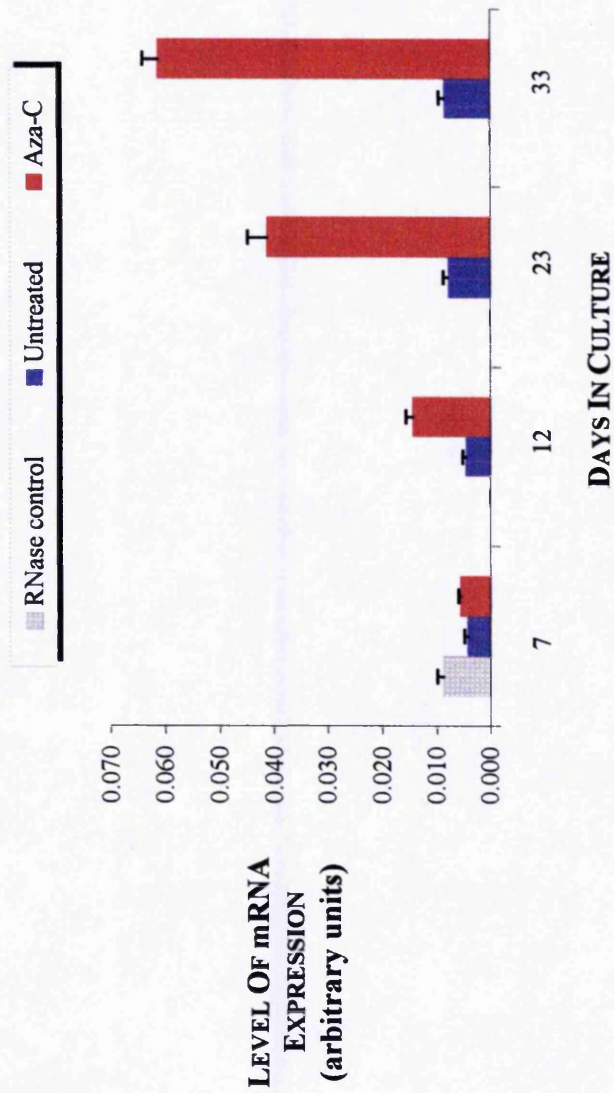


Figure 5.6. mRNA expression of *bax* in foetal bovine epiphyseal chondrocyte cultures. Aza-C treated and untreated chondrocytes were collected at selected time-points and processed for *in situ* hybridisation analyses. A human cDNA probe specific for *bax* was used to study its mRNA expression in culture. An RNase control was included whereby the chondrocytes were treated with RNase to eliminate any hybridisation signal (+aza-C: day 23). All results were quantified using a Quantimet 600S image analysis system. Values represent mean \pm SEM (n=20, where n represents the number of fields analysed).

Data obtained from the *in situ* hybridisation studies on the expression of *bcl-2* and *bax* in aza-C treated chondrocyte cultures were used to determine the ratio of *bcl-2/bax* expression at each time-point with a view to examine the variation of the *bcl-2/bax* ratio with time in culture. As shown in Table 5.1, the ratio of *bcl-2/bax* expression decreased steadily in value with time in culture. The *bcl-2/bax* ratio was in favour of *bcl-2* early in culture and shifted gradually in favour of *bax* at later time-points in culture. It should be noted that the quantification of these results are valid and meaningful since the same labelled probe and the same exposure times were used at all different time-points chosen for the analysis of the marker concerned. In addition, the expression of both *bax* and *bcl-2* was analysed in the same *in situ* experiment using a single *bcl-2* and a single *bax* radiolabelled probe. Although the incorporation of the radiolabel in the probes was not measured, the *bcl-2* and *bax* ratio is valid since a single probe for each marker was used. The ratio only gives an indication of the relationship between *bcl-2* and *bax* expression. Therefore, the quantification procedure does not depend on the level of radioactive incorporation in each probe and the length of the probe. All quantification is based solely on the number of messages present per unit area of cells, which is represented by the number of grains per unit area of cells (grain density).

5.6. DISCUSSION

A large body of evidence has shown apoptosis to be the fate of terminal hypertrophic chondrocytes during endochondral ossification (EO) in the growth plate (Farnum and Wilsman, 1989; 1987; Zenmyo *et al.*, 1996). Ultrastructural and morphological evidence include cell condensation with the plasma membrane attached asymmetrically to the last transverse septum (Farnum and Wilsman, 1987), nuclear condensation and cell retraction from the pericellular matrix (Zenmyo *et al.*, 1996). Results obtained in this study seem to agree with those previous findings. Foetal bovine epiphyseal chondrocytes were treated with 15 µg/ml of 5-azacytidine (aza-C) for 48 hours only, left in culture, and collected at selected time-points to test for the occurrence of apoptosis. DAPI fluorescent staining indicated the presence of apoptotic chondrocytes in aza-C treated cultures collected on the fourth week in

TABLE 5.1.
RATIO OF THE LEVEL OF EXPRESSION OF *bcl-2* AND *bax* IN FOETAL BOVINE EPIPHYSEAL
CHONDROCYTE CULTURES UNDER 5-AZACYTIDINE TREATMENT

DAY	DENSITY OF HYBRIDISATION SIGNAL		RATIO <i>bcl-2/bax</i>
	<i>Bcl-2</i>	<i>Bax</i>	
7	0.0263	0.0056	4.70
12	0.031	0.0145	2.14
23	0.0297	0.0413	0.72
33	0.0253	0.0614	0.41

culture. This observation was confirmed by transmission electron microscopy whereby aza-C treated chondrocytes at the same time-points (fourth week) exhibited a highly condensed chromatin, nuclear fragmentation, and intact cytoplasmic organelles and plasma membrane. Further support for the apoptotic death of aza-C treated chondrocytes was obtained by the detection of exposed phosphatidylserine on the outer surface of the plasma membrane of apoptotic cells with the use of annexin V-FITC. In aza-C treated cultures, chondrocytes that were undergoing apoptosis (annexin V positive, propidium iodide [PI] negative) were identified on day 26. In addition, it was observed that some chondrocytes stained positive with annexin V and PI, indicating that these cells had already died by apoptosis (results not shown). The presence of these dead chondrocytes (annexin V positive, PI positive) indicates the lack of clearance of apoptotic cells by phagocytosis by neighbouring chondrocytes in culture. This observation raises the question of how apoptotic chondrocytes in the terminal hypertrophic zone of the growth plate are cleared *in vivo*. The use of annexin V-FITC revealed no indication of apoptotic chondrocytes in aza-C treated cultures analysed prior to the fourth week in culture (days 13 and 20). No sign of apoptosis was observed in all untreated cultures analysed throughout the culture period, although some necrotic cell death (annexin V negative, PI positive) was found at later time-points in culture. The absence of apoptosis in untreated cultures at four weeks in culture confirms that apoptosis in the aza-C treated cultures was not a consequence of senescence and prolonged culture. Therefore, it is tentative to speculate that the fourth week in culture marks the terminal hypertrophic stage of chondrocyte differentiation *in vitro*. It is interesting to note that aza-C has been shown recently to induce apoptosis in hepatic cancer cell lines (Wang *et al.*, 1998) and developing neuronal cells *in vitro* (Hossain *et al.*, 1997). Findings in this study provide another example of aza-C promoting apoptosis in foetal bovine epiphyseal chondrocytes that have undergone differentiation.

It was interesting to analyse the expression of *bcl-2* and *bax* in the cell culture model described in this study. *Bcl-2* and *bax* are known to be key players in the determination of cell survival or apoptotic death (Oltvai *et al.*, 1993; Hockenberry *et al.*, 1990). In this present study, it was found that *bcl-2* was expressed highly in aza-C treated chondrocyte cultures during the first week in culture until day 12. Its

expression decreased slightly subsequently until day 33. These observations are not surprising since *bcl-2* is a cell survival gene and inhibits apoptosis (Hockenberry *et al.*, 1990). The expression pattern of *bcl-2* in culture also correlates with the proliferation stage of chondrocyte differentiation (days 0-11) obtained in the studies described in Chapter Two. Conversely, *bax* was not expressed early in aza-C treated cultures until its expression was increased markedly on day 23 until day 33. *Bax* is known to be a cell death inducer and to promote apoptosis (Oltvai *et al.*, 1993). Its high expression during the fourth week in culture is consistent with the occurrence of apoptosis in aza-C treated cultures observed microscopically at the same period in culture. The patterns of expression of *bcl-2* and *bax* are consistent with their expressions the growth plate *in vivo* (Amling *et al.*, 1997; Hillarby *et al.*, 1996). *Bcl-2* is present abundantly in the proliferative and maturing zones of the growth plate, while the expression of *bax* increases progressively towards the hypertrophic zone (Amling *et al.*, 1997). In addition, the pattern of expression of *bcl-2* is similar to that of PTHrP obtained in Chapter Four, which supports indirectly the current view (discussed in Chapter Four, Section 4.6) that PTHrP regulates the expression of *bcl-2* (Amling *et al.*, 1997).

Previous studies have shown that the determining factor for cell survival or the occurrence of apoptosis is the ratio of the level of expression of *bcl-2* and *bax* (Oltvai *et al.*, 1993). Quantification of data obtained by *in situ* hybridisation analyses enabled the determination of the *bcl-2/bax* ratio at several time-points in culture. These results showed a gradual and steady shift in the ratio in favour of *bax* with time in culture, particularly in the fourth week. *Bcl-2* and *bax* are known to heterodimerise with each other whereby apoptosis is suppressed only when half or more of available *bax* is associated with *bcl-2* (Oltvai *et al.*, 1993). The higher expression of *bax* compared to that of *bcl-2* in the fourth week in culture may provide a plausible explanation for the shift in the ratio in favour of *bax*, and consequently the apoptotic death of aza-C treated chondrocytes. In addition, a similar shift in the *bcl-2/bax* ratio has been reported in previous studies (Amling *et al.*, 1997). Taken together, these findings indicate strongly that the fourth week in culture marks the terminal hypertrophic stage of differentiation and apoptosis of foetal bovine epiphyseal chondrocytes that had been treated initially with aza-C. These results also show that treatment of foetal

bovine epiphyseal chondrocytes with aza-C under the conditions used throughout these studies induced the entire differentiation pathway that occurs in the growth plate *in vivo*.

Chapter Six

CHAPTER SIX

EFFECTS OF PARATHYROID HORMONE-RELATED PEPTIDE ON 5-AZACYTIDINE INDUCED ENDOCHONDRAL OSSIFICATION

6.1. INTRODUCTION

As described previously (Chapter Four, Section 4.1.1.), parathyroid hormone-related peptide (PTHrP) is a crucial regulator of endochondral ossification (EO). Any dysregulation of its expression in the growth plate results in a severe disruption of growth plate structure leading to profound skeletal abnormalities (Weir *et al.*, 1996; Amizuka *et al.*, 1994; Karaplis *et al.*, 1994). *In vivo*, PTHrP has been shown to play a major role in delaying the progress of growth plate chondrocytes to hypertrophy and in promoting cell proliferation (Weir *et al.*, 1996). *In vitro* studies have also indicated the potent stimulatory effect of PTHrP on the proliferation of chick growth plate chondrocytes, and its inhibitory effect on alkaline phosphatase activity in the same system (Loveys *et al.*, 1993). In addition, it has been shown recently that PTHrP inhibits specifically the expression of type X collagen with no significant effect on type II collagen expression in short-term cultures of chick growth plate chondrocytes (O'Keefe *et al.*, 1997). Clearly, both *in vivo* and *in vitro* findings seem to be in agreement on the inhibitory effects of PTHrP on the differentiation of growth plate chondrocytes to hypertrophy.

6.2. AIMS

The present study is focused on the determination of the effect of PTHrP on hypertrophic chondrocytes in cultures that had been treated initially with 5-azacytidine (aza-C). The approach taken was the analysis of the mRNA expression of type X collagen in aza-C treated cultures after varying lengths of exposure to a fixed concentration of PTHrP (10^{-7} M). This concentration was used because it has been cited in the literature to produce maximal effects of PTHrP (O'Keefe *et al.*, 1997;

Tsukazaki *et al.*, 1996). In addition, the N-terminal of PTHrP (1-34) was used in this study since it has been shown that the biological activity of PTHrP resides in this fragment (O'Keefe *et al.*, 1997; Loveys *et al.*, 1993). This study would show if the hypertrophic stage of differentiation of aza-C treated chondrocytes is under the influence of PTHrP, as it is the case in the growth plate *in vivo*. The strategy of investigation is outlined in figure 6.1.

6.3. MATERIALS

Gibco BRL: Paisley, Scotland.

Dulbecco's phosphate buffered saline (without calcium and magnesium), foetal calf serum, gentamycin, *L*-glutamine, minimal essential medium (with Earle's salts and *L*-glutamine), penicillin-streptomycin solution, sodium bicarbonate (7.5%), trypsin (0.25% w/v in Gibco Solution A).

Sigma Chemicals Co. Ltd: Dorset, U.K.

Acetic anhydride, 5-azacytidine (50X; lyophilised and sterilised), bacterial collagenase type 1A, bovine serum albumin, dextran sulphate, diethylpyrocarbonate, dithiothreitol, Ficoll, glycine, *L*-ascorbic acid, paraformaldehyde, polyvinylpyrrolidone, salmon sperm DNA, Sephadex G50.

BDH Laboratory Supplies: Dorset, U.K.

Hydrogen peroxide (30%), methanol, sodium chloride, EDTA, concentrated hydrochloric acid, sodium citrate, formamide.

Boehringer Mannheim: East Sussex, U.K.

RNase A.

Amersham Pharmacia Biotech Ltd: Buckinghamshire, U.K.

³⁵S-dCTP, Amersham Megaprime™ DNA Labelling System

Ilford Imaging U.K. Ltd.: Cheshire, U.K.

K-photographic emulsion, Hypam rapid fixer, Phenisol high contrast film developer, and Ilfostop Pro low-odour stop bath.

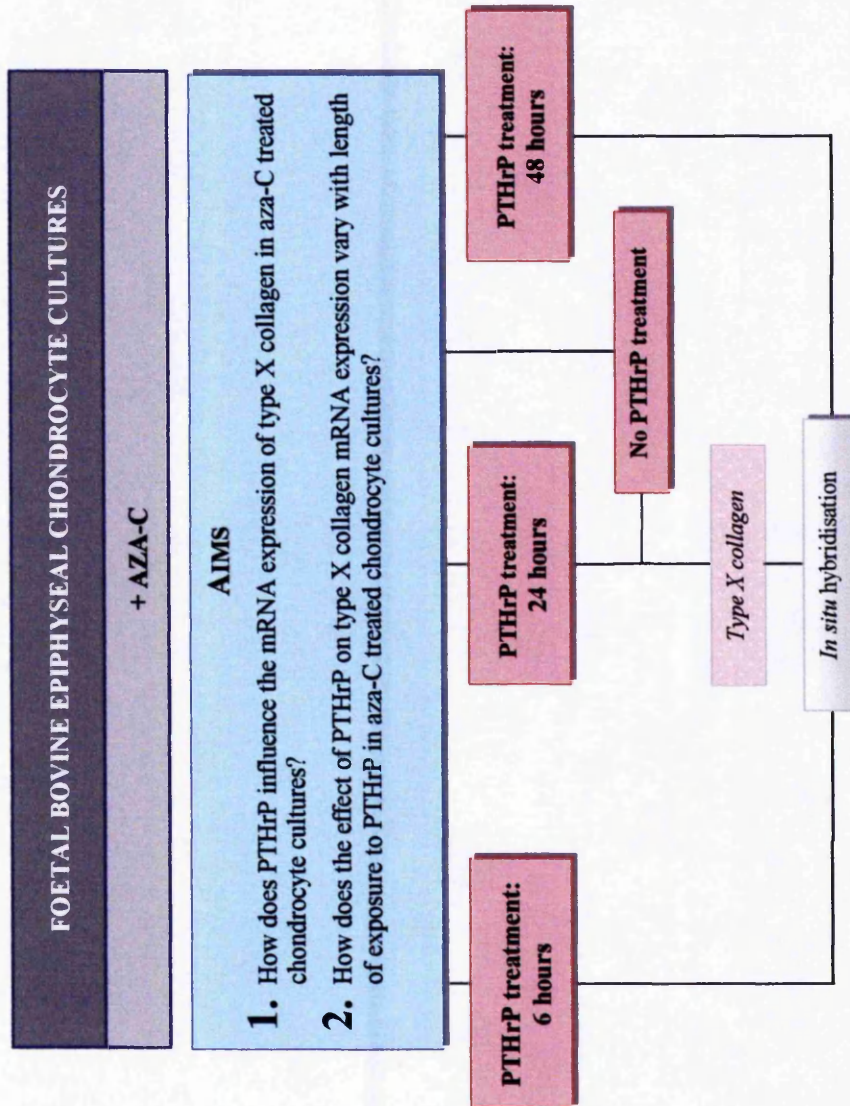


Figure 6.1. Schematic diagram showing the strategies of investigation into the effect of parathyroid hormone-related peptide on the mRNA expression of type X collagen in foetal bovine epiphyseal chondrocyte cultures under 5-azacytidine treatment.

ICN Biochemicals Inc.: Ohio, U.S.A.

Tris (Ultra-Pure).

Peninsula Laboratories: Merseyside, U.K.

Parathyroid hormone-related protein (1-34, human)

Loctite: Hertfordshire, U.K.

Loctite® engineering adhesive.

The cDNA probe for type X collagen was kindly provided by Dr. Judith Hoyland (University of Manchester).

All reagents were of the highest grade commercially available.

6.4. METHODS

6.4.1. BOVINE CHONDROCYTE CULTURE

The procedures are as described in Chapter Two, Section 2.4.1.

6.4.2. 5-AZACYTIDINE TREATMENT OF CHONDROCYTE CULTURES

The procedures are as described in Chapter Two, Section 2.4.2.

6.4.3. PTHrP TREATMENT OF CHONDROCYTE CULTURES

Chondrocyte cultures initially treated with 5-azacytidine (15 µg/ml) were selected for PTHrP treatment on day 14. The cultures were supplemented with freshly prepared PTHrP at a concentration of 10^{-7} M, and incubated for 6, 24 and 48 hours at 37°C in 5% CO₂/95% O₂ atmosphere. The treatment was stopped by removal of the culture medium followed by cell trypsinisation.

6.4.4. TRYPSINISATION OF CHONDROCYTE CULTURES

The procedures are as described in Chapter Three, Section 3.4.3.

6.4.5. IN SITU HYBRIDISATION

The procedures are as described in Chapter Three, Section 3.4.9. A human cDNA probe for type X collagen was used to identify its mRNA expression in cultured chondrocytes (see Appendices).

6.4.6. IMAGE ANALYSIS

The procedures are as described in Chapter Three, Section 3.4.12.

6.5. RESULTS

6.5.1. mRNA EXPRESSION OF TYPE X COLLAGEN IN 5-AZACYTIDINE-INDUCED CHONDROCYTE CULTURES AFTER EXPOSURE TO PARATHYROID HORMONE-RELATED PEPTIDE

Foetal bovine epiphyseal chondrocyte cultures were treated for 48 hours with 15 µg/ml 5-azacytidine (aza-C) one hour after plating. On day 14, the aza-C treated chondrocyte cultures were exposed to parathyroid hormone-related peptide (PTHrP) (10^{-7} M) for various lengths of time (6, 24, and 48 hours). Some aza-C treated cultures were not supplemented with PTHrP as positive controls for type X collagen expression. All cultures were trypsinised after the appropriate length of exposure and processed accordingly for radioactive *in situ* hybridisation analyses using a ^{35}S -labelled cDNA probe for type X collagen. The level of mRNA expression of type X collagen was quantified, as previously published (Mee *et al.*, 1997; Walsh *et al.*, 1993) by the Quantimet image analysis system described under Methods. For each time-point, ten fields per slide were chosen randomly and the number of grains counted. Since duplicate slides were analysed for each time-point, the mean grain counts of twenty fields was calculated. The level of mRNA expression of type X collagen was expressed as the mean grain density per unit area of cells.

The level of type X collagen mRNA expression in aza-C treated cultures obtained in the absence of PTHrP exposure on day 14 was high and similar to the level obtained

at the same time-point in previous experiments (Chapter Three). Exposure of aza-C treated chondrocyte cultures to 10^{-7} M PTHrP for 6, 24, and 48 hours on day 14 resulted in a dramatic decrease in the level of mRNA expression of type X collagen (Figure 6.2). After 6 and 48 hours of exposure to PTHrP, a 78% reduction in type X collagen expression was obtained while a 24-hour exposure to PTHrP resulted in an 87% decrease in the level of mRNA expression of type X collagen (Figure 6.2). These results indicate that a 24-hour exposure of aza-C treated chondrocyte cultures to PTHrP produced the most effective suppression of type X collagen expression.

6.6. DISCUSSION

The previous chapters discussed so far have focused on the characterisation of a cell culture model based on the induction of the differentiation of foetal bovine epiphyseal chondrocytes with the use of 5-azacytidine (aza-C) *in vitro*. The study described in the present chapter was designed to bring additional support to the validity of the cell culture model as a true representation of endochondral ossification (EO) *in vitro*. The study aimed at determining if the presence of exogenous parathyroid hormone-related peptide (PTHrP) in aza-C treated chondrocyte cultures would influence the mRNA expression of type X collagen, which is expressed in hypertrophy only. The strategy of investigation used was to expose aza-C treated chondrocytes to PTHrP soon after they had reached hypertrophy and synthesised type X collagen in culture. The time-point chosen for exposure of aza-C treated cultures to PTHrP was day 14 since results obtained in Chapter Three have shown that the hypertrophic stage of differentiation started on day 11, as indicated by the expression of type X collagen.

The major finding in this study is that exogenous PTHrP at a concentration of 10^{-7} M suppressed the mRNA expression of type X collagen by 87% in hypertrophic chondrocytes that had been treated previously with aza-C. This inhibitory effect was obtained after a 24-hour exposure to PTHrP. Other studies have also obtained a similar level of suppression of type X collagen in short-term cultures of chick growth plate chondrocytes at the same concentration and for the same length of exposure to PTHrP (O'Keefe *et al.*, 1997). In addition, results obtained in this study also show a

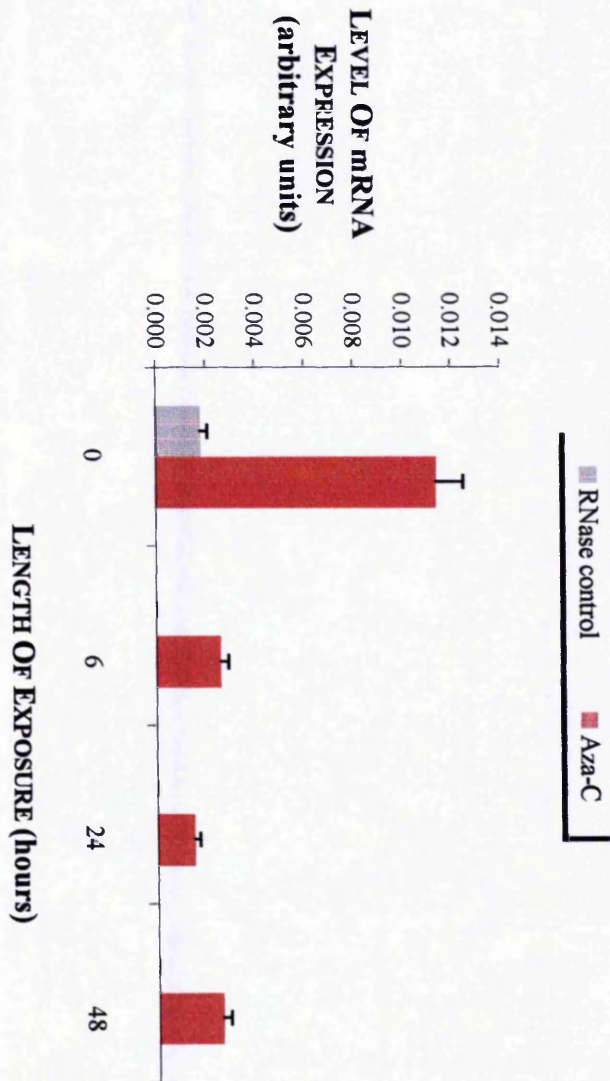


Figure 6.2. Effect of PTHrP on the mRNA expression of type X collagen in foetal bovine epiphyseal chondrocyte cultures under 5-azacytidine treatment. Aza-C treated chondrocyte cultures were exposed to PTHrP (10^{-7} M) for 6, 24, and 48 hours on day 14. The chondrocytes were collected subsequently and processed for *in situ* hybridisation analyses. A human cDNA probe specific for type X collagen was used to study its mRNA expression after various lengths of exposure to PTHrP. An RNase control was included whereby the chondrocytes were treated with RNase to eliminate any hybridisation signal (+aza-C; day 14, no PTHrP). A positive control was also included where no PTHrP was added to the aza-C treated cultures. All results were quantified using a Quantimet 600S image analysis system. Values represent mean \pm SEM ($n=20$, n represents the number of fields analysed).

marked decrease in type X collagen expression (78% reduction) after exposing aza-C treated cultures to PTHrP for 6 and 48 hours.

It would be interesting to investigate the mechanism by which PTHrP could mediate its inhibitory action of type X collagen expression in this culture model. As discussed in Chapter Four, it is speculated that the PTHrP receptor was not expressed after day 9 in aza-C treated cultures, although no analysis was carried out at this time-point. Therefore, *in situ* hybridisation experiments should be carried out to analyse the receptor expression after day 9. If it is found that the receptor is not expressed after day 9, it is possible that exogenous PTHrP does not mediate its action via its receptor since PTHrP exerted its inhibitory effect on day 14. Alternatively, there is also the possibility that PTHrP is able to autoregulate the expression of its receptor and therefore, induces the receptor expression upon the addition of PTHrP to the culture. In the case of *in situ* studies showing that PTHrP receptor is expressed after day 9, experiments could be conducted whereby the receptor expression could be knocked out by anti-sense technology at time-points after day 9 and exogenous PTHrP added to the culture on day 14 as described in this chapter. Analyses of the expression of type X collagen upon such treatment would indicate whether PTHrP inhibited type X collagen expression by acting via its receptor or not in this culture system.

The inhibitory effects of PTHrP on the terminal differentiation of growth plate chondrocytes are apparent from numerous studies carried out both *in vitro* and *in vivo*. PTHrP has been shown to exert a potent mitogenic effect on chick growth plate chondrocyte cultures whilst inhibiting the activity of alkaline phosphatase, which occurs only in hypertrophy (Loveys *et al.*, 1993). *In vivo*, PTHrP-deficient mice exhibit disorganised growth plate structure, reduced cell proliferation, and premature chondrocyte differentiation to hypertrophy (Amizuka *et al.*, 1994; Karaplis *et al.*, 1994). Conversely, transgenic mice overexpressing PTHrP develop cartilaginous skeletons as a result of a delayed EO (Weir *et al.*, 1996).

The findings in the present study are consistent with all the observations described above and confirm further the critical role of PTHrP in the regulation of EO by

controlling the progress of growth plate chondrocytes to hypertrophy. They also show that the hypertrophic stage of differentiation induced by aza-C treatment of foetal bovine epiphyseal chondrocytes is regulated, at least in part, by PTHrP, as is the case in the growth plate *in vivo*. In addition, the results indicate that PTHrP exerts its influence on the expression of type X collagen at the transcriptional level, as suggested previously (O'Keefe *et al.*, 1997). Although aza-C is known to be a DNA demethylating agent, the mechanism by which aza-C induces the chondrocyte differentiation pathway in this cell culture model is not known. It is possible that aza-C switches on the expression of EO genes individually by its demethylating action, as shown in previous studies involving a number of different genes in various systems (Gotzinger *et al.*, 1996; Thompson *et al.*, 1991; Clough *et al.*, 1982). It is also possible that aza-C acts on a single gene involved in the early stages of the differentiation pathway, which in turn triggers the whole cascade of chondrocyte maturation. Alternatively, aza-C may trigger the entire maturation cascade of chondrocytes by an unknown mechanism or mechanisms, instead of acting on each gene involved in EO separately. Whatever the mechanism of action of aza-C is, the fact that the hypertrophic stage of differentiation in this cell culture model can be influenced by the presence of exogenous PTHrP in a fashion similar to that in the growth plate *in vivo* suggests strongly that the stage of hypertrophy has not been induced by aza-C in a manner that is separate from the other stages of chondrocyte differentiation. Rather, the hypertrophic stage is part of the entire differentiation pathway of chondrocytes induced in this cell culture system and is closely related to and influenced by all the other differentiation stages and stage-specific regulatory molecules involved in the EO pathway.

Chapter Seven

CHAPTER SEVEN

GENERAL DISCUSSION

7.1. GENERAL DISCUSSION

During embryonic development, long bone formation occurs by endochondral ossification (EO) in the growth plate cartilage. EO consists of discrete stages of chondrocyte differentiation, which are under strict spatial and temporal regulation. The molecular mechanisms that initiate the entry of growth plate chondrocytes into the differentiation pathway still remain obscure. In addition, the role(s) of regulatory factors involved in the differentiation pathway and their intricate interplay in the control of the developmental programme of growth plate chondrocytes is not fully understood. Understanding the precise mechanisms of regulation of EO is crucial since an abnormal EO leads to severe, and often lethal, skeletal disorders. The work presented here describes the construction and characterisation of a cell culture model, which should prove useful in the studies of the factors controlling the differentiation pathway. The cell culture system is based on the treatment of epiphyseal chondrocytes derived from long bones of bovine fetuses with a potent DNA demethylating agent, 5-azacytidine (aza-C), with a view to inducing EO in culture.

As with all cell culture models, the development of the aza-C based system requires a number of culture conditions for maximal cell viability and the maintenance of a cell phenotype characteristic of chondrocytes. Growing foetal bovine epiphyseal chondrocytes in monolayers on plastic necessitates a minimum plating density of 0.5×10^6 cells per cm^2 in order to avoid chondrocyte dedifferentiation. Maximal cell viability was ensured by using aza-C at a concentration of 15 $\mu\text{g/ml}$. Under the culture conditions described in Chapter Two, all cells were viable with intact organelles and showed a normal proliferation status. Cultured cells exhibited morphological characteristics typical of chondrocytes and elaborated an extracellular matrix similar to that in cartilage *in vivo*, composed mainly of proteoglycans and collagen types II and VI. Once the cartilage characteristics of the chondrocyte

cultures had been established, the system was ready to be characterised in order to determine its validity as a true representation of mammalian EO. Several markers involved in each stage of chondrocyte differentiation were selected for subsequent analyses in time-course experiments. The determination of their patterns of expression enabled the construction of an expression profile for each marker with time in culture (Figure 7.1).

The expression of the markers of maturation, PTHrP and its receptor, were analysed. As discussed in Chapter Four, although PTHrP was present at all time-points studied, its higher expression during the period "days 5-9" suggests that the maturation stage could possibly be on days 5-9. A high expression of PTHrP receptor was also obtained during the same period. However, as already discussed, further experiments are needed in order to analyse the receptor expression after day 9 to confirm the period "days 5-9" as the maturation period. Another possible indicator of the period of maturation is the onset of an increase in cell size from days 4-5 (Chapter Two). This finding is consistent with the increase in cell size which occurs during maturation *in vivo* (Poole, 1991). In agreement with the expression pattern in the growth plate *in vivo*, type X collagen, a marker of hypertrophy, was expressed from day 11 onwards, i.e. after maturation. The expression of type X collagen correlated with a marked increase in cell size (13-fold increase by day 10), similar to that in the system *in vivo*. The hypertrophic period in culture was also marked by the expression of alkaline phosphatase (days 14-21). As alkaline phosphatase activity increased and reached a peak on days 20-21, a corresponding increase in calcification was obtained when aza-C treated cultures were supplemented with 10mM calcium β -glycerophosphate (days 14-25).

Aza-C treated chondrocytes died by apoptosis in the fourth week in culture, as indicated by several parameters characteristic of apoptotic death, namely nuclear condensation and fragmentation and the presence of exposed phosphatidylserine on the plasma membrane surface. The apoptotic suppressor, bcl-2, was expressed highly early in culture (days 7-12) with a slight decrease in expression level at subsequent time-points in culture, although the expression level remained higher

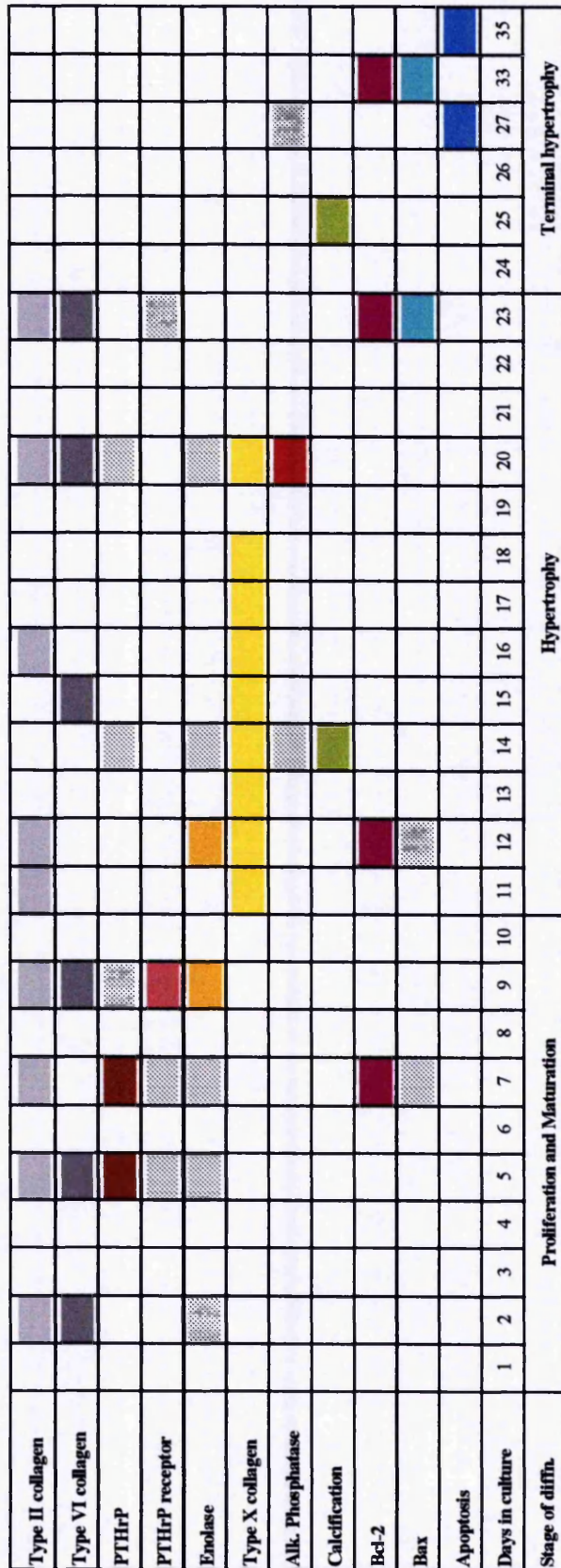


Figure 7.1. Expression profiles of markers of differentiation in the aza-C based foetal bovine epiphyseal chondrocyte culture system. Note that time-points shown for each marker listed represent time-points at which the marker was analysed. Also note that for each marker shown, colour-filled bands represent a high expression level of the marker and dotted bands represent a low level of expression of the same marker.

than that of untreated cultures. On the other hand, the apoptotic inducer, *bax*, was expressed highly only in the fourth week. Consequently, the ratio of the level of expression of *bcl-2* and *bax* shifted in favour of *bax* with time in culture, thereby favouring the occurrence of apoptosis. There is a large body of evidence of chondrocytes undergoing apoptotic death in the terminal hypertrophic stage in the growth plate (Zenmyo *et al.*, 1996). The occurrence of apoptosis in the fourth week in culture indicates that aza-C treated chondrocytes had reached terminal hypertrophy on week 4. It could be argued that apoptosis occurred in culture as a consequence of aza-C treatment itself as has been observed in other cell types (Wang *et al.*, 1998) and not as a consequence of the induction of the differentiation pathway. However, the reduction of *bcl-2* expression and the upregulation of *bax* at later stages in culture are most likely a consequence of EO induction and not aza-C treatment. In order to distinguish between the expression of *bcl-2* being a consequence of the induction of the differentiation pathway as opposed to being a consequence of aza-C treatment, the time-course culture experiments could be performed with one or more of the genes involved in the chondrocyte differentiation pathway knocked out, e.g PTHrP or type X collagen, with a view to breaking the pathway and preventing hypertrophy. Any subsequent apoptosis must be due to aza-C treatment and not a consequence of the induction of the differentiation pathway. In addition, in the presence of a *bcl-2* gene knock-out, an instant induction of apoptosis should be obtained if *bax* is already present. This observation would confirm the importance of *bcl-2* expression in maintaining cell survival in EO in culture. Therefore, although the expression of *bcl-2* may be a direct consequence of aza-C treatment, it remains that its expression is essential for the differentiation pathway in culture. It is unknown which genes are switched on by aza-C in this model and whether these genes are involved in the differentiation pathway. The induction of *bcl-2* expression by aza-C treatment may be one of the initiating factors in the culture model.

Since several novel genes have been found recently in the growth plate (Hillarby *et al.*, 1996), it was interesting to use the cell culture model to define the expressions of these novel genes *in vitro* and to confirm the suitability of the model for the characterisation of these genes. The gene analysed was α -enolase, which was identified recently in the proliferative zone of the growth plate (Chapman, 1998). α -

enolase was expressed highly during the proliferative/maturation period in aza-C treated cultures (Chapter Four), which is consistent with its expression *in vivo*. It should be noted that the stage of proliferation is not clearly distinguishable from the maturation stage in the aza-C based culture system, which explains why these two stages are expressed as one. One possible way to determine and define the presence of an independent proliferative stage would be to analyse the expression of *c-myc*, which is specific to proliferation.

The fact that proliferation and maturation are not clearly distinguishable in aza-C cultures limits the use of the model in the study of genes specific to either of these two stages of EO. However, the aza-C based system still provides exciting opportunities for the characterisation of the newly identified genes which are differentially expressed between hypertrophy and proliferation/maturation. For example, genes can be switched off at specific time-points in culture by using anti-sense technology and the subsequent effect(s) on the chondrocyte differentiation pathway determined by analysing the expression of relevant markers of differentiation in time-course experiments. As mentioned earlier, one indicator of chondrocyte maturation is the increase in cell size. Therefore, the culture model is best suited for the study of the maturation and later stages of EO.

The cell culture model can also be used to study the effect(s) of factors involved in the regulation of the chondrocyte differentiation pathway. In the study described here, the effect of PTHrP on chondrocyte differentiation *in vitro* was examined by exposing hypertrophic chondrocytes in aza-C treated cultures to exogenous PTHrP. As is the case *in vivo*, the presence of exogenous PTHrP suppressed the expression of type X collagen in aza-C treated chondrocytes that had already entered hypertrophy (Chapter Six). Chondrocyte differentiation is known to be under the control of many regulatory factors (Chapter One, Section 1.6) but the precise roles of these factors are still not fully understood. The cell culture system described in this report offers the possibility to study the influence of these regulatory factors on chondrocyte differentiation *in vitro*. Exogenous factors can be added individually at varying concentrations and lengths of exposure at selected time-points in culture and their effects on each stage of differentiation examined by analysing the expression of

markers of differentiation. In addition, a cocktail of two or more exogenous factors can be added with a view to having a better understanding on the complex interactions of the added factors and their combined effects on chondrocyte differentiation *in vitro*.

In the light of the results obtained in the work presented here, it was possible to map the various stages of chondrocyte differentiation with the cell culture growth profile (Figure 7.2). On a preliminary basis, it can be proposed tentatively that days 0-10 correspond to the proliferation and maturation stages; days 11-23 correspond to the hypertrophic stage; and days 24-34 correspond to the stage of terminal hypertrophy. Calcification occurs in weeks 2-3 after the onset of hypertrophy. It is still too early to predict this aza-C based system to be a faithful recapitulation of the differentiation pathway in EO. Further experiments already mentioned above and in previous chapters are required in order to show and confirm its validity as a true representation of EO. It should also be noted that even if this culture model is shown to recapitulate all stages of chondrocyte differentiation, the weaknesses and limitations of the aza-C based model should also be considered. For example, as discussed above, the culture model does not allow the distinction between proliferation and maturation as two separate differentiation stages. This culture model also does not take into account the spatial and temporal regulation of chondrocyte differentiation and the interaction of chondrocytes at different stages of their differentiation and the effect(s) of such cell interactions on the differentiation pathway. Equally important is the presence of matrix mineralisation, which seems to occur only sparsely in the matrix, which might only indicate non-specific mineralisation. There is also the possibility that the effects of PTHrP in this culture system may not be mediated via its receptor as is the case *in vivo*. In addition, the culture model does not accurately represent the time span over which the various changes associated with chondrocyte differentiation occur as *in vivo*.

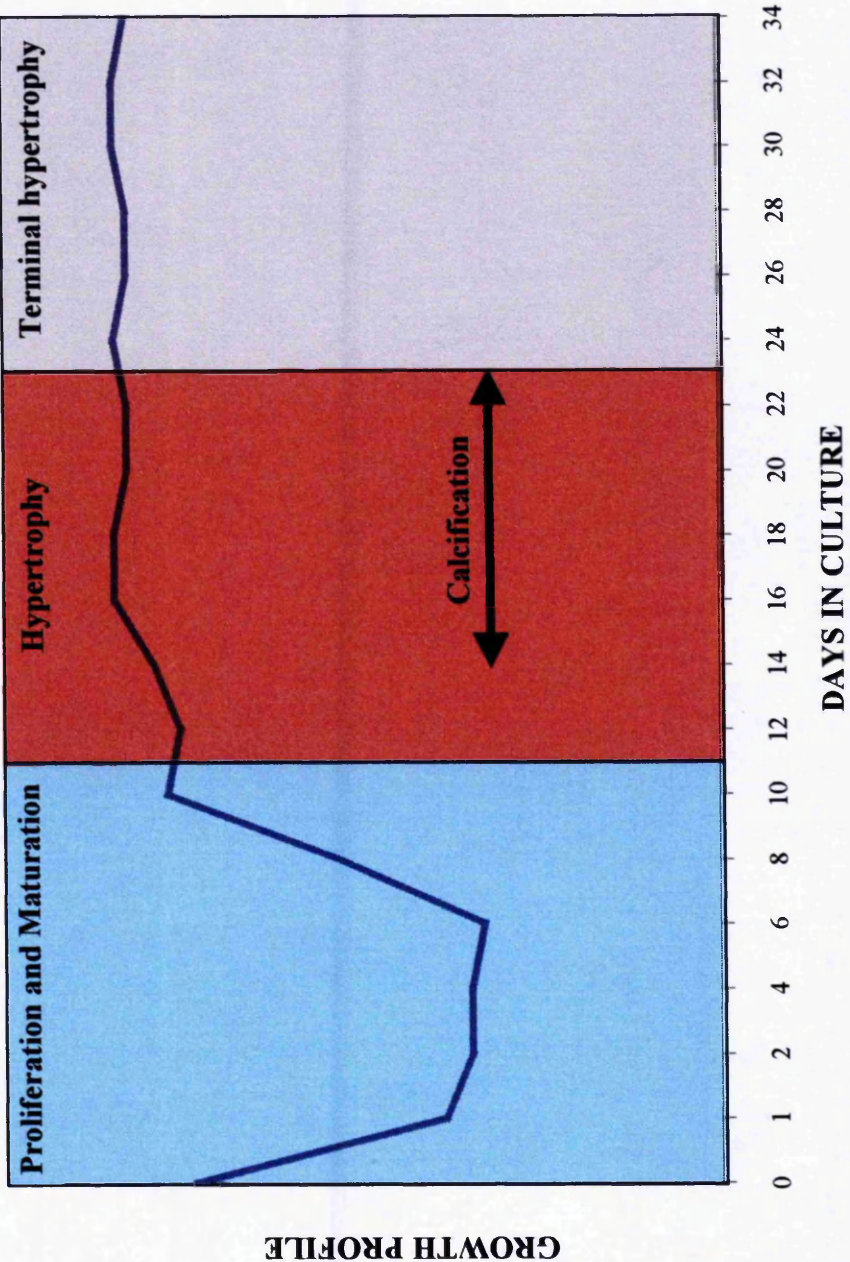


Figure 7.2. Characterisation of the cell culture model of mammalian endochondral ossification into differentiation stages of the chondrocytic pathway in the growth plate over a four-week culture period.

Despite these limitations, the system would prove useful in the studies of the molecular and hormonal regulation of EO. If proved to represent faithfully mammalian EO, this aza-C based culture system would be simple to set up and use. A better understanding of the process of EO is crucially important, especially since any dysregulation in this tightly controlled process is known to cause a wide spectrum of chondrodysplasias, many of which are severe and lethal.

Appendices

Appendices

APPENDIX A

1. PREPARATION OF BUFFERS AND REAGENTS: cDNA SYNTHESIS

dNTP mix

dATP, 100 mM	20 µl
dCTP, 100 mM	20 µl
dGTP, 100 mM	20 µl
dTTP, 100 mM	20 µl
Distilled water	Make up to 1000 µl

cDNA Strand Buffer

AMV RT Buffer (5X)	4 µl
dNTP mix	2 µl
Random hexamers	1 µl
RT	1 µl
DEPC-treated water	Make up to 20 µl

2. PREPARATION OF BUFFERS AND REAGENTS: PCR

PCR Buffer (10X)

MgCl ₂ , 1 M	0.15 ml
KCl, 1 M	5 ml
Tris, 1 M, pH 8.3	1 ml
Distilled water	Make up to 10 ml

dNTP mix

dATP, 100 mM	20 µl
dCTP, 100 mM	20 µl
dGTP, 100 mM	20 µl
dTTP, 100 mM	20 µl
Distilled water	Make up to 1000 µl

TBE Buffer (10X)

Tris	108 g
Orthoboric acid	55 g
EDTA, 0.5 M	40 ml
Distilled water	Make up to 1 litre

3. PREPARATION OF BUFFERS AND REAGENTS: IMMUNOHISTOCHEMISTRY

TBS Buffer (10X)

Tris	121.1 g
NaCl	170.0 g
Distilled water	Make up to 2 litres
Adjust pH to 7.4-7.6.	

4. PREPARATION OF BUFFERS AND REAGENTS: *IN SITU* HYBRIDISATION

STE Buffer

Tris-HCl, pH 8.0	10 mM
NaCl	100 mM
EDTA, pH 8.0	1 mM
Make up with DEPC-treated water and autoclave.	

SSC (20X)

NaCl	175.3 g
Sodium citrate	88.2 g (1 litre)
Adjust pH to 7.0.	

Developer

Phenisol developer	1 part
Deionised water	4 parts

Stop Bath Solution

Ilfostop Pro stop bath	1 part
Deionised water	19 parts

Fixer

Hypam fixer	1 part
Deionised water	4 parts

Scott's tap water substitute

Potassium bicarbonate	2 g
Magnesium sulphate	20 g
Deionised water	1000 ml

5. PREPARATION OF BUFFERS AND REAGENTS: ALCIAN BLUE STAINING

1 M Acetate Buffer

Glacial acetic acid, 1.2% 2.5 ml
 Sodium acetate, 0.2 M 47.5 ml
 Adjust pH to 5.8, and make up to 100 ml with distilled water.

Salt Solutions

<i>Molarity of $MgCl_2$ (M)</i>	0.05	0.5	0.9
<i>1M acetate buffer (ml)</i>	2.5	2.5	2.5
<i>5M $MgCl_2$ (ml)</i>	1	10	18

Staining Solutions

Salt solution 25 ml
 Alcian blue in distilled water, 0.1% 25 ml

Rinse Solutions

Salt solution 25 ml
 Distilled water 25 ml

APPENDIX B

PRIMER DNA SEQUENCES

Type II collagen, bovine.

5' primer GGA CCC AAG AAC TTT CCA ATC

3' primer GAA TAG CGC CGT TGT GTA GGA

The total length of DNA fragment amplified by RT-PCR is 321 bases.

Below is the cDNA sequence of bovine type II collagen taken from Sangiorgi *et al.* (1985). The sequences recognised by the 5' primer and 3' primer are indicated in red and blue respectively. The full cDNA fragment amplified by RT-PCR is underlined. Numbers refer to the nucleotide positions.

1	ACCCTGGATG	CCATGAAGGT	TTTCTGCAAC	ATGGAGACTG	GCGAGACCTG
51	CGTCTACCCC	AACCCGGCCA	GCGTCCCCAA	GAAGAACTGG	TGGAGCAGCA
101	AGAGCAAGGA	CAAGAAACAC	ATCTGGTTTG	GAGAAACCAT	CAACGGTGGC
151	TTCCAATTCA	GCTATGGAGA	TGACAACCTG	GCTCCCAACA	CCGCCGACGT
201	CCAGATGACC	TTCTGCGCC	TGCTGTCCAC	CGAGGGCTCT	CAGAACATCA
251	CCTACCACTG	CAAGAACAGC	ATTGCCTACC	TGGACGAAGC	TGCTGGCAAC
301	CTCAAGAAGG	CTCTGCTCAT	CCAGGGCTCC	AACGACGTGG	AGATCCGGGC
351	TGAGGGCAAC	AGCAGGTTCA	CATATACCGT	TCTGAAGGAT	GGCTGCACGA
401	AACACACCGG	TAAGTGGGGC	AAGACTATGA	TCGAGTACCG	GTCACAGAAG
451	ACCTCCCGTC	TGCCCATCAT	TGACATTGCA	CCCAYGGACA	TAGGAGGGCC
501	CGAGCAGGAA	TTCGGTGTGGA	ACATAGGGCC	TGTCTGCTTC	TTGTAAAAAC
551	CCGAACCCAG	AACCAACACA	ATCCATTGCA	AACCCAAAGG	<u>ACCCAAGAAC</u>
601	<u>TTTCCAATCC</u>	<u>CAGTCACTCT</u>	<u>AGGACTCTGC</u>	<u>ACTGAATGGC</u>	<u>TGACCTGACC</u>
651	<u>TGACGCCCAT</u>	<u>TCATCCCACC</u>	<u>CTCTCACAGT</u>	<u>TCGGACTTTG</u>	<u>CTCCCCTCTC</u>
701	<u>TAAGAGACCT</u>	<u>GAACTGGGCA</u>	<u>GACTGCAAAA</u>	<u>TCAAATCTCG</u>	<u>GTGTTCTATT</u>
751	<u>TATTTATTGT</u>	<u>CTTCCTGTAG</u>	<u>GACCTTTGGG</u>	<u>TCAAGGCAGA</u>	<u>GACAGGAAAC</u>
801	<u>TAAGTGGAGT</u>	<u>GAGTCAAACG</u>	<u>CCCCCTGAGT</u>	<u>AACTACCCCC</u>	<u>CGGCCCAAGC</u>
851	<u>AAGGGGCCCC</u>	<u>TGCAGGTGCC</u>	<u>GGGCGCAGGG</u>	<u>ACTGCGCGCG</u>	<u>TCCTACACAA</u>
901	<u>CGGCGCTATT</u>	<u>CTGTGTCAAA</u>	CACCTCTGTA	TTTTTTAAAA	CGTCAATTGA
951	TATTAATAAC	AAAAAATTA	TTGGAAAGT		

Type X collagen, bovine.

5' primer GAA ACA TTC GGG AGA TGT CAT
 3' primer TTT CAT GAG GCA CAG CTT AAG

The total length of DNA fragment amplified by RT-PCR is 555 bases.

Below is the partial cDNA sequence of bovine type X collagen taken from Thomas *et al.* (1991). The sequences recognised by the 5' primer and 3' primer are indicated in red and blue respectively. The full cDNA fragment amplified by RT-PCR is underlined. Numbers refer to the nucleotide positions.

2151	GATTCTTGGT	GGCTCCAATG	TGAGCATATT	CTCACCGAGC	TAACACAAAT
2201	CTGCTTGAAA	AGGCATTCCC	CAACTCCACC	CCATCCCACG	AAATGCATAT
2251	GGAGGTAGGC	TGAAAAAAAT	GTGACTAATT	TTCCAAAATA	CAGGTCTGAG
2301	CTCTCAGATA	CGGTAAACAT	ATGTGAAGGC	CCTCTTGAGT	TTCTAGTCAG
2351	CAATCCTAAA	ACTCCTAAAA	GTCTTCTGTG	AACTCCTTCA	GTATTTAAAA
2401	ATTTTCATCCT	GCTAGGCTGA	AAAAACGATC	GCAAAAAACC	CTGAAATGTG
2451	ATGCTAAATT	ATGTCAAATT	TGATTTCAGA	AGTTCAGCAT	TTCTTTTAA
2501	AAATCAGTCT	GTTCTGACAA	TTAACAGGAA	AATTTCCAGG	<u>AAACATTCGG</u>
2551	<u>GAGATGTCAT</u>	<u>ATCTTTATGG</u>	<u>GACTTAAATA</u>	<u>CTTGAATATT</u>	<u>CAAATTTAAA</u>
2601	<u>AAACACTACA</u>	<u>TACCCTGAGA</u>	<u>TCTTTCTGAT</u>	<u>GGTGCATTAC</u>	<u>TCAAAGGTCT</u>
2651	<u>AAGTGGCCCC</u>	<u>TTTTGTCAAT</u>	<u>ATCTATTCAA</u>	<u>GTATACAGGT</u>	<u>GCATATAGAC</u>
2701	<u>TTTTTACAGC</u>	<u>TCTCATAAGA</u>	<u>AACCCAAAAT</u>	<u>ATTAATGCTA</u>	<u>AAGTTAATCT</u>
2751	<u>GAAATGCAAG</u>	<u>GTGCTTTTGT</u>	<u>CATGAACCTC</u>	<u>TTCAAACTTT</u>	<u>TCTGTGGATT</u>
2801	<u>GCTGAAAGCT</u>	<u>TTCTATATAC</u>	<u>CCTTTACAAC</u>	<u>TTGGAAATGG</u>	<u>TGTCTAACCT</u>
2851	<u>ATTTTATTTA</u>	<u>TTTGACACAA</u>	<u>GTGTGATTAA</u>	<u>TTTGCTTTAA</u>	<u>TGACTACTTG</u>
2901	<u>AGTCTTATAT</u>	<u>AATCTTAATA</u>	<u>TGATTTTGTG</u>	<u>GGTTTATAGA</u>	<u>GTATTAGCAT</u>
2951	<u>ATGTACCTTG</u>	<u>GGCCTCCCAT</u>	<u>TCCAGTGAAA</u>	<u>TTGTAATATC</u>	<u>AAGGGTTTCA</u>
3001	<u>AAATCTGACT</u>	<u>AGAAATGAAA</u>	<u>AGATATTATT</u>	<u>TATTCTGCT</u>	<u>CTGTACTGTA</u>
3051	<u>TTTTAATGTT</u>	<u>TCTGTTTTAA</u>	<u>ACTCTTAAGC</u>	<u>TGTGCCTCAT</u>	<u>GAAATGTTTT</u>
3101	ATCCACTCCT	TATTTACAAT	GCAATAAAAT	AACATCAATA	CCG

APPENDIX C**CDNA PROBES USED IN *IN SITU* HYBRIDISATION STUDIES**

<i>PROBE</i>	<i>ORIGIN (SPECIES)</i>	<i>LENGTH (bp)</i>
Alkaline phosphatase	Human	2500
Bax	Human	600
Bcl-2	Human	1000
α -Enolase	Bovine	
PTHrP	Human	1200
PTHrP receptor	Human	1800
Type X collagen	Human	700

APPENDIX D

REAGENT KITS

Annexin V-FITC Apoptosis Detection Kit (*Oncogene Research Products*)

Annexin V-FITC
5X Binding Buffer
Media Binding Reagent
Propidium Iodide

Sigma Diagnostics Alkaline Phosphatase Kit (*Sigma*)

Sigma 104® Phosphatase Substrate
221 Alkaline Buffer Solution
p-Nitrophenol Standard Solution

Amersham Megaprime™ DNA Labelling System (*Amersham*)

Primer
Reaction Buffer
dATP
dTTP
dGTP
dCTP
Klenow fragment

Publications

Publications

1. Cheung, J., Ayad, S., Hoyland, J.A., Grant, M.E., and Hillarby, M.C. (1998) A novel cell culture model of mammalian endochondral ossification. *Submitted*: American Society for Bone and Mineral Research Meeting, San Francisco, U.S.A.
2. Cheung, J., Ayad, S., Grant, M.E., and Hillarby, M.C. (1998) Effects of 5-azacytidine on chondrocyte differentiation in culture. *Bone*. **22** (No. 3 Suppl): 36S.
3. Cheung-Wo-Yuen, J.O.P., Hillarby, M.C., Boot-Handford, R.P., Ayad, S., and Grant, M.E. (1998) Effects of 5-azacytidine on chondrocyte differentiation in culture. *Int. J. Exp. Path.* **79**(2): A17.

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