Biology of the Articular Cartilage

Progenitor Cells

A thesis submitted to the University of Wales
for the degree of Doctor of Philosophy.

by

Joanna Charlotte Bishop B.Sc. (Hon.)

Cardiff School of Biosciences,
Biomedical Buildings,
University of Wales, Cardiff.

April 2004
Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed...........................................(Candidate)
Date...........................................

19/10/04

Statement 1

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed...........................................(Candidate)
Date...........................................

19/10/04

Statement 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed...........................................(Candidate)
Date...........................................

19/10/04
ABSTRACT

It is known that articular cartilage grows by apposition from the articular surface, which contains a population of slowly cycling cells suggestive of the existence of a chondroprogenitor cell population. Within this study, we have identified and confirmed the existence of a chondrocyte progenitor population by their rapid adhesion to fibronectin and their ability to form large numbers of colonies from a low seeding density. We further characterised the progenitor population derived from 7-day-old bovine articular cartilage by cloning single colonies and expanding them in monolayer culture. We were able to generate $10^{12}$ cells from a single progenitor that continued to express high levels of Sox9 and were still capable of chondrogenesis. A total of 50 population doublings (PD), 15 more PDs than had been predicted for chondrocytes. We also demonstrated the existence of a chondroprogenitor population within mature bovine tissue which gives hope to its existence within mature human tissue. We were able to reduce the progenitors doubling time by supplementing the media with various growth factors without compromising their ability to synthesise a cartilaginous matrix. Within this study we also assessed the differentiation potential of the progenitors \textit{in vivo} by injecting them into the proximal limbs of stage 19-21 chick embryos. Chondroprogenitor cells derived from both early and late passaged cultures have the ability to integrate into various tissues following their injection into the developing limb bud. Differentiation of the clonally derived chondroprogenitors' toward the chondrogenic and adipogenic lineage but not osteogenic was demonstrated \textit{in vitro} as was the surprising differentiation towards a neuronal phenotype. Chondrocytes retained their zonal characteristics and continued to synthesise cartilaginous matrix when cultured in basic media containing only 10% FCS. Transplantation of chondroprogenitor pellets reduced the cell death at the wound margins of partially wounded articular cartilage explants in culture. Therefore, the existence of a progenitor cell that could be potentially isolated from the articular cartilage by a minimally invasive procedure, expanded in culture and produce cartilaginous tissue is an exciting prospect to cartilage repair research.
LIST OF CONTENTS

Title..................................................................................................................i
Declaration........................................................................................................ii
Abstract.............................................................................................................iii
List of Contents................................................................. iv
List of Figures....................................................................................................vii
List of Tables.....................................................................................................xiv
Acknowledgements..........................................................................................xv
Abbreviations....................................................................................................xvi

Chapter 1: General Introduction.................................................................1
  1.1 SYNOVIAL JOINT.................................................................1
  1.2 ARTICULAR CARTILAGE..................................................1
  1.3 COMPOSITION OF ARTICULAR CARTILAGE......................3
  1.3.1 Chondrocyte.............................................................................3
  1.4 STRUCTURAL MACROMOLECULES.......................................6
  1.4.1 Cartilage collagens...............................................................6
  1.4.2 Cartilage proteoglycans.......................................................10
  1.4.3 Non-collagenous cartilage matrix proteins and glycoproteins14
  1.4.4 Interstitial tissue fluid...........................................................16
  1.5 STRUCTURE OF ARTICULAR CARTILAGE–MORPHOLOGICAL
    HETEROGENEITY.............................................................16
  1.5.1 Superficial zone.................................................................16
  1.5.2 Middle zone............................................................................17
  1.5.3 Deep zone...............................................................................19
  1.5.4 Calcified cartilage...............................................................19
  1.5.5 MATRIX REGIONS.........................................................19
  1.6 BIOCHEMICAL HETEROGENEITY WITHIN ARTICULAR
    CARTILAGE...........................................................................21
  1.7 CARTILAGE MATRIX TURNOVER.....................................22
    1.7.1 Proteoglycan synthesis......................................................22
    1.7.2 Proteoglycan catabolism......................................................22
    1.7.3 Collagen synthesis.............................................................23
    1.7.4 Collagen catabolism............................................................24
1.8 CHONDROCYTE-MATRIX INTERACTIONS ...................................................... 24
1.9 Receptors involved in chondrocyte-matrix interaction ............................ 26
1.10 CARTILAGE DEVELOPMENT ................................................................. 29
1.11 DEVELOPMENT OF THE SYNOVIAL JOINT ........................................... 30
1.12 DEVELOPMENT OF AC ............................................................................ 31
1.13 DEGRADATION OF AC ............................................................................ 36
1.13.1 Osteoarthritis ..................................................................................... 37
1.14 ARTICULAR CARTILAGE REPAIR .......................................................... 40
1.14.1 Intrinsic response of articular cartilage following wounding ................. 40
1.14.2 Extrinsic repair of articular cartilage .................................................. 41
1.15 STEM CELLS .......................................................................................... 46
AIMS ............................................................................................................. 51

CHAPTER 2: Characterisation of chondrocytes derived from the superficial,
middle and deep zones of articular cartilage .............................................. 52
2.1 Introduction ............................................................................................. 53
2.2 Materials and methods ........................................................................... 55
2.3 Results ..................................................................................................... 64
2.4 Discussion ............................................................................................... 101

CHAPTER 3: Isolation and characterisation of articular cartilage progenitor
cells ............................................................................................................ 115
3.1 Introduction ............................................................................................. 116
3.2 Materials and methods ........................................................................... 121
3.3 Results ..................................................................................................... 131
3.4 Discussion ............................................................................................... 171

CHAPTER 4: The effects of growth factors on the expansion and differentiation
of articular cartilage progenitor cells ........................................................... 179
4.1 Introduction ............................................................................................. 180
4.2 Materials and methods ........................................................................... 186
4.3 Results ..................................................................................................... 189
4.4 Discussion ............................................................................................... 214

CHAPTER 5: The plasticity of articular cartilage progenitor cells in vitro ......... 219
5.1 Introduction ............................................................................................. 220
5.2 Materials and methods ........................................................................... 223
5.3 Results ..................................................................................................... 229
LIST OF FIGURES

Figure 1.1. Schematic representation of the diarthrodial joint .........................1
Figure 1.2. The organisation of articular cartilage ....................................2

Figure 1.3. Cartilage Collagens ......................................................................8
Figure 1.4. Schematic model of aggrecan ..................................................12
Figure 1.5. Schematic representation of the structure of articular cartilage
SZP ........................................................................................................14
Figure 1.6. Schematic representation of adult articular cartilage ..................18
Figure 1.7. Schematic representation of the changes in distribution of proliferating
chondrocytes, syndecan-3 and tenascin-C in skeletal elements at early and late stages
of embryogenesis ..............................................................................32
Figure 1.8. Diagram summarising hypothetical cell lineage of articular cartilage.....35
Figure 1.9. Similarities and differences between embryonic and adult stem cells ......49

Chapter 2
Figure 2.1 Schematic illustration showing the overall strategy for chondrocyre
isolation and pellet formation .................................................................57
Figure 2.2. Photomicrographs of the gross morphology of pellet ..................69
Figure 2.3. Scanning electron micrographs of normal articular cartilage and pellets..71
Figure 2.4. Photomicrographs of pellets stained with safranin O ....................73
Figure 2.5. Photomicrographs of pellets immunolabelled for type II collagen......74
Figure 2.6. Photomicrographs of pellets immunolabelled for type IX collagen ......75
Figure 2.7. Photomicrographs of pellets immunolabelled for link protein ...........76
Figure 2.8. Photomicrographs of pellets immunolabelled for chondroitin-4-sulphate
and dermatan sulphate ...........................................................................77
Figure 2.9. Photomicrographs of pellets immunolabelled for type I collagen ......78
Figure 2.10. Photomicrographs of pellets immunolabelled for SZP ...................79
Figure 2.11 Photomicrographs of pellets immunolabelled for clusterin .............80
Figure 2.12 Photomicrographs of pellets immunolabelled for type X collagen ......81
Figure 2.13 Photomicrographs of pellets immunolabelled with ethidium homodimer
to assess cell death .............................................................................82
Figure 2.14. Graph representing the DNA content in pellets ..........................84
Figure 2.15. Graph representing the GAG content in pellets ..........................86
Figure 2.16. Graph representing the collagen content in pellets................................. 88
Figure 2.17. Graph representing the GAG content in pellets and representative native tissue.................................................................90
Figure 2.18. Graph representing the collagen content in pellets and representative native tissue................................................................. 92
Figure 2.19. Photomicrograph of representative sections of stratified pellets (S/D)....93
Figure 2.20. Photomicrograph of representative sections of stratified pellets (S/D) stained with safranin O.........................................................94
Figure 2.21. Photomicrograph of representative sections of stratified pellets (S/M/D).................................................................................95
Figure 2.22. Photomicrograph of representative sections of stratified pellets (S/M/D) stained with safranin O.........................................................96
Figure 2.23. Photomicrograph of representative sections of surface zone and deep pellets and mixed pellets (S&D)..............................................97
Figure 2.24. Photomicrograph of representative sections of surface zone and deep pellets and mixed pellets (S&D) stained with safranin O............98
Figure 2.25. Photomicrograph of representative sections of stratified pellets (S/D) labelled for type X collagen......................................................... 99
Figure 2.26. Photomicrographs of representative positive control sections of primary antibodies used.................................................................100

Chapter 3
Figure 3.1. Schematic illustration showing the bovine skeleton and bovine metacarpalphalangeal joint.................................................................122
Figure 3.2. Montage of safranin O stained articular cartilage taken from 7-day, 18-month and over 30-month old bovine metacarpalphalangeal joints............137
Figure 3.3. Graph showing the initial number of cells isolated from 7-day bovine metacarpalphalangeal joint articular cartilage adhering to various ligands........ 139
Figure 3.4. Graph showing the CFE of surface and deep zone chondrocytes derived from 7-day bovine MCP joints following initial adhesion to various ligands........141
Figure 3.5a. Graph showing the initial number of cells isolated from 18-month bovine metacarpalphalangeal joint articular cartilage adhering to fibronectin..............143
Figure 3.5b. Graph showing the CFE of surface and deep zone chondrocytes derived from 18-month bovine MCP joints following initial adhesion to fibronectin........143
Figure 3.6a. Graph showing the initial number of cells isolated from 18-month bovine metacarpal-palangeal joint articular cartilage adhering to fibronectin.

Figure 3.6b. Graph showing the CFE of surface and deep zone chondrocytes derived from 18-month bovine MCP joints following initial adhesion to fibronectin.

Figure 3.7. Typical morphology of a 32+ cell clone.

Figure 3.8. Morphological differentiation of isolated clones following 264 days in culture.

Figure 3.9. Morphological differentiation of isolated chondroprogenitor enriched following 166 days in culture.

Figure 3.10. Morphological differentiation of isolated middle/deep chondrocytes following 65 days in culture.

Figure 3.11. Morphological differentiation of isolated full depth chondrocytes following 66 days in culture.

Figure 3.12. Graph representing the cell population growth curve of 8 clones.

Figure 3.13. Graph representing the cell population growth curve of 5 chondroprogenitor enriched cultures.

Figure 3.14. Graph representing the cell population growth curve of 3 middle/deep derived articular chondrocyte cultures.

Figure 3.15. Graph representing the cell population growth curve of 5 full depth derived articular chondrocyte cultures.

Figure 3.16. Graph representing the growth rate of clones, the chondroprogenitor enriched population, middle/deep population and the full depth derived cultures.

Figure 3.17. Graph representing the relative quantity of Sox9 mRNA normalised to control within clonally derived cultures.

Figure 3.18. Graph representing the relative quantity of Sox9 mRNA normalised to control within chondroprogenitor enriched cultures.

Figure 3.19. Graph representing the relative quantity of Sox9 mRNA normalised to control within middle/deep derived cultures.

Figure 3.20. Graph representing the relative quantity of Sox9 mRNA normalised to control with full depth derived cultures.

Figure 3.21. Photomicrographs of cultures stained for β-galactosidase to detect lysosomal β-Gal and cell senescence associated β-Gal.

Figure 3.22. Graph representing percentage of senescence associated β-Gal positive cells.
Figure 3.23. Photomicrographs of clonally derived pellets stained with safranin O. ................................................................. 163
Figure 3.24. Photomicrographs of clonally derived pellets immunolabelled for collagen type I and II ........................................... 164
Figure 3.25. Photomicrographs of chondroprogenitor enriched derived pellets stained with safranin O ........................................... 165
Figure 3.26. Photomicrographs of chondroprogenitor enriched derived pellets immunolabelled for collagen type I and II ............... 166
Figure 3.27. Photomicrographs of middle/deep derived pellets stained with safranin O ................................................................. 167
Figure 3.28. Photomicrographs of middle/deep derived pellets immunolabelled for collagen type I and II ....................................... 168
Figure 3.29. Photomicrographs of full depth derived pellets stained with safranin O ................................................................. 169
Figure 3.30. Photomicrographs of full depth derived pellets immunolabelled for collagen type I and II ....................................... 170

Chapter 4

Figure 4.1. Comparative morphology of clones cultured for 12 days in control medium + 1% FCS or control medium containing various growth factors ................................................................. 193
Figure 4.2. Comparative morphology of clones cultured for 12 days in control medium + 10% FCS or control medium containing various growth factors ................................................................. 195
Figure 4.3. Comparative morphology of passage 2 clones cultured for 6 days in control medium + 1% FCS or control medium containing various growth factors ................................................................. 197
Figure 4.4. Comparative morphology of passage 2 clones cultured for 6 days in control medium + 10% FCS or control medium containing various growth factors ................................................................. 199
Figure 4.5. Graph representing the time in hours for cells expanded in control medium + 1% FCS/10% FCS or in the presence of various growth factors during passage 1 ............................................................................ 201
Figure 4.6. Graph representing the time in hours for cells expanded in control medium + 1% FCS/10% FCS or in the presence of various growth factors during passage 2 ................................................................. 203
Figure 4.7. Graph representing the relative quantity of Sox9 mRNA for cells expanded in control medium + 1% FCS/10% FCS or in the presence of various growth factors during passage 1 ................................................................. 205
Figure 4.8. Graph representing the relative quantity of Sox9 mRNA for cells expanded in control medium + 1% FCS/10% FCS or in the presence of various growth factors during passage 2 ................................................................. 207
Figure 4.9. Photomicrographs of pellets stained with safranin O ................................................................. 209
Figure 4.10. Photomicrographs of pellets immunolabelled for collagen type II ................................................................. 211
Figure 4.10. Photomicrographs of pellets immunolabelled for collagen type I ................................................................. 213

Chapter 5
Figure 5.1. Schematic illustration showing the overall strategy for chondrocyte isolation, expansion and subsequent stimulation with differentiation media .......... 224
Figure 5.2. Phase microscopic appearance of representative clones, SZ, MZ, DZ and following 21 days in culture in adipogenic media ................................................................. 231
Figure 5.3. Oil red O staining of clones, SZ, MZ and DZ cells cultured in adipogenic differentiation media ................................................................. 233
Figure 5.4. Graph representing the expression of mRNA for lipoprotein lipase in cultures exposed to both control and adipogenic treatment ................................................................. 235
Figure 5.5. Phase microscopic appearance of representative clones, SZ, MZ, DZ and following 21 days in culture in osteogenic media ................................................................. 236
Figure 5.6. Graph representing the expression of mRNA for osteocalcin in cultures exposed to both control and adipogenic treatment ................................................................. 239
Figure 5.7. Photomicrographs of clones following 24 hours in pre-induction or control medium and exposure to neuronal induction media for up to 6 hours ................................................................. 242
Figure 5.8. Photomicrographs of SZ derived cells following 24 hours in pre-induction or control medium and exposure to neuronal induction media for up to 6 hours ................................................................. 244
Figure 5.9. Photomicrographs of MZ derived cells following 24 hours in pre-induction or control medium and exposure to neuronal induction media for up to 6 hours.................................................................246

Figure 5.10. Photomicrographs of DZ derived cells following 24 hours in pre-induction or control medium and exposure to neuronal induction media for up to 6 hours........................................................................248

Figure 5.11. Photomicrographs of clones, SZ, MZ and DZ cells expanded with neurogenic media and immunolabelled for the neuronal protein tau......................................................................................250

Figure 5.12. Photomicrographs of clones, SZ, MZ and DZ cells expanded with neurogenic media and immunolabelled for the neuronal protein marker β-III tubulin........................................................................250

Figure 5.13. Photomicrographs of representative positive control sections of primary antibodies used.................................................................................................................................254

Chapter 6

Figure 6.1. Micrographs showing preparation of the egg prior to injections (A,B) and typical hindlimb anatomy for embryos.................................................................269

Figure 6.2. Micrograph of GP293 cells 72 hours following transfection with the lacz gene and stained with x-gal and micrographs of early and late passaged chondroprogenitors and early middle/deep derived chondrocytes cultures (transfected with the pseudovirus) stained for the β-gal enzyme.................................................................275

Figure 6.3. Typical endogenous labelling following x-gal staining for the β-gal enzyme.................................................................................................................................276

Figure 6.4. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21........278

Figure 6.5. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21........280

Figure 6.6. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21........282
Figure 6.7. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21.  
Figure 6.8. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a long period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21.  
Figure 6.9. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a long period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21.  
Figure 6.10. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a long period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21.  
Figure 6.11. Middle/deep derived chondrocytes, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21.  
Figure 6.12. Middle/deep derived chondrocytes, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21.  

Chapter 7

Figure 7.1. Wounding full depth 7-day bovine excised explant with drill bit and partial thickness defect tool.  
Figure 7.2. Full depth explants wounded with a drill bit.  
Figure 7.3. Full depth explant wounded with a partial thickness defect tool and stained with safranin O.  
Figure 7.4. Full depth 7-day bovine articular cartilage explants wounded with a partial thickness tool and labelled with ethidium homodimer.  
Figure 7.5. Wounded representative full depth explant transplanted with chondroprogenitor enriched derived pellets and stained with safranin O.  
Figure 7.6. Wounded representative full depth explant transplanted with chondroprogenitor enriched derived pellets and labelled with ethidium homodimer.  
Figure 7.7. Wounded representative full depth explant transplanted with middle zone derived pellets and stained with safranin O.
Figure 7.8. Wounded representative full depth explant transplanted with middle zone derived pellets and labelled with ethidium homodimer.................................317
Figure 7.9. Wounded representative full depth explant transplanted with deep zone derived pellets and stained with safranin O...........................................318
Figure 7.10. Wounded representative full depth explant transplanted with deep zone derived pellets and labelled with ethidium homodimer.................................319
Figure 7.11. Wounded representative full depth explant transplanted with full depth derived pellets and stained with safranin O...........................................320
Figure 7.12. Wounded representative full depth explant transplanted with full depth derived pellets and labelled with ethidium homodimer.................................321
Figure 7.13a. Diagrammatic representation of a full depth wounded explant........323
Figure 7.13b. Graph representing the percentage of dead cells found within region A at a depth of 0-100μm.................................................................323
Figure 7.13c. Graph representing the percentage of dead cells found within region A at 100-200μm.................................................................................323
Figure 7.14a. Diagrammatic representation of a full depth wounded explant........325
Figure 7.14b. Graph representing the percentage of dead cells found within region B at a depth of 0-100μm.................................................................325
Figure 7.14c. Graph representing the percentage of dead cells found within region B at 100-200μm.................................................................................325

LIST OF TABLES

Table 2.1. Spectral characteristics of the fluorescent Cell Tracker probes..........56
Table 2.2. Monoclonal/polyclonal antibodies used.........................................62
Table 3.1. Concentration of ligands used in the differential adhesion assay........121
Table 3.2. Primers used to generate PCR products........................................128
Table 3.3. Quantitative PCR primer and probe sequences and reaction concentrations for Sox9.................................................................130
Table 5.1. Antibodies used during immunohistochemistry..............................226
Table 5.2. Quantitative PCR primer and probe sequences for osteocalcin and lipoprotein lipase.................................................................228
Table 5.3. Quantitative PCR primer and probe reaction concentrations.............228
ACKNOWLEDGEMENTS

I would like to thank Professor Charlie Archer for his supervision, encouragement ("It is character building") and support during my PhD.

For their help during this work I would like to thank everyone within the Preclinical library, Dr Darrell Evans for his assistance with the in ovo work, Dr Hann for the SEM work, Derek Scarborough during histological processing and both Guy and Viv for their help with photographic work.

I would like to thank the following for their continuous encouragement (tea making and cake buying!) during my PhD at Cardiff: Rhiannon, Samantha, Samuel, Kirsty, Becs, Sarah, Ilyas and Elaine.

I am indebted to my parents and all of my family for their support and to Leighton for his untiring patience and reassurance.

This work was funded by Smith & Nephew.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>Adipogenic medium</td>
</tr>
<tr>
<td>APES</td>
<td>3-Aminopropyltriethoxysaline</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>β-Gal</td>
<td>Beta galactosidase</td>
</tr>
<tr>
<td>BM</td>
<td>Basement Membrane</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CE</td>
<td>Chondroprogenitor enriched</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage Oligomeric matrix protein</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin Sulphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DPX</td>
<td>Distrene plasticizer xylene</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan Sulphate</td>
</tr>
<tr>
<td>DZ</td>
<td>Deep zone</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>EthHD</td>
<td>Ethidium homodimer</td>
</tr>
<tr>
<td>F12</td>
<td>Ham’s F12</td>
</tr>
<tr>
<td>FACIT</td>
<td>Fibril-Associated Collagens with interrupted triple helices</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxyfluorescein</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>FD</td>
<td>Full depth</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methyl xanthine</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin like Growth Factor</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>KS</td>
<td>Keratan Sulphate</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MAPC</td>
<td>Multipotent adult progenitor cells</td>
</tr>
<tr>
<td>MCP</td>
<td>Metacarpalphalangeal Joint</td>
</tr>
<tr>
<td>MMP</td>
<td>Metalloproteinases</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MZ</td>
<td>Middle zone</td>
</tr>
<tr>
<td>NBFS</td>
<td>Neutral Buffered Formal Saline</td>
</tr>
<tr>
<td>NM</td>
<td>Neuronal induction medium</td>
</tr>
<tr>
<td>NT</td>
<td>Native tissue</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OM</td>
<td>Osteogenic medium</td>
</tr>
<tr>
<td>OTM</td>
<td>Over-30-month</td>
</tr>
<tr>
<td>P</td>
<td>Passage</td>
</tr>
<tr>
<td>PD</td>
<td>Population doubling</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell nuclear Antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycans</td>
</tr>
<tr>
<td>PLA</td>
<td>Processed lipoaspirate</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SA-β-Gal</td>
<td>Senescent associated beta galactosidase</td>
</tr>
<tr>
<td>S/M/D</td>
<td>Stratified pellets, SZ atop MZ, MZ atop DZ</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>S/D</td>
<td>Stratified pellets, SZ atop DZ</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SFM</td>
<td>Chemically defined serum-free media</td>
</tr>
<tr>
<td>SFMB</td>
<td>SFM supplemented with 10ng/ml BMP-2</td>
</tr>
<tr>
<td>SFMT</td>
<td>SFM supplemented with 10ng/ml TGF-β1</td>
</tr>
<tr>
<td>SFMTLD</td>
<td>SFM supplemented with TGF-β1 and 10⁻⁷M dexamethasone</td>
</tr>
<tr>
<td>SSM</td>
<td>Serum supplemented media</td>
</tr>
<tr>
<td>SZ</td>
<td>Surface zone</td>
</tr>
<tr>
<td>SZP</td>
<td>Surface zone Protein</td>
</tr>
<tr>
<td>TAMRA</td>
<td>6-carboxy-tetramethyl-rhodamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor- β</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinase</td>
</tr>
<tr>
<td>VD</td>
<td>1,25-dihydroxyvitamin D₃</td>
</tr>
</tbody>
</table>
CHAPTER 1
General Introduction
INTRODUCTION

1.1 SYNOVIAL JOINTS

The synovial joint provides vertebrates with both mobility and dexterity. Many distinct tissues combine to make up this important structure including bone, ligament, meniscus, metabolically active synovium, hyaline articular cartilage and the enclosing fibrous joint capsule (fig 1.1). This joint varies throughout the body not only in size and shape but also in its range of motion. The articular cartilage provides a smooth low friction and load-bearing surface that enables the articulating bones to transmit high loads whilst maintaining contact stresses at an acceptably low level, and to move against each other with minimal frictional resistance (Freeman, 1979).

![Synovial membrane](image1.jpg)

**Figure 1.1.** Schematic representation of the diarthrodial joint adapted from (Seer, 2003)

1.2 ARTICULAR CARTILAGE

Articular cartilage is a multiphasic material consisting of a fluid phase comprising both water (65-80% of wet mass) and electrolytes and a solid phase of which collagen (10-30% of wet mass), proteoglycans (5-10% of wet mass), glycoproteins and chondrocytes are components (fig 1.2A).

Articular cartilage thickness, cell density, matrix composition and mechanical properties all vary dramatically within the same joint. The remarkable property of this thin tissue is its "stiffness to compression, resilience, and ability to distribute loads
Figure 1.2. The organisation of articular cartilage with respect to depth from the articular surface: A) Histologic view of mature rabbit articular cartilage; (adapted from Cormack 1993) B) Schematic representation of the distribution of chondrocytes; C) Schematic representation of the collagen ultrastructure (adapted from Ratcliffe and Mow, 1998).
which minimises peak stresses on the underlying subchondral bone” (Mow and Rosenwasser, 1988). During adult life its thickness remains unchanged. It does not thin simply as a result of physiological joint activity, thus, providing the majority of the general population with normal joint function for up to 80 years.

Normal articular cartilage has a slick “glossy” appearance (Meachim and Stockwell, 1979) with a firm inert surface (‘chondromalacie’) that resists deformation. Light microscopic analysis reveals that articular cartilage has a relatively low cell density. It is devoid of nerves and is primarily avascular, but does have a few blood vessels in the deepest parts adjacent to the subchondral bone. The cells are sustained by nutrient and gas diffusion, which occurs over a large surface area (Stockwell, 1978). In comparison to bone and tendon the tissue itself has a low level of metabolic activity.

Morphological and biological studies on articular cartilage reveal that it is an intricate tissue having a highly organised structure. The chondrocytes and surrounding matrix are functionally dependent on one another to maintain the tissue through many complex interactions. Thus, it can be said that the physiological and mechanical properties of cartilage depend upon the physical properties of its matrix and the matrix in turn, is dependent for its composition and maintenance upon the chondrocytes (Freeman, 1979).

1.3 COMPOSITION OF ARTICULAR CARTILAGE
1.3.1 Chondrocyte

Cartilage is a unique tissue as it contains only one cell type; the highly specialised chondrocyte surrounded by extracellular matrix (Buckwalter and Mankin, 1998; Stockwell, 1978; Stockwell, 1979b). The chondrocytes are responsible for synthesising both major structural components: type II collagen and large aggregating proteoglycans, and other less abundant non-collagenous proteins. Chondrocytes also organise the surrounding matrix into its final 3-dimensional network.

Articular chondrocytes vary in size, shape and distribution and in metabolic activity depending on their location within the tissue (Aydelotte et al., 1996). With increasing distance from the articular surface they appear not only to increase in size but also
become widely scattered in the matrix. In the superficial zone they are found singly, discoidal in shape with a diameter of 10-20µm, in the middle zone they are spherical with a diameter of 10µm or more and are found both individually and in groups. Deep zone cell sizes are more variable and are found in columns perpendicular to the surface.

Ultrastructurally, chondrocytes predominantly contain the organelles that are required for matrix synthesis and secretion e.g. rough endoplasmic reticulum, golgi apparatus transport vesicles which are more abundant in growing cartilage chondrocytes. Lysosomes function not only to remove unwanted intracellular material but also play a role in extracellular matrix turnover. Mitochondria are frequently found more abundantly in immature tissue cells compared to mature due to the chondrocytes low respiratory activity (Stockwell, 1978). The chondrocyte contains intermediate filaments, which may contribute to structural strength of the cell although it has been proposed that large masses of these could be a sign of cell degeneration (Meachim and Roy, 1967). Glycogen is a common component of the chondrocyte and possibly provides a store of raw chemical material for matrix synthesis and energy. There are large amounts of lipid within the chondrocyte, the size of the fat globules increases with maturation and fat globlets are smaller in articular cartilage compared to hyaline cartilages. Short processes/microvilli extend from the cell into the matrix functioning to sense changes in the matrix. Chondrocytes do not normally form cell-to-cell contacts but instead surround themselves with ECM. Articular chondrocytes appear hypo-active, although this is deceptive, due to their apparent unchanged location, appearance and activity over many years.

Chondrocytes primarily depend on anaerobic metabolism (energy requirements coming from glycolysis) due to the low oxygen tension within the tissue (ranging from 10% at the surface to <1% in the deep layers). This is due to the avascular nature of cartilage and the large diffusion distances. Nutrients must pass from the synovial capillaries through the synovial membrane and synovial fluid and then through extensive extracellular matrix before reaching the chondrocytes. It is remarkable that chondrocytes are able to synthesise and secrete components in this avascular environment under such low oxygen tensions. The types and concentrations of macromolecules produced by the chondrocytes and their assembly and organisation
into a highly ordered macromolecular framework provide the mechanical properties of articular cartilage. Maintenance involves continual turnover of matrix macromolecules, replacing degraded proteins by synthesising new and incorporating these into the pre-existing ECM. The cells sense changes in matrix composition due to degradation of macromolecules and mechanical compression via calcium ions, cyclic AMP and signal transduction e.g. integrins. The manufacture of both collagens and proteoglycans (see section 1.8) are dependent on the supply of precursors, which largely depends on the physiochemical state of the matrix (Maroudas, 1979).

Articular chondrocytes differ both in activity and function during skeletal development, growth and maturation. Local functional heterogeneity among chondrocytes within zones could be cyclical, transient or permanent. Some cells produce chondroitin sulphate rich rather than keratan sulphate rich proteoglycans whilst others are more active in proteoglycan synthesis as well as showing heterogeneity in degradative enzymes released. Within a growing individual, chondrocytes produce new tissue, which expands and remodels the articular surface. However, within the skeletally mature individual, macromolecules are synthesised and the tissue remodelled without significant change in tissue volume (Buckwalter and Martin, 1995).

Cartilage increases in size by both appositional and interstitial growth. Mitotic rates are important in chondrocytes residing in the growth plate to increase the length of the long bone whilst other cartilages are dependent on appositional cell proliferation in later stages of growth. Studies have demonstrated that mitotic rates in articular cartilage were 1/20th those of the growth plate- showing no growth within adult (Mankin, 1964). However, cell proliferation is resumed following cartilage damage (Dustmann et al., 1974) or if cells are isolated and grown in culture (Sokoloff, 1983). There is a sevenfold reduction in cellularity in human cartilage during development from birth to adult (Stockwell, 1967). This reduction is due to secretion and deposition of new matrix, and of cell death. The cell distribution is not uniform within adult tissue with cells lying singly or in isogeneous groups, Stockwell (1975) hypothesised that ‘the maximum cell density found in the superficial zone of articular cartilage must be attributed to proximity to synovial fluid and its nutrients and other contents’. Cell density of different adult cartilages varies considerably e.g. femoral condylar cartilage
showing a 25-fold difference between small species and larger species, like man. The same pattern is seen between small and larger joints of the same species, Thus, overall cellularity is inversely related to cartilage thickness.

1.4 STRUCTURAL MACROMOLECULES

1.4.1 Cartilage collagens
The major structural macromolecules of the articular cartilage's ECM are the collagens (50-60% of articular cartilage's organic dry weight), which function to withstand tensile and shear forces and the large aggregating proteoglycans that withstand compressive forces. During development and ageing the comparative concentrations of these macromolecules change. This change is due not only to location but to their functional requirements (For review see Royce and Steinmann, 2002).

Articular chondrocytes synthesise and secrete up to ten genetically distinct collagens, types I, II, III V, VI, IX, X, XI, XII and XIV however, twenty seven genetically distinct collagen are known to exist (Royce and Steinmann, 2002). Type II collagen is predominantly found in articular cartilage (90%), forming the basic fibrillar structure of the ECM. Type II collagen is cartilage specific, other cartilage specific collagens found in less abundance include type IX and XI (fig 1.3). These have been proposed to form hybrid molecules with type II and are important functional components of tissue stability (Duance et al., 1999) (fig 1.3A). Collagens provide high tensile strength to the cartilage matrix. In different zones of the tissue, collagen fibrils have varying diameters that are governed by the proportion of both predominant and minor collagens and, also, the content of collagen-binding molecules. The biomechanical properties that are essential for normal joint functioning are due to heterogeneous differences in ECM composition at varying depths (Mow et al., 1992).

Type II Collagen
Type II collagen is a classical fibril forming collagen (fig 1.3 A). It is synthesised as a homotrimer consisting of 3 identical polypeptide chains, known as the α chains. The α chains are synthesised procα1 chains with non-collagenous N and C propeptides. Extracellular processing by N-terminal and C-terminal proteinases then produces mature triple helical molecules that assemble into striated fibrils by a quarter
staggering of the individual molecules. The staggered arrangement facilitates the helical structure. It has has two alternatively spliced variants, type IIA and type IIB collagen (Ryan and Sandell, 1990). Type IIB collagen is associated with prechondrogenic condensations and type IIA is the most common splice variant, and is associated with mature tissues (Sandell et al., 1991). Small diameter (10-25nm) fibrils are found pericellularly, and the larger fibrils (300nm) aggregate in the territorial and interterritorial matrix (Eyre et al., 1987).

**Type IX** is a member of the ‘Fibril-Associated Collagens with Interrupted Triple helices’ (FACIT) family of collagens and is the only cartilage specific collagen (fig 1.3B). It is covalently linked with the surface of type II collagen and has been proven to modulate fibril formation by preventing close parallel alignment of collagen II fibrils (Wotton et al., 1988). Thus, type IX collagen is associated with small diameter unbanded fibres explaining its high abundance in foetal cartilage (10% decreasing to 1-5% in the adult), (Duance and Wotton, 1991). Type IX also has a function in linking type II collagen fibrils (Smith and Brandt, 1992) and bridging proteoglycans together stabilising the fibrillar network. Type IX comprises three distinct chains the α1(IX), α2(IX) and α3(IX) chains. Unlike fibrillar collagens it undergoes no processing before deposition into the ECM. The molecule consists of three collagenous domains (COL1-3) and four non-collagenous domains (NC1-4) in articular cartilage. It also possesses a large globular N-terminal domain. There are both proteoglycan and non-proteoglycan forms of the type IX collagen synthesised as discrete populations (Ayad et al., 1991) in some cases the α2(IX) chain has a chondroitin/dermatan sulphate chain attached at the NC3 domain. Type IX collagen is preferentially located around the chondrocytes in adult mammalian articular cartilages especially with increasing distance from the surface.

**Type XI collagen**, a minor fibrillar collagen representing around 3% of the collagen found in cartilage forms heterotypic fibrils with types II and IX collagens, limiting fibrillar size (fig 1.3C) (Smith et al., 1985). It is synthesised as a heterotrimeric procollagen comprising three distinct proα1 (XI), proα2(XI) and proα3(XI) chains. Type XI collagen has 5 major domains, NC1-NC3 and COL 1 and 2. High glycosylation of its triple helix creates a ‘bulkier’ molecule that alters intermolecular
Figure 1.3. Cartilage collagens. A) A heterotypic fibril consisting of types II, IX and XI collagen. B) Type IX collagen molecule. C) Type XI collagen molecule (Adapted from Duance et al. (1999)).
spacing in the fibril. Molecular packing influences its role in fibril formation as does the N-terminal domain by sterically hindering interactions of certain molecules onto its surface. Type XI collagen also interacts with proteoglycans (Vaughan-Thomas et al., 2001) and the chondrocytes themselves. Thus, it is involved directly in cell-matrix interactions.

**Type VI collagen** and type X collagen unlike the other cartilage collagens are not fibrillar and do not participate in the formation of striated fibrils. Collagen type VI is essentially a glycoprotein with a short collagenous central domain (Ayad, 1998). It is synthesised mainly as a heterotrimer comprising three genetically distinct chains α1(VI)α2(VI)α3(VI) (Ayad, 1998) Found in most connective tissues, it may interact with macromolecules such as hyaluronan, fibronectin and type II collagen and possibly cell surface receptors (McDevitt et al., 1988). In cartilage, it is found largely in the pericellular matrix (Poole et al., 1988). A possible role is that it anchors chondrocytes to the surrounding matrix and helps protect the chondrocyte by forming a lattice structure around it (Keene et al., 1988).

**Type X collagen** is a short chain collagen also synthesised as a homotrimer comprising 3 identical α1X chains. It has a short triple helix, approximately 132nm in length, a small N terminal domain and a large C terminal globular domain. Its synthesis by chondrocytes is restricted to the calcified cartilage zone hence it is often used as a marker for terminal differentiation (Schmid and Linsenmayer, 1987). It appears to have a hexagonal organisation in vivo and undergoes no ECM processing. Although its function is unknown in regards to interactions with cells and other matrix macromolecules its localisation suggests it has functional roles in endochondral ossification. One hypothesis is that it may help prevent the local collapse of cells when the matrix is removed during endochondral ossification (Schmid and Linsenmayer, 1987).

**Type I collagen** is present in porcine articular cartilage (Wardale and Duance, 1993), and human articular cartilage (Aigner et al., 1993). It is also present in repair tissue. Like collagen type II, type I collagen is a fibrillar collagen, which is reinforced with calcium hydroxyapatite within bone providing tensile strength (Ayad, 1998). It is synthesised as a heterotrimeric procollagen, which comprises two pro-α1 (I) chains
and one pro-α2 (I) chain. Type I collagen is also present at the articular surface, during development and during osteoarthritis.

**Type III** collagen is primarily associated with type I collagen throughout the body although its function is unknown it has been found to co-localise and cross-link with type II collagen (Wu et al., 1996). It is also widely distributed during development and repair processes (Young et al., 1995; Young et al., 2000).

**Collagens types XII and XIV** are FACIT collagens with homology to type IX collagen. They have been localised in foetal bovine articular cartilage in a proteoglycan form whereby they possessed an attached chondroitin sulphate chain (Watt et al., 1992). Although they do not have an affect on fibril diameter, it is possible that “these collagens may bridge adjacent fibrils or be involved in mediating matrix deformability through interactions of their NC3 domains with other tissue components” (Duance et al., 1999).

### 1.4.2 Cartilage proteoglycans

Proteoglycans are a diverse family of molecules consisting of one or more glycosaminoglycans (GAGs) side-chains covalently bound to a protein core. GAGs are unbranched polysaccharide chains consisting of repeating disaccharides that contain an amino sugar, normally either a hexuronate, hexosamine or galactose unit. Sulphate substitution occurs in various positions, which increases diversity of this already complex carbohydrate both in chemical and physical properties. Diversity is also caused by disaccharide bonding. Each disaccharide unit has a negatively charged carboxylate or sulphate group. This configuration creates a highly negatively charged molecule. These negative charges repel each other and other negatively charged molecules but attract cations e.g. water providing a swelling pressure. The swelling, however, is restricted due to its entrapment within the collagen three-dimensional network. The GAGs found within articular cartilage are hyaluronan (HA), chondroitin sulphate (CS), keratan sulphate (KS) and dermatan sulphate (DS). CS can exist as one of three isoforms-CS, C4S or C6S (Ratcliffe et al., 1984).
Proteoglycans are ubiquitously distributed amongst animal cells and are involved in a wide range of biological processes. There are two major classes of proteoglycans found within articular cartilage. Large aggregating proteoglycans; aggrecan and versican which have a high affinity for other matrix components forming stable structures with these other molecules soon after synthesis and secretion. Small proteoglycans or non-aggregating proteoglycans include decorin, biglycan, and fibromodulin that can exist independently within the matrix. A third sub-group exists in the form of cell associated proteoglycans; glypican and syndecan which are integrated into the plasma membrane. Type IX collagen is also classified as a proteoglycan due to its GAG component.

**Aggrecan** accounts for 5-10% of the cartilage dry weight (Heinegard and Oldberg, 1989) (Fig 1.4). As aggregates, aggrecan molecules may form macromolecular complexes of 300-400 x 10^6 kDa. These macro-molecular aggregates are formed by the interaction of hyaluronan and link protein. Up to 100 aggrecan molecules can bind to a single HA chain. Each aggrecan molecule has a mass of 1-4 x 10^6 Da consisting of large numbers of chondroitin sulphate and keratan sulphate chains attached to a protein core filament. The protein core has a molecular weight of ~220,000 with 3 distinct regions namely the globular 1 (G1), globular 2 (G2) and the globular 3 (G3) domain. G1 found at the N-terminus has a binding site for hyaluronan which is stabilised by link protein also interacting with both HA and G1. Between G1 and G2 lies an interglobular domain (IGD). The G2 domain contains sites for attachment of KS chains (20-50) and two regions that are enriched with CS chains (100). The G3 domain lies at the C-terminus. The heterogeneity of the proteoglycan within the cartilage is due to post-translational modifications (Hascall et al., 1999).

**Versican** is an aggregating CS proteoglycan which is found transiently in prechondrogenic condensations (Hall and Miyake, 1995). Following the formation of cartilage, versican is rapidly removed and replaced with aggrecan. Versican also has the ability to aggregate with both type I collagen and fibronectin suggesting involvement with cartilage morphogenesis as they are also major components found within prechondrogenic condensations (Kimata et al., 1986).
Figure 1.4. Schematic model of aggrecan. The determined amino terminus (residue 1) and the predicted carboxyl terminus (residue 2395) are indicated. The long lines denote locations of –serine-glycine- in the sequences each of which may contain a chondroitin sulphate chain. They also denote the limits of the chondroitin sulphate-rich domain, which was initially subdivided into two regions (CS-1 and CS-2) based upon different internal repetitive sequence motifs. The short lines indicate site and theonine sites in the keratan sulphate-rich domain each of which may contain either an O-linked oligosaccharides or a keratan sulphate chain (Taken from Hascall et al., 1999).

Small proteoglycans: These non-aggregating proteoglycan’s or small leucine-rich proteoglycans (SLRPs) are associated with most connective tissues (Heinegard and Oldberg, 1989; Waddington et al., 2003). They account for only a small percentage of the ECM within articular cartilage. Biglycan and decorin are members of a structurally related proteoglycans called the small CS/DS proteoglycans. Biglycan within cartilage has a molecular weight of approximately 100kDa, and a protein core of 38kDa with two extended glycosaminoglycans chains. DS accounts for 40-50% and the rest is CS. Decorin has a single GAG chain, which can be DS or CS. Decorin has been associated with collagen fibrils and can inhibit collagen fibrillogenesis. It also binds transforming growth factor β (TGFβ) and neutralises its activity (Yamaguchi et al., 1990). Fibromodulin belongs to the KS proteoglycan group and consists of a core protein with several attached KS groups (Plaas et al., 1990). Fibromodulin can interact with fibrillar collagens (Hedbom and Heinegard, 1989) and fibronectin (Bidanset et al., 1992).

Superficial zone protein/proteoglycan (SZP): SZP was first described by Schumacher et al., (1994) and is an approximately 345kDa proteoglycan that only chondrocytes residing in the surface zone and some synovial surface lining cells synthesise in the adult but is widely distributed during development. It has been classified as a novel proteoglycan due to the following characteristics which indicate that it is distinctly different from any known PGs: (i) it is not retained in the ECM
following synthesis (following culture procedures of explants and cells, SZP is predominantly found in the media), (ii) its molecular weight of 345 kDa is not altered substantially following removal of its constituent CS and KS GAGs (indicates that it has only small GAG chains on its core protein and is not an aggrecan metabolite) (iii) only partial degradation to a 315kDa catabolite ensues a pepsin digestion at 4 °C (due to O-linked oligosaccharides- see below) however, it is extensively degraded by trypsin (due to lysine residues), papain or pronase (iv) SZP is also present in synovial fluid suggestive that it is actually secreted there by the superficial zone chondrocytes. SZP shares sequence homology with the precursor protein for megakaryocyte stimulating factor (MSF) (Jay et al., 2001).

The articular cartilage SZP/MSF precursor (Fig 1.5) contains large (76-78 repeats) and small (6-8 repeats) mucin-like O-linked oligosaccharide-rich repeat domains flanked by cysteine-rich N- and C-terminal domains that are homologous to the somatomedin B and hemopexin domains of vitronectin, respectively. The precursor proteins contain three potential sites for N-linked oligosaccharide substitution (at Asn^{206}, Asn^{1159} and Asn^{1289}) and another site for GAG attachment to serine (..^{220}DEAGSG^{225}...). Native SZP occur as proteins with extensive post-translational modifications.

![Diagram of the structure of articular cartilage SZP](image)

**Figure 1.5.** Schematic representation of the structure of articular cartilage SZP based on biochemical analysis and primary sequence information. Functional domains are indicated by the patterned boxes, and sequences and/or sites potentially involved in carbohydrate binding or substitution are indicated. Adapted from (Adapted from Flannery et al., 1999).

Although the precise biological function of SZP is unknown, the different structural motifs within the SZP/MSF precursor molecule potentiate i) cell proliferation (N- and C-terminal vitronectin-like domains), ii) cytoprotection (vitronectin somatomedian B can bind plasminogen activator inhibitor-1 (Seiffert, 1997) and vitronectin synthesised by rheumatoid synovial cells inhibits plasmin generation (Tomasini-Johansson et al.,
1998). Together, these attributes during joint articulation would play a vital role in maintaining the integrity of the articular surface and thus, protect the deeper layers. During disease, these functional properties are compromised. Studies have also shown that interleukin-1 (IL-1), which is increased in diseased tissue inhibits SZP biosynthesis.

1.4.3 Non-collagenous cartilage matrix proteins and glycoproteins
Within normal adult articular cartilage, there are a wide variety of non-collagenous proteins and glycoproteins that have been found throughout the matrix. However, to date, their specific functions have been poorly studied (compared to collagens and proteoglycans). It is possible that they function to help in organisation and in maintenance of the macromolecular structure and cell matrix interactions (for a review see (for a review Neame et al., 1999).

Fibronectin is a widely distributed structural glycoprotein found in most tissue extracellular matrices, as well as in serum. It is a major multifunctional adhesive protein involved in cellular interactions such as migration during development and wound healing, but is also fundamental in regulating cell growth, differentiation, and haemostasis/thrombosis. Although a normal product of chondrocytes, it is also found in the synovial fluid at low concentrations (Lust and Burton-Wurster, 1992). The production of fibronectin by both chondrocytes and synoviocytes is elevated during disease states e.g. osteoarthritis (Burton-Wurster et al., 1986) and in rheumatic joints (Clemmensen et al., 1982). Fibronectin can be considered a major contributor to cartilage biology in organising a biomechanically stable matrix. Due to its modular structure and discreet domains, it has the ability to interact with many matrix components e.g. collagen, heparin GAGs, cell surface integrins and itself (Burton-Wurster et al., 1997).

Laminin
Laminin belongs to a family of large glycoproteins that are a major component of basement membranes (BMs) (Timpl and Brown, 1994). It has multi-functional roles in development, differentiation and migration. This multifunctionality is largely due to its ability to interact with cells via cell-surface receptors (integrins) and other basement membrane components (type IV collagen and heparin sulphate
proteoglycan). Laminin has been identified in articular, epiphyseal, growth plate and meniscal cartilage and suggests a role in development (Durr et al., 1993; Salter et al., 1995).

**Tenascin**

Tenascin is a large ECM glycoprotein belonging to a multi-member family which include tenasin C, X and R (Chiquet-Ehrismann, 1990; Erickson, 1993). Tenascin C in its native form consists of six subunits associated into a disulphide-bonded hexabrachion structure (fig 1.8). Each subunit has a molecular mass of 220-320 kD containing a series of structural domains homologous to other proteins Although sharing structural similarities to fibronectin, tenascin possesses anti-adhesive and anti-spreading properties. In vitro and in vivo studies revealed tenascin-C within the pericellular matrix of cartilage, thus, it is possible that it functions by interactions with receptors such as integrins and syndecans (Salmivirta et al., 1991) found on developing and mature articular chondrocytes (Salter et al., 1992). Tenascin is present transiently during embryonic development and is absent in most normal adult tissue; this is true for hyaline cartilage. However, it is expressed at low levels in normal articular cartilage (Chevalier et al., 1994) being confined to the surface zone (Salter, 1993) and perichondrium (Mackie et al., 1987). Its presence in articular cartilage and not hyaline cartilage could be related to articular cartilage’s unique load bearing function or because of the phenotypic heterogeneity of articular chondrocytes (Archer et al., 1990). Its expression is greatly increased in damaged or diseased joints, however its function is unknown (Chevalier et al., 1994; Salter, 1993). Syndecan-1 is a cell surface PG that interacts with tenascin which in turn serves as receptors for growth factors. Tenascin, therefore, has a tightly regulated pattern of expression involving morphogenetic events that include wound healing, tumourogenesis and embryonic migration.

**Cartilage oligomeric matrix protein (COMP)** is a high molecular weight acidic cartilage matrix glycoprotein (Hedbom et al., 1992) consisting of five disulphide-linked 100kDa sub-units. Although its functions are unknown it is preferentially
localised in the territorial matrix of cartilage and thus, could be important in cell adhesion via the αVβ3 integrin (Hedbom et al., 1992). Recent studies did demonstrate that zonal variations in equine COMP distribution were influenced by loading (Murray et al., 2001).

1.4.4 Interstitial tissue fluid
Within the tissue the intermolecular spaces are filled with water and dissolved electrolytes. The water content within cartilage ranges from 60 to 85%. Maroudas (1979) estimated that articular pore size range from 200 to 600nm. This size still allows the movement of large molecules, which include PGs through the ECM. The total water contained intracellularly or within the infrasartillar compartment varies depending on age and species analysed (Katz et al., 1986). Physical factors such as swelling pressure (that is dependent on fixed charge density of proteoglycan), collagen network (stiffness and strength) and material properties of the proteoglycan-collagen matrix affects the amount of water present (Guilak et al., 1994; Mow et al., 1992).

1.5 STRUCTURE OF ARTICULAR CARTILAGE-MORPHOLOGICAL HETEROGENEITY
Composition, organisation, mechanical properties of the matrix as well as cell morphology and synthetic activities vary with depth and with distance from the cell (figs 1.2 and 1.6). Tissue composition and organisation are also affected by age and pathology e.g. osteoarthritis, joint inflammation and disuse atrophy.

It is possible to identify four zones due to morphological features of the resident chondrocytes and the matrix with relative depth from the surface: the superficial zone, the middle zone, the deep zone and the zone of calcified cartilage. There are no distinct boundaries between the upper three zones with the morphological changes occurring gradually across a region. Each region varies according to developmental stage, joint and species analysed (Stockwell, 1979a).

1.5.1 Superficial zone
This is the thinnest zone, it has been postulated that its unique structure and composition, is responsible for its specialised mechanical and probable biological properties (figs 1.2 and 1.9). It consists of two layers, an acellular layer, composed, of
amphorous material (Jurvelin et al., 1996), sometimes referred to as ‘lamina splendens’ (MacConail, 1951), having fine fibrils and a small amount of polysaccharide, and a deeper cellular layer. The chondrocytes within the cellular layer are flattened and ellipsoid in shape within a largely collagenous matrix. They are arranged so that their longest axis is parallel to the articular surface. The matrix within this zone is predominantly composed of collagen with a low percentage of proteoglycans synthesised compared to the subsequent zones. The content of fibronectin and water is also greatest in this zone. Fibronectin has been observed lining serrations of the most superficial layer possibly having a role in joint lubrication and in protecting the cartilage by binding to hyaluronan which is present within synovial fluid (Clemmensen et al., 1982; Nishida et al., 1995).

The collagen fibrils have a small diameter and are arranged parallel to the joint surface. In this configuration the collagen fibrils not only provide mechanical properties to the tissue but also regulate the molecules, which may pass in and out of the cartilage. Thus, this zone has greater tensile stiffness and strength than the deeper zones that enable the tissue to resist shear forces generated during joint use.

The superficial zone also contributes to articular cartilage’s compressive behaviour, which has been shown during in vitro experiments. Its removal not only increases permeability but also the amount of load put onto the macromolecular framework in the deeper zones during loading. It was demonstrated that during experimentally induced articular cartilage degeneration the first detectable change occurred in the superficial zone with remodelling of the collagenous matrix (McDevitt and Muir, 1976; McDevitt et al., 1975). Thus, it is possible that osteoarthrosis may develop due to alterations in this zone leading to mechanical failure of the whole tissue. Shaw and Martin (1962) claimed that the superficial zone chondrocyte had few if any rough endoplasmic reticulum conversely Mankin, (1963) found that almost all the cells in the tissue incorporated $^3$H-cytidine indicating that the cells are not quiescent as previously believed.

1.5.2 Middle zone

This zone is sometimes referred to as the transitional zone, as its matrix composition and morphology is intermediate between the superficial and deep zone (figs 1.2 and
Figure 1.6. Schematic representation of adult articular cartilage showing the relationship of the chondrocytes to the fibrillar components of the matrix and the organisation of these components. The pericellular matrix compartments surrounds individual chondrocytes and varies in width; it is free of fibrillar components but contains and abundance of isotropically arranged cross-banded filaments which extend throughout all matrix compartments. The territorial matrix contains a basket-like arrangement of collagen fibrils and makes chondrones distinct morphologic entities. The interterritorial matrix form the bulk of the tissue and contains two populations of fibrils and fibril bundles. The first population consists of fibrils with a highly oriented parallel arrangement that form the arcade-like structures. The second population consists of more randomly oriented fibrils (Adapted from Buckwalter 1999).
1.8). Its volume is greater than the superficial zone. The resident chondrocytes have more synthetic organelles, endoplasmic reticulum and Golgi than those residing in the superficial zone. The cells are spheroidal in shape synthesising a matrix that has collagen fibrils with a larger diameter and higher proteoglycan content but lowered water and relative collagen content compared to the superficial zone.

1.5.3 Deep zone
The chondrocytes residing in the deep zone (radial zone) are larger, spheroidal in shape and are aligned perpendicular to the articular surface in columns. Within this zone are the collagen fibres with the largest diameter, the greatest concentration of proteoglycan and the least amount of water. The collagen bundles provide a strong anchoring system for the tissue by crossing the tidemark and inset within the calcified cartilage (Redler et al., 1975).

1.5.4 Calcified cartilage
The calcified cartilage zone separates the uncalcified cartilage from the subchondral bone (figs 1.2 and 1.9). The cells within this thin zone have a smaller volume than those of the deep zone containing little endoplasmic reticulum or Golgi, indicating a low level of metabolic activity. A prominent feature at the interface between calcified and non calcified hyaline layers of articular cartilage is the articular tidemark (Bullough and Jagannath, 1983; Green et al., 1970). The tidemark behaves as a mineralization front realising the steady creeping advancement of the calcified layer to the articular surface (Havelka and Horn, 1999). At the light microscope level it is observed as a thin band (5-10μm) that is basophilic in haematoxylin-eosin stained sections.

1.5.5 MATRIX REGIONS
There are three regions/compartment with variations in matrix organisation within each zone: the pericellular, territorial and interterritorial matrix regions (fig 1.8). The pericellular and territorial regions provide a protective and an interactive role to the chondrocyte. Protective, in that it prevents damage occurring to the cells during deformation and loading of the tissue. Interactive, as it binds the cell’s membrane to matrix molecules, cell-matrix interactions. Mechanical signals may be transmitted to
the chondrocytes during deformation. The interterritorial matrix provides mechanical properties to the tissue.

1.5.5.1 Pericellular matrix

A pericellular matrix compartment surrounds each chondrocyte surface. The cell’s membrane is attached to the thin rim of pericellular matrix. The compartment varies in width. Although rich in proteoglycan, non-collagenous matrix proteins and the non-fibrillar collagen type VI, it is free of fibrillar components. The chondron is the chondrocyte with its immediate pericellular matrix bounded by a “felt-like” capsule of fine fibrous material. Poole et al. (1988) using porcine articular cartilage and rat chondrosarcoma revealed strong type IX collagen labelling in the capsule of chondrons. It was also demonstrated concentric arrangement of capsules surrounding both individual and clusters of chondrocytes. It was hypothesised that chondrons protect the chondrocytes from excessive loads and their development correlates with mechanical load that the cartilage is subjected to (Poole et al., 1988). Type IX collagen is preferentially located in the pericellular region of the chondrocyte in the adult articular cartilage whereas, in the foetal calf cartilage, it was distributed throughout the matrix (Irwin et al., 1985). Distribution therefore appears to be age related. Ultrastructural and immuno-electron microscopy studies (Duance et al., 1982; Wotton et al., 1988) confirmed that the pericellular matrix is composed of a fine meshwork of fibres compared to the inter-territorial matrix, which contains predominantly large fibre bundles.

1.5.5.2 Territorial Matrix

Territorial matrix can be seen to either envelope individual chondrocytes or their pericellular matrix or enveloping pairs and sometimes chondrocytic clusters such as the columns found within the deep zone. Thin collagen fibres of the territorial matrix which are juxtaposed to the chondrocytes adhere themselves to the pericellular matrix. With distance from the cell, they form ‘basket like’ structures by decussating their fibrils at various angles. It has been postulated that this collagenous basket “may provide mechanical protection for the chondrocytes during loading and deformation of the tissue” (Buckwalter, 1999). The boundary between the territorial and interterritorial matrices is marked by the collagen fibrils increasing in diameter and conforming to a parallel arrangement.
1.5.5.3 **Interritorial Matrix**

Within adult articular cartilage the interterritorial matrix makes up most of the volume. Within this compartment are found the largest diameter collagen fibrils. There are two populations of fibrils and fibril bundles, which are either arranged parallel to the surface that form arcade-like structures or those that are randomly oriented. The orientation is dependent on the distance from the articular surface. In the superficial zone fibrils are smaller in diameter and lie parallel to the joint surface, in the middle zone collagen fibrils lie obliquely and in the deep zone fibrils align themselves perpendicularly to the articular surface. This structural organisation was first described by Benninghoff (1925) and is known as ‘Benninghoff arcades’.

**1.6 BIOCHEMICAL HETEROGENEITY WITHIN ARTICULAR CARTILAGE**

The superficial zone contains the highest relative concentration of collagen, decreasing by 15% from the superficial to the deep zone (Muir et al., 1970) however the deep zone possesses the fibrils with the greatest diameter (Poole et al., 1984). Wardale and Duance (1993) in their study on porcine articular cartilage revealed immunohistochemically that types I and VI collagen differentially localised in the surface zone and that labelling for type IX were greater with distance from the articular surface. Type X collagen is found in the calcified cartilage zone (Schmid and Linsenmayer, 1987) and is temporarily expressed in the surface during development (Morrison et al., 1996).

However, in contrast, it has been shown that the glycosaminoglycans content of cartilage increases with depth by 50% (Maroudas, 1979). Ratcliffe et al. (1984) showed that proteoglycan content is highest in the middle zone and lowest in the superficial zone. Aggrecan is found throughout the cartilage but its highest concentration is in the middle zone and preferentially distributed pericellularly. Aggrecan aggregates are smaller at the surface compared with deeper parts of the tissue. Poole et al. (1992) also showed differential distribution for link protein. Although, primarily found within the superficial zone, decorin is also found in the pericellular regions of the middle and deep zones during articular cartilage development. CS and KS are found heterogeneously throughout the tissue. The concentration of CS are highest in the middle zone while the concentration of KS
increases with distance from the surface (Bayliss et al., 1983; Zanetti et al., 1985), however, these distributions vary with maturity.

1.7 CARTILAGE MATRIX TURNOVER

Synthetic and degradative events, that maintain the tissue, include synthesis of matrix components, incorporation and organisation of these components into the matrix and degradation and loss of components from the matrix. These events are orchestrated solely by the chondrocytes, and throughout life, cartilage matrix is kept balanced.

1.7.1 Proteoglycan synthesis

The synthesis of aggrecan (and link protein) involves the transcription of mRNA from a specific gene followed by translation of the specific mRNA generating a protein core to which GAG chains are substituted post-translationally. The molecules (monomeric form) are then released into the ECM pericellular matrix. HA is synthesised separately at the plasma membrane. Aggrecan aggregates only form in the ECM, whereby aggrecan and HA come together and are stabilised by the link protein. Hardingham and Fosang (1992) showed that the ratio of aggrecan and link protein is 1:1. They are all secreted independently from the cell, on secretion the G1 domain of the proteoglycan is not functional (Bayliss and Roughley, 1985). Thus, allowing the proteoglycans to move away from the cell before forming aggregates - the new proteoglycan will now be able to interact/reside within the territorial and interterritorial matrix. This phenomenon is known as ‘delayed aggregation’.

1.7.2 Proteoglycan catabolism

Key enzymes involved in the breakdown of cartilage ECM and synthesised by the chondrocytes themselves are the proteinases (endopeptidasases). Both exopeptidasases and glycosidases play secondary roles. The 4 major classes of endopeptidasases are metallo, serine, cysteine and aspartate (Barrett et al., 1998).

Proteoglycans are continuously broken down and released from the matrix. Proteoglycan catabolism has been studied using aggrecan. Ratcliffe et al., (1988) demonstrated that the major proteolytic cleavage site of enzymes (released by the chondrocytes) on aggrecan is found between its G1 and G2 domain (interglobular domain) and at amino acids 373-374 by the action of the metalloproteinase(s)
"aggrecanase-1 and -2" (Sandy et al., 1991). The consequence of this cleavage is to separate the G1 domain (which is essential for aggregation) from the region containing GAGs. The large non-aggregating fragment then proceeds to pass through the matrix where it is lost to the synovial fluid. G1 domain and link protein undergo further degradation and then are released. Common cleavages are seen at amino acids 341 and 342 by many of the metalloproteinases (MMPs) and by cathepsin B (Fosang et al., 1992; Mort, 1998).

Aggrecanases-1 and aggrecanases-2 are both cartilage aggrecanases and members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) gene family (Tang, 2001) which are now currently referred to as ADAMTS-4 and ADAMTS-5, respectively. It is believed that aggrecanases play a key role in the early stages of rheumatoid arthritis and in osteoarthritis (for review see Arner, 2002; Nagase and Kashiwagi, 2003).

Tissue inhibitors of metalloproteinases (TIMP) are also produced locally by the chondrocytes, which inhibit the MMP activity. It is the molar ratio of TIMP that determines whether degradation occurs (for review see Cawston, 1998).

The loss of ECM macromolecules from the cartilage results in serious impairment of joint function and by analysing the synovial fluid and its content of proteoglycan fragments, the rates of cartilage degradation in cartilage disease can be determined (Sandy et al., 1992).

1.7.3 Collagen synthesis

To date there are 27 distinct collagens with complex gene organisation which are complexes of 3 polypeptides. Although type II and X are derived from a single gene, type VI, IX and XI collagen are formed from gene products of 3 different genes. Like other secreted glycoproteins, collagens are synthesised by transcription of specific mRNA's followed by translation to α chains that contain signal peptides, collagenous domains and the non-collagenous domains at the N-C terminal ends. Within the endoplasmic reticulum, the signal peptide is removed. Following peptide formation proline and lysine are hydroxylated. Triple helix formation starts with disulfide bonds forming at the carboxyl terminal – the procollagen molecules pass into the Golgi
complex where carbohydrates are added. Collagen molecules are secreted into the matrix in their proforms, followed by controlled production of factors that trim non-helical regions (propeptidases) producing collagen molecules which then aggregate into collagen fibrils and fibril bundles. An orderly event ensures the incorporation of collagen into the matrix. The resultant tropocollagen molecules organise themselves into quarter-staggered arrays to form fibrils. During secretion collagen microfibrils are modified enzymatically to allow for intrafibrillar crosslinks. The aggregation of collagen fibrils is known as fibrillogenesis. The mechanism, which specifically controls fibril formation and the huge array of variation seen within this unique tissue, is unknown. However, large and small proteoglycans and other minor collagens (IX and XI) are thought to be involved.

1.7.4 Collagen catabolism

Collagens have a higher resistance to degradation than the proteoglycans due to their tightly apposed fibrils, crosslinks and their interactions with glycoproteins. Type II collagen is broken down initially by the metalloproteinases collagenase 1 and 3 (MMP1 and MMP13) by cleaving the fibrillar structure approximately 3/4 of the way along from the N-terminus. Proteolytic enzymes e.g. gelatinase A (MMP2) and stromelysin 1 (MMP3) (which also cleaves type IX) then further degrade the remaining the molecule (Wu et al., 1991a).

1.8 CHONDROCYTE-MATRIX INTERACTIONS

It was originally believed that mature chondrocytes were biologically senescent due to their isolation from the physiological environment. This belief of course is not the case as chondrocytes are active and respond to environmental changes that may occur including physiochemical events, stress, strain, flow velocities, osmotic, hydraulic pressure and electric currents/potentials. Chemical factors to which the cells may respond include interleukins, growth factors and pharmaceutical agents. Chondrocytes are also affected by the matrix composition.

Structural organisation of articular cartilage ensures that although protected from joint loading the cells can respond to their mechanical environment due to the buffering effect of the pericellular matrix. Studies have demonstrated that cyclic loading induces matrix synthesis while static loading causes matrix degradation (Quinn et al., 1998).
The mechanism by which chondrocytes receive extracellular signalling is unclear. It is possible that metabolic activity could be regulated due to shape changes- as compression would involve volume as well as shape changes thus indicating the involvement of mechanoreceptors. Such mechanoreceptors could come in the form of integrins (Ingber, 1991) and stretch activated ion channels (Sachs, 1991). Various forms of integrins are expressed by chondrocytes. It is possible that the cytoskeleton transmits extracellular deformation to intracellular organelles inducing changes in gene expression-Tensegrity (see below).

The chondrocytes organise the pericellular matrix by the binding of hyaluronan to their cell surface via the receptor CD44 (Knudson and Knudson, 1991). Again this receptor with its close association with the actin cytoskeleton may transmit mechanical information into the cell.

Growth factors and cytokines are a major influence on chondrocyte activity. Interleukin-1 (IL-1) has the ability to accelerate proteoglycan degradation, however, at a lesser concentration can decrease proteoglycan synthesis (Benton and Tyler, 1988). Growth factors regulate chondrocyte activity, playing a major role during development. They function by binding specific receptors on the chondrocyte surface and activate intracellular messenger pathways. The resultant response is dependent on the overall concentration of the growth factor and the number of receptors on the cell surface. Examples include basic fibroblast growth factor (bFGF), which acts as a powerful mitogen. Insulin-like growth factor (IGF), which stimulates DNA synthesis and matrix production (important in adult tissue by maintaining matrix stability). Transforming growth factor beta (TGFβ) initiates cartilage and bone formation by the periosteum. It also potentiates the induction of DNA synthesis by other growth factors, stimulates proteoglycan synthesis and suppresses type II collagen synthesis as well as influencing the synthesis of TIMP. Luyten et al., (1992) showed that bone morphogenetic proteins (BMPs) have the ability to stimulate proteoglycan synthesis and decrease proteoglycan breakdown.
1.9 RECEPTORS INVOLVED IN CHONDROCYTE-MATRIX INTERACTION

Integrins are transmembrane heterodimeric glycoprotein receptors formed by noncovalent bonds between \( \alpha \) (120-180kDa) and \( \beta \) (90-110kDa) subunits, which behave as cell surface receptors when bound to specific ECM macromolecules (Hynes, 1992). At present there are 8\( \beta \) and 15\( \alpha \) subunits characterised all representing distinct gene products. Only a limited number of combinations have been identified _in vivo_, to date approximately 23 (Ivaska et al., 1998) although the potential for other combinations is far greater.

Integrins have a wide tissue distribution and as their name implies are capable of providing the cells with information on cell-cell interaction and cell-matrix interactions. They are also essential for adhesion/anchorage and thus, important for signal transduction, controlling differentiation and regulation of function (Hynes, 1992). Hirsch et al. (1996) showed by immunohistochemistry that chick chondrocytes form cell matrix attachment complexes (CMAX) that include the integrin molecules (\( \alpha 2\beta 1 \), \( \alpha 5\beta 1 \), \( \alpha 3\beta 1 \)) and cytoskeletal proteins (f-actin, vinculin, focal adhesion kinase, actinin, zyxin and paxillin). Integrins recognise and bind their ligands by a specialised cell attachment site often having the tripeptide sequence, Arg-Gly-Asp (RGD) (Pierschbacher and Ruoslahti, 1984). Using fragments taken from fibronectin containing the RGD sequence they showed that these fragments promoted cell attachment while synthetic RGD peptides would inhibit this.

A number of studies have confirmed the presence of integrins in cartilage. However very little has been published concerning chondrocyte matrix receptors in general and the actual integrin types present have been rather contradictory. Enomoto et al.(1993) have found that isolated chick chondrocytes express, \( \beta 1 \), \( \alpha 2 \), \( \alpha 3 \), \( \alpha 5 \), Durr et al (1993) found that human foetal chondrocyte expresses \( \beta 1 \), \( \alpha V \), \( \alpha 5 \), \( \alpha 1 \), \( \alpha 2 \), \( \alpha 6 \). Salter et al., (1995) confirmed Durr's work by showing that foetal chondrocytes express a different integrin profile from that seen in previous adult studies, in particular the expression of \( \alpha 6 \) subunit. This disparity indicates a difference both in species and age of the tissue studied. He demonstrated that adult articular chondrocytes express \( \beta 1 \), \( \alpha 5 \), \( \alpha V \) strongly, \( \alpha 1 \) and \( \alpha 3 \) weakly and do not express \( \alpha 2 \) or \( \alpha 6 \) (Salter et al.,
1992). These contradictory results possibly depend on detection techniques, whether enzymatic digestion was carried out allowing epitope unmasking during immunofluorescence studies on cartilage sections. Integrins are localised to the cell surface, hidden by the pericellular matrix, thus, epitope access is limited as most integrin antibodies recognise the extracellular domain.

Durr et al., (1993) demonstrated that α2 was not expressed in intact cartilage but was found in cultured foetal chondrocytes suggesting that α2β1 integrin may be involved in a 'repair response' mediating chondrocyte-pericellular collagen matrix interactions during reconstruction of a new pericellular matrix in vitro but was not involved in chondrocyte-collagen interaction in intact cartilage (Cancedda et al., 1995). Salter et al (1995) in their study also showed that foetal chondrocytes in anatomically distinct cartilages around the knee vary in their expression of integrin molecules. Thus, altered cell-matrix interactions mediated by these molecules may be involved in chondrocyte function and differentiation.

Another class of potential matrix binding proteins are the annexins. Annexins are a group of proteins encoded by 94 unique genes, with a diverse range of functions which includes anti-inflammatory by inhibition of phospholipase A2, coagulation of blood, signal transduction, calcium ion channelling and cell-matrix interaction (Mollenhauer, 1997). It has been hypothesised that they may play a role in cartilage metabolism. In chondrocyte three types have been identified annexin II, V (anchorin CII) and VI (Mollenhauer and von der Mark, 1983). This research group first identified annexin within chick cartilage as a type II collagen-binding protein. Immunohistochemically, it has been identified at the cell surface (Mollenhauer et al., 1984) and in the matrix vesicles of growth plate cartilage (Wu et al., 1991b) where it may contribute to calcification. Recent immunohistochemistry studies revealed annexin V within human cartilage, preferentially within the superficial and upper zone chondrocytes (Mollenhauer et al., 1999). Flow cytometric analysis of bovine articular chondrocytes revealed that the percentage of superficial zone cells labelling positive was 2-fold greater than those in the deeper zones, with anti-α5 and anti-β1 showing a similar trend although not significantly so (Dowthwaite et al., 2004; Reid et al., 2000). Thus, both experiments show that the results correlate with metabolic variations between the layers. Cell attachment, collagen binding and receptor analysis on bovine
articular chondrocytes revealed that annexin V is the quantitatively predominant type-II collagen receptor and may also have a role in regulating chondrocyte metabolism (Reid et al., 2000).

CD44 is expressed by chondrocytes and binds hyaluronan to the surface directing assembly of the chondrocyte pericellular matrix and possibly plays a role in hyaluronan internalisation (Knudson, 1993). CD44 is an integral membrane protein, found in a number of molecular weight isoforms that differ in their degree of glycosylation and which can contain CS chains (Lesley et al., 1993). It is present within intact cartilage (Knudson et al., 1995) and in isolated chondrocytes. It interacts with the cytoskeleton via ankyrin as well as members of the ezin, radixin and moesin family (Tsukita et al., 1994). It may transduce signals to the cell in a similar fashion as integrins.

The majority of chondrocytes express β1 and α5 integrins α5β1 is a classical fibronectin receptor, suggesting interactions with fibronectin are important and affect the metabolism of the cells in vivo (Enomoto-Iwamoto et al., 1997). Despite cartilage containing a high proportion of collagen, there is heterogeneity of immunoreactivity for α1 and α2, the major collagen receptor subunits. The pericellular environment expresses a number of adhesion glycoproteins and thus, separates the cell from the bulk of hyaline cartilage (Poole, 1992). It is possible that fibronectin functions as a bridge between the chondrocyte and the collagenous matrix in the territorial matrix, and that, indeed, annexin V and α2β1 are the predominant collagen receptors. Localisation of α1 and α2 could be involved in chondrocyte-tenascin interactions (Sriramarao et al., 1993). However, the presence of the subunits does not ensure that they are functional. Loeser (1993) demonstrated that although appropriate receptors were expressed in freshly extracted adult articular chondrocytes they showed poor adherence to a variety of adhesion proteins including collagen and fibronectin. However following a period in monolayer culture the mature chondrocytes adhered, indicating that these chondrocytes express low-affinity receptors that are active in culture conditions. Therefore, high affinity receptors may be expressed by chondrocytes in vivo only when the appropriate stimuli are present.
Chondrocytes show heterogeneity in their expression of integrin and other cell adhesion molecules depending on the source and at which developmental stage studied both in vivo and in vitro. It will be important to understand how these molecules are regulated both in expression and in activity when adjacent ECM components (ligands) are altered and thus, influence chondrocyte metabolism during development, trauma and disease processes.

1.10 CARTILAGE DEVELOPMENT

Within the embryo, chondrocytes are either mesodermal in origin and contribute towards the elements of the limb or are derived from the neural crest which gives rise to the skeleton of the face. In higher vertebrates, secondary chondrocytes from the ‘periosteal’ layer, that surrounds membranous bone, form cartilage in the head and clavicular regions in response to mechanical stimulation. Cartilage develops from chondroprogenitor cells within the limb bud, which condense to form a cartilaginous model of bone. The orchestrated events that produces such a unique tissue involves proliferation and commitment to the chondrocyte lineage (chondrogenesis) followed by acquisition of the cartilage phenotype. These synchronised events are controlled genomically, by the local environment and by the cell itself. Although the precise mechanisms involved during condensation are uncertain, it is known that the cell adhesion molecules N-CAM and N-cadherin have important roles (reviewed by DeLise et al., 2000). An important transcription factor during condensation is Sox9 as it precedes the expression of the alternatively spliced isoform of collagen II – type IIa collagen (Akiyama et al., 2002). Endochondral ossification is the process by which a cartilage anlagen of the ‘to be’ bone is replaced in the embryo. This process involves cell proliferation followed by matrix production, chondrocyte hypertrophy (terminally differentiated state) and finally death or transdifferentiation to osteoblast-like-cells – converting surrounding matrix from cartilage to bone. There are many similarities between resident chondrocytes of articular cartilage, permanent cartilage and the growth plate including metabolism and markers such as collagens. However, there are important biochemical and physiological differences due to their local environment. The fate choice made by the chondrocytes varies depending on their origin and location, what determines this has yet to be elucidated.
1.11 DEVELOPMENT OF THE SYNOVIAL JOINT

The joint develops from the skeletal blastema - a primitive avascular, densely packed cellular mesenchyme (Bernays, 1878; Mitrovic, 1978). In the middle of the blastema, cartilage nodules appear and become surrounded by perichondrium. The growth of the cartilage anlagen is due to cell division and apposition of the ECM and the chondroblastic proliferation of the inner chondrogenic layer of the perichondrium. A narrow band of densely packed cellular blastema delineates the future site of joint formation – the interzone. It is, therefore, in between the cartilaginous nodules that the interzone differentiates into three distinct layers: two chondrogenic layers that are perichondrium covering the cartilaginous articulating surfaces, and an intermediate layer that is composed of loose cellular tissue. Before the onset of cavitation it has been observed that blood vessels appear at the periphery of the joint in the capsulosynovial blastemal mesenchyme. Joint cavitation is a complex process and, thus, many theories exist on how it is achieved including programmed cell death in the interzone (Mitrovic, 1971), vascular invasion followed by enzymatic degradation of matrix of the interzone (Mitrovic, 1974), mechanical disruption of the interzone cells by muscle tone (Drachman and Sokoloff, 1966) and forces due to surrounding tissues having varying growth rates. More recently it has been suggested that biochemical changes in the matrix itself may play a role as a hyaluronan rich matrix replaces a collagen rich matrix (Craig et al., 1990; Edwards et al., 1994; Pitsillides et al., 1995). Cavitation is then observed to start moving laterally toward the centre of the joint.

The mechanisms involved both in specifying a joint area and in spacing are not understood. Growth differentiation factors 5, 6 and 7 (GDF 5/6/7) represent a distinct subgroup within the BMP family of secreted signalling molecules. GDF5 a member of the TGF-β superfamily, is specifically expressed in developing joints in transverse stripes across developing skeletal elements and is the earliest known marker of joint formation during embryonic development. A null or loss of function mutations in this gene disrupts the formation of some bones and joints in the limb, skull and axial skeleton both in mice and in humans (Polinkovsky et al., 1997; Storm and Kingsley, 1999). Ectopic application of GDF5 inhibited chondrogenesis (Francis-West et al., 1999) and, therefore, functions downstream of joint specification. The gene suggested to have an inductive role in joint formation is the gene encoding Wnt-14, a secreted molecule belonging to Wnt family (for review see Sanz-Ezquerro and Tickle, 2003).
Wnt-14 is expressed at the sites of joint formation (Hartmann and Tabin, 2001) and when overexpressed is able to inhibit chondrogenesis and induce changes. Morphological changes include producing gaps in chondrogenic condensation and at the molecular level, activation of joint markers characteristic of joint formation. It was observed that ectopic joint like regions are able to suppress formation of adjacent endogenous joints. This has led to the proposal that an auto-inhibition process could account for spacing of joints. Therefore, once a joint has been specified, above a certain threshold a secreted product would inhibit the induction of a new interzone in the area. As the condensation grows, the negative influence would be lost and this positive signal for joint induction would proceed, thus, forming a new joint. BMPs have a positive influence in interdigital areas and the BMP inhibitor chordin or noggin have a negative influence (Zou et al., 1997).

1.12 DEVELOPMENT OF ARTICULAR CARTILAGE

The development of articular cartilage has not been widely studied nor has the ability of residing chondrocytes to resist entering the endochondral pathway and remain at each extremity of a developing long bone been examined (Pacifici, 1995; Pacifici et al., 1999). It is possible that this is achieved by intrinsic mechanisms, external influence (structures, factors or neighbouring cell types) or a combination of both.

Tenascin-C is likely to be involved with the mechanism that separates the epiphyseal ends coinciding with morphogenetic events during the establishment of physical boundaries (fig 1.7). Although initially present in presumptive periosteum surrounding the diaphysis of 6.5-day-old chick embryo metacarpal element, tenascin-C transcript levels were low within the incipient epiphyses. However, syndecan-3, a PG associated with cell surface that possibly functions to regulate chondrocyte proliferation (Shimazu et al., 1996), was present. By day 10 of embryogenesis, there is a gradual increase of tenascin-C at the epiphyses with levels falling at the diaphysis. Tenascin levels were found to be maximal in the epiphyses during separation of the skeletal elements by the interzone mesenchyme creating a non-adhesive 'barrier'. Mesenchymal cells in contact with most-epiphyseal chondrocytes and the potential articular chondrocytes showed strong tenascin-C expression. Although lacking in the growth plate and the rest of the cartilaginous element, tenascin-C was present in the joint capsule and other joint associated structures. At day 18, the definitive shape of
Figure 1.7. Schematic representation of the changes in distribution of proliferating chondrocytes (black), syndecan-3 (blue) and tenascin-C (red) in skeletal elements at early and late stages of embryogenesis. (A) At early stages, there is a near coincidence between proliferating chondrocytes (which occupy the entire epiphysis and extend into the underlying metaphysis) and the gene expression patterns of syndecan-3 and tenascin-C. Note that at these stages tenascin-C gene expression is particularly strong at the most epiphyseal border. (B) At late stages, however, the proliferating chondrocytes from the top proliferating zone of the growth plate, which extends from the metaphysis toward the diaphysis (dia); only syndecan-3 gene expression characterises proliferating chondrocytes while tenascin-C gene expression is restricted to, and extremely abundant in, the entire population of epiphyseal articular chondrocytes (Adapted from Buckwalter 1999).
the long bone has been established and the articular chondrocytes with their round phenotype and low mitotic activity can be distinguished from non-cartilaginous tissue and the flat proliferating cells of the growth plate. Although undetectable in growth plate chondrocytes, there was an abundant expression of tenascin-C in the articular cartilage matrix precisely delineating the boundary between the two structures. Tenascin-C, therefore, functions by maintaining articular chondrocytes in their stable round configuration due to its ability to prevent attachment and spreading. Thus, allowing retention of the normal phenotype throughout postnatal life (Pacifci et al., 1999).

Growth mechanisms of articular cartilage and the generation of its structure - the shift from an immature isotropic structure to a highly anisotropic structure in mature tissue remain to be elucidated. Temporo-spatial patterns of matrix components during growth have been studied (Archer et al., 1996; Morrison et al., 1996). Cartilage thickness and cellular metabolism vary between species, between individual joints and between areas of differing loads. Stereological analysis has revealed that when articular cartilage matures tissue thickness and cell density decreases (for review see Stockwell, 1979a). Studies on the rabbit femoral trochlear groove, revealed a decrease in both cell and tissue thickness between 6 and 8 weeks, however, the biggest decline was in the surface zone (Oreja et al., 1995).

Studies by Mankin (1962) concluded that articular cartilage grew by a combination of appositional and interstitial growth. Chondrocyte proliferation was studied using tritiated thymidine. Experiments using an immature rabbit knee revealed 2 bands of proliferative activity, the first was beneath the articular surface and the second detected above the resident hypertrophic chondrocytes. Recent studies (Archer, 1994) using the marsupial Monodelphis domestica an animal model which exploits the early developmental stages at which the animals are born (Archer et al., 1996), confirms the hypothesis that articular cartilage growth is appositional from the articular surface in a mechanism not dissimilar to the epiphyseal growth plate. The study used antibodies to IGF I and II, TGFβ1, 2 and 3 and proliferating cell nuclear antigen (PCNA), (proliferating chondrocytes). Spatio-temporal patterns were established which revealed that prior to secondary centre of ossification formation, all factors were distributed throughout the cartilage elements. However, following ossification, the
factors were localised in the presumptive articular cartilage and in the upper cell layers of the tissues two months before skeletal maturity. Appositional growth would require the existence of an articular cartilage progenitor or stem cell to reside at the articular surface and the resorption of tissue at the base via endochondral ossification forming the subchondral plate (fig 1.8). Studies by Hayes et al. (2001) confirmed such an assumption of appositional growth and the establishment of zones, by the abolishment of the transitional layer after the treatment of the joint with bromodeoxyuridine (BrDU) which blocks chondrocyte proliferation. Histological analysis revealed that the cartilage comprised a superficial and basal hypertrophic layer only, producing thinning of the articular cartilage. Itayem et al. (1999) showed that an application of TGFβ into rat knees increased the number of chondrocytes in the intermediate zones of the patellae, therefore, it is possible that TGFβ is associated with the non-terminally proliferated state of articular chondrocytes (Hayes et al., 2001). The expression of many growth factors at the articular surface is also suggestive that this region represents an important centre (Archer, 1994; Hayes et al., 2001).

Notch receptor family members have been shown to be expressed in the superficial zone of bovine articular cartilage (Dowthwaite et al., 2003; Dowthwaite et al., 2004). The regulation of many asymmetric developmental cell fate decisions are carried out by the Notch family of cell signalling molecules. This highly conserved family can either have an inductive function as observed during the specification of arterial endothelial cells (Lawson et al., 2001) or inhibitory during neurone specification within Drosophila (Artavanis-Tsakonas et al., 1995). Within cartilage, Delta-Notch 2 signalling pathway regulates the prehypertrophic chondrocytes to hypertrophic chondrocytes during chick growth plate elongation (Crowe et al., 1999). A subpopulation of chondrocytes derived from the surface zone specifically expresses Notch-1. These Notch 1 positive cells may contain a chondrocyte progenitor subpopulation (Archer et al., 2002; Dowthwaite et al., 2003; Dowthwaite et al., 2004). Recently the distribution of Notch receptors (1,2 and 4) and their ligands (delta and jagged) during articular cartilage development have been characterised (Hayes et al., 2003). The results highlighted the complex Notch signalling interactions that result in the formation of the heterogeneous articular cartilage and allow for the co-ordinated
Figure 1.8. Diagram summarising hypothetical cell lineage of articular cartilage. Progenitor cells in the articular surface divide to give 2 daughter cells, one being another progenitor cell and the second being a transit amplifying unit cell within the transitional zone. The transit amplifying cell can undergo further cell divisions along the chondrocyte differentiation pathway. Not that the maturing chondrocytes do not migrate through the matrix. Rather, as the articular cartilage grows through apposition, the relative position of the original transit amplifying cells moves relative to the original progenitor cells which remain at the articular surface. (Adapted from Hayes et al., 2001)
ossification and elongation of the growth plate. Notch-1 is expressed by murine chondrocytes at the articular surface before birth, expression becomes restricted to deeper layers after birth. It has been proposed that Notch-1 may either function to determine differentiation along the chondrocyte pathway or maintain cells in a proliferative state. Recent studies have also demonstrated that blocking Notch-1 signalling disabled the ability of these cells to form chondrocyte colonies (Dowthwaite et al., 2004), suggestive that Notch-1 signalling functions to determine proliferation rather than differentiation of surface zone chondrocytes. Notch signalling within cartilage require activation by cell surface ligands, however, there is little cell-cell contact and, therefore, Notch signalling must be activated by soluble ligands however the precise mechanisms have yet to be elucidated.

It was postulated by Tonna (1961) that cell proliferation at the marginal transitional zone contributes to the growth of the articular surface. Still to be explained is the incorporation of tritiated thymidine by cells above the subchondral plate as cell proliferation halts at skeletal maturity (Makin, 1962; Makin, 1963a; Makin, 1963b). Makin (1963b) postulated that mitosis cannot be found in adult cartilage due to infrequent cell division, making the phenomenon virtually impossible to observe.

1.13 DEGRADATION OF ARTICULAR CARTILAGE

The quality of articular cartilage can be affected following traumatic injury or by a number of diseases including rheumatoid arthritis and osteoarthritic degeneration. Cartilage composition, microstructure, metabolic and material properties are all affected and thus, function is compromised. Articular cartilage has a limited ability to repair beyond normal maintenance (metabolic synthesis and replacement) and, thus, tissue degeneration ensues. This situation was first reported centuries ago - “an ulcerated cartilage is universally allowed to be a very troublesome disease and then when destroyed, it is never recovered” (Hunter, 1743). Over the last fifty years investigators have attempted to repair these defects to no avail. The articular cartilage shows potential to repair itself during osteoarthritis, however, the repair matrix is functionally unstable leading to degeneration. This has been postulated to be due to morphogenesis (Archer, 1994). However, to date the mechanisms which give rise to the characteristic structural and biochemical heterogeneity of articular cartilage are unknown. The ability of the homogeneous rounded chondrocyte of the embryo to
develop into the unique structure is due to both intrinsic factors (cell shape and matrix organisation) and extrinsic factors (mechanical influences). However, the formation of Benninghoff arcades (Benninghoff, 1925), the collagen leaf structure as well as the chondron, which are clearly important in tissue function, remains unknown.

1.13.1 Osteoarthritis:

Osteoarthritis (OA) or degenerative joint disease can be defined as a "progressive loss of articular cartilage that begins with fraying or fibrillation of the articular surface and progresses to exposure of the subchondral bone: attempted repair of articular cartilage, remodelling of subchondral bone, with osteophyte formation accompanying the articular cartilage degeneration" (Buckwalter, 1999). This in short leads to joint failure, affecting the individual and is also costly to society.

Although osteoarthritis can occur in any diarthrodial joint it commonly occurs within knees or hips. The aetiology of the disease is difficult to evaluate and cannot be pinpointed to one factor, it is possible that sex, age, diet, environment, physical trauma or in fact genetic predisposition of a patient may contribute to the initiation/onset of OA. OA can be classified as 'primary OA' where the cause is not known or 'secondary OA' which occurs as a result of injury, infection or physiological disorder (Freeman and Meachim, 1979).

Buckwalter and Lane (1997) in their most recent review have simplified the progression of OA into three overlapping stages. The first is the disruption or alteration of the cartilage matrix due to trauma, metabolic changes or increased degradation of the ECM due to a response to inflammation. Studies using animal models of OA (Guilak et al., 1994; Pond and Nuki, 1973) and the increasing popularity of using monoclonal antibodies to various epitopes have shown that initially the macromolecular framework is disrupted at the molecular level with decreasing PG aggregates, aggrecan macromolecules and GAG chain length followed by an increase in tissue hydration due to increased permeability. Although the type II collagen concentration remains constant, there are alterations in minor collagen-collagen fibril relationship. The second stage is the response of chondrocytes to disruption or alteration of the matrix which may be due to alterations in osmolarity, tissue damage. The response by the chondrocytes is to increase synthesis and
degradation of the matrix resulting in proliferation in an attempt to restore and stabilise the tissue. Histological analysis reveals that OA tissue characteristically contains cluster of proliferating cells in the superficial and transitional zones that are surrounded by newly synthesised matrix (Telhag, 1972; Telhag, 1973). In response to mechanical and chemical stimuli it has been shown that chondrocytes produce nitric oxide (Amin et al., 1995), which diffuses through the matrix inducing the production of IL-1. This cytokine stimulates the expression of metalloproteases (Lohmander et al., 1993) that degrade the ECM molecules. Although enzymatic degradation is detrimental in digesting intact molecules (breaking cell-matrix interactions in the process) in addition to damage they also release anabolic cytokines, which are stored in the matrix, bound to small proteoglycans e.g. decorin which can influence proliferation. Therefore, in an attempt to repair, the chondrocytes synthesise altered populations of proteoglycans and matrix adhesive proteins including excess amounts of fibronectin which is detected in the synovial fluid. Fibronectin fragments induced through cytokines (by increased chondrocyte activity) may also cause cartilage tissue damage (Homandberg and Hui, 1994). Concurrent with this, is the degradation of the minor collagens types IX and XI that function to stabilise collagen-fibril meshwork. Therefore disruption leads to an expansion of aggregcans and further increase in water content. The resultant weaker matrix cannot withstand/recover from compressive forces (Muir, 1986) leading to further break-up of matrix.

The third stage is the inability to stabilise or restore the tissue. Thus, in an attempt to return the articular cartilage to normal the repair response is insufficient to ongoing to withstand the ongoing insult the articular cartilage receives. There is a progressive loss of articular cartilage concurrent with the decline of chondrocytic anabolic and proliferative response due to mechanical damage and chondrocytic death. It is at this stage when patients first show symptomatic pain and loss of mobility and joint function. The response of the underlying subchondral bone to articular cartilage loss is subchondral sclerosis (increased density) and the formation of cyst-like bone cavities, which contain fibrous or cartilaginous tissue.

The end stage of the disease is characterised by complete loss of articular cartilage, which leaves two articulating surface of thickened dense subchondral bone resulting in misshaped, unstable joint and shortening of the limb.
Therefore, the disease progresses by the disruption of homeostasis within the cartilage with regards to metabolic and catabolic activities. There is a net loss of ECM molecules caused by an increased production of enzymes by the chondrocytes which degrade both collagen and proteoglycans.

Interestingly, as well as upregulation of molecules normally found within articular cartilage e.g. fibronectin, tenascin, and collagen type II during early OA, chondrocytes synthesise a number of ‘novel’ components. An example of which is the synthesis of type I collagen. The significance of this molecule in the repair response is unclear; however, it is likely that the disease affects the phenotype of the chondrocyte, which could be a sign of de-differentiation. In conjunction with synthesising novel proteins, the chondrocytes synthesise a matrix which is different to that of normal cartilage e.g. small proteoglycans, containing different patterns of glycosylation (reduced chondroitin-6/4-sulphate ratio), with GAG chains increased in length (Carney et al., 1985), fibronectin ED-A (F-EDA), (Salter et al., 1992) and type X collagen (normally expressed by hypertrophic chondrocytes), which reside in the calcified cartilage zone (until adolescence) is re-expressed by the osteoarthritic chondrocytes. It is apparent that many of these molecules are present normally during developmental stages e.g. prechondrogenic condensations or foetal cartilages, and are mis-expressed by the chondrocytes in the diseased joint. Again, the significance is unclear, but possibly indicates an attempt to return to an embryonic phenotype, which may aid repair and possibly reinitiate growth (Archer et al., 1990). Another example is cell proliferation (see above), which is a major feature of the developing tissue (prechondrogenic condensation) that also occurs during the repair response. However, the cells do not separate as little matrix is produced. Another interesting observation is the formation of osteophytes at the marginal zone that has been compared to the callus during fracture repair. It has also been resembled to that of immature growing cartilage (Sokoloff, 1983). However, the tissue is structurally and biochemically heterogeneous lacking the order of normal growth.

Current therapies for OA are primarily at symptom relief rather than disease modification or cure. Research institutes are attempting to induce the damaged cartilage to heal to prevent the self-perpetuating process of OA. Targets for
therapeutic intervention are the MMP inhibitors mannosamine and BB-16. The omega-3 class fatty acid (found in cod liver oil) has also been found to have chondroprotective effects by inhibiting MMPs (Curtis et al., 2002).

1.14 ARTICULAR CARTILAGE REPAIR

1.14.1 Intrinsic response of articular cartilage following wounding
Articular cartilage defects are classified as either full or partial-thickness dependent on whether or not the subchondral bone is penetrated. Partial thickness defects can be compared to fissures or clefts that occur in the early stages of osteoarthritis that are contained within the articular cartilage. The defects seldom if ever repair spontaneously and grow larger (Mankin, 1974a; Mankin, 1974b). Full-thickness defects do heal transiently and produce a reasonably functional cartilage tissue (Shapiro et al., 1993). This repair is due to an inflammatory wound-healing response whereby cells from the marrow are recruited to the reaction site similar to that found elsewhere in the body. A fibrin clot is formed and then is invaded by fibroblast like cells (marrow derived), forming a fibroblastic repair tissue. The defect is then rapidly filled with a cartilaginous or fibrocartilaginous tissue type, containing chondrocyte like cells and metachromatic ECM. Biopsy samples reveal the presence of type I and type II collagens. The resulting fibrocartilaginous plug does not resemble the original cartilage either biochemically or biomechanically which may be essential for normal articular cartilage function and longevity.

Partial-thickness defects do not heal because they do not have access to macrophages, endothelial cells and mesenchymal cells that are found within the bone marrow. Cartilage is also avascular and is largely sheltered from any immunological recognition that could occur due to damage. However, a repair response (to some extent) has been visualised which involves the chondrocytes themselves and synoviocytes (Hunziker and Rosenberg, 1996) in terms of cell migration, proliferation and matrix synthesis up-regulation.

Classical studies by Stockwell (1979) on postwounded articular cartilage revealed a pattern of events that occurred at the wound edge following trauma. Articular cartilage was typically wounded using either scalpels or borers to create partial or full-depth injuries for experimental analysis. Histological analysis revealed a 'zone of necrotic
cells' at the wound edge, as early as one day after wounding. This remained uniform in width up to 200μm deep (Mankin, 1962). After numerous weeks, the zone contained empty lacunae and a loss of metachromasia in the ECM. Although the actual cause of cell death is unknown, a few hypotheses have been put forward. It is possible that the cell death is due to mechanical perturbation during the wounding process itself or that a diffusion based mechanism, whereby cell death proceeds a loss of nutrients or survival factors, due to the uniformity of the zone (Calandruccio and Gilmer, 1962). Behind the zone of necrosis is a zone of cellular proliferation (Calandruccio and Gilmer, 1962, Mankin 1962) and, thus, an apparent attempt by the tissue to repair as revealed by ^3H-thymidine incorporation into DNA. However, the proliferating cells do not migrate into the defect and, instead, form cell clusters with increasing amounts of metachromatic matrix surrounding them. Elevated synthetic activity and elaboration is also indicated by the incorporation of ^3H-proline (Carlson, 1957). The signal for proliferation is unknown however; it could be stimulated by either the loss of proteoglycan or possibly as a result of mechanical strain during localised compression (Stockwell, 1979).

1.14.2 Extrinsic repair of articular cartilage

There are a number of potentially productive avenues being followed to date, which are both active and exciting. Current treatment options for damaged or lost cartilage are referred to as the four R’s, restored, replaced, relieved and resected (O’Driscoll, 1998). Restoration is when the joint surface (hyaline AC and subchondral bone) is healed or partially regenerated by either enhancing intrinsic capacity of the tissue to heal itself or transplantation of chondrocytes or chondrogenic cells, which have cartilage generation potential. Replacement is accomplished by prosthesis or an allograft. Relief involves decreasing the stresses placed on the joint by an osteotomy. Resection is the final option that may or may not include interposition arthroplasty.

Restoration attempts have utilised the recruitment of pleuripotential stem cells from the bone marrow by penetrating the subchondral bone. Mechanical, electrical and laser treatment have also been used in an attempt to stimulate healing along with scaffolds, with or without bioactive agents in the form of growth factors and cytokines. Subchondral drilling (Meachim and Roberts, 1971), abrasion (Altman et al., 1992) or microfractures (Steadman et al., 1997) are attempts at recruiting
pleuripotent stem cells from the marrow by penetrating the subchondral bone inducing a healing response. There are some benefits seen with small defects but not large defects, osteoarthritic joints or older adults.

Continuous passive motion was introduced by Salter et al. (1980) and has been confirmed as beneficial on the regeneration of cartilage after periosteal transplantation, in defects smaller than three millimetres (O'Driscoll et al., 1986).

Electrical stimulation is a popular technique in fracture healing but not so for cartilage healing (Lippiello et al., 1990). Using pulsed direct current for four hours per day, improved healing of cartilage defects in rabbit knees has been observed. However, the exact role of electrical stimulation was unclear. The results of laser treatment to induce cartilage repair are contradictory and, thus, its contribution therapeutically remains to be established (Athanasiou et al., 1995; Hardie et al., 1989).

Pharmaceutical agents may be administered systemically or locally (intra-articularly). To date, there is no systemic agent that can counteract the generalised symptoms of OA. There are three basic categories of intra-articular injections 1) corticosteroids which have conflicting results in that some investigators show enhanced healing (Olah and Kostenszky, 1976) whilst more recent studies reveal impaired cartilage physiology and arthropathy induction (Shoemaker et al., 1992). 2) Hyaluronan has been used has a viscosupplement, although having lubricant properties, it may have a direct biochemical effect providing articular cartilage with a protective coating (Smith and Ghosh, 1987). 3) Growth factors which include TGFβ, IGF-1, and BMP's have been injected intra-articularly due to their chondrogenic effects in vitro (Cuevas et al., 1988; van Beuningen et al., 1993; van Beuningen et al., 1994). The usefulness of this technique is unknown although gaining popularity, the data however are relatively sparse. For the full potential of the growth factors and bioactive agents to be fully exploited, a delivery system must be constructed to maintain the extract during the healing process. Such a construct might involve the implantation of scaffolds containing growth factors.

Scaffolds have also been used for healing joint defects, alone and with added growth factors that act as shape and guidance templates for tissue development. Scaffolds
permit 3D immobilisation of cells and maintain the differentiated phenotype of chondrocytes, their mechanical properties are insufficient for tissue transplantation. Examples of material used include nonabsorbable materials such as carbon fibre (Brittberg et al., 1994), dacron, teflon (Messner and Gillquist, 1993) and porous metal plugs. These are not successful in restoring cartilage when inserted alone with merely fibrous tissue forming. Adsorbable polymers/copolymers (Puelacher et al., 1994), which include polyglycolic/polylactic acid, have shown potential when transplanted with cells and growth factors. Fibrin clots were used containing mitogenic growth factors and when combined with enzymatic treatment (see above), there was an increase in the number of cells adhering (from the synovium) (Hunziker and Kapfinger, 1998; Hunziker and Rosenberg, 1996).

The use of a collagen-sponge was first described in the 1970's (Holmes et al., 1975). Initially, it was used as a scaffold whereby cells from the defect would migrate, grow and synthesise extracellular components. It was then developed as a carrier in which chondrocytes could be transplanted and maintained within the articular defects (Wakitani et al., 1989). Other natural polymeric gels have been used successfully such as hyaluronic acid, alginate and chitosan.

Advances in this field have included injectable in situ crosslinkable polymeric preparations that entrap cells as well as techniques that combine both porous fibre structures and gels (Silverman et al., 1999; Sittinger et al., 1994). Development of 'smart scaffolds' has focused on incorporating inflammatory inhibitors or antibiotics. The rationale is that the slow and controlled release of the bioactive molecules will provide sufficient time for the new cartilage to adapt in a hostile in vivo environment, as well as preventing infection following the surgical procedure.

Current studies are targeting specific zones (cartilage and subchondral bone) in an attempt to generate vertical organisation by using growth factors, which are released at specific time intervals. It is proposed that such scaffolds and matrices combined with delivered bioactive agents and transplanted cells will be important in cartilage regeneration/tissue engineering although, to date, the evidence is not compelling (Athanasiou et al., 1997; Sellers et al., 1997).
Thus, tissue engineering has been defined “as the application of engineering science and technology to the combined field of cellular and molecular biology with the goal of regulating the growth, differentiation, and metabolic activity of cells that are either transplanted or recruited to heal or regenerate a joint surface” (O’Driscoll, 1998).

The regeneration/growth of new cartilage by transplanted cells into a defect was first demonstrated by Bentley and Greer (1971). To date, as well as using chondrocytes to improve healing, stem cells (Wakitani et al., 1994) or tissue having chondrogenic potential such as periosteum and perichondrium have also been utilised.

Using cells cultured in vitro increases the number of cells from a relatively small source. A problem arises when trying to maintain the cells in the defect, this has been overcome by using tissue grafts shaped to the articular defect size which are then sutured in place - biological resurfacing with periosteum or perichondrium. The use of periosteum and perichondrium in cartilage repair has been studied worldwide, and, thus, their chondrogenic potential as well as their survival and ongoing growth in organ culture have been confirmed (Engkvist et al., 1975; Rubak, 1982). Periosteal arthroplasty carried out by O’Driscoll et al. (1986) on rabbit knees showed encouraging results with grafts healing with predominantly hyaline cartilage, which was enhanced by postoperative continuous passive motion. After one year, there was no degeneration of the regenerated tissue and the subchondral bone had been completely restored. The importance of this result lies in the biomechanics of cartilage with alterations in the underlying bone affecting the stability of the overlying cartilage. This procedure has been used on humans for over a decade, the majority of patients having no pain and arthroscopy imaging revealing cartilaginous tissue (Niedermann et al., 1985). Perichondrial arthroplasty has also been used therapeutically in humans with contradictory results (Bouwmeester et al., 1997; Homminga et al., 1990). Vachon et al., (1989) demonstrated that there was significantly more chondrogenesis observed in periosteal grafts than perichondrial autogeneous grafts. Another consideration is that periosteum is readily accessible with less morbidity than perichondrium, (which is incised from the rib) and, thus, by most is the preferable tissue choice for biological resurfacing of the joint.
Partial regeneration of cartilage (fibrocartilage) can occur using fully differentiated chondrocytes or by using undifferentiated (incompletely differentiated) chondrocyte precursors or pleuripotent mesenchymal stem cells. Worldwide research groups have investigated both with promising results.

Differentiated chondrocytes only produce cartilage when cultured in appropriate conditions and are limited to repairing chondral defects only. However, many defects involve the underlying subchondral bone, and chondrocytes do have the capacity to induce bone healing during fracture healing. Sources of multipotent cells are the bone marrow, and periosteum, which can potentially regenerate both cartilage and underlying bone. Van Dyk et al., (1998) demonstrated that correcting the defect within the bone using impacted autogeneous cancellous bone into large defects showed marked improvement in the repaired surface. They hypothesised that the graft provided a scaffold to which mesenchymal stem cells from the bone marrow could adhere and differentiate into chondrocytes and synthesise matrix.

Transplantation of autogeneous or autologous chondrocytes involves isolation of chondrocytes from biopsies and then their subsequent expansion in culture to increase number. Once suspended in liquid medium, they are placed beneath a periosteal graft sewn over the defect (Brittberg et al., 1996; Grande and Pitman, 1988; Grande et al., 1987). Brittberg et al., (1996) showed that compared to controls, rabbits treated with transplanted autogeneous chondrocytes had a better repair tissue. However, integration into the defect was incomplete although over a one-year period, the hyaline-like tissue matured and contained more pronounced columnarization. Preliminary results of transplantation of autogeneous chondrocytes in humans showed clinical results of good to excellent and forty-six months postoperative studies revealed healed defects, although visible edges were noted (Brittberg et al., 1994). Recent studies show clear disparity between subjective clinical results and the actual biological results, which tends to leave doubt on the contribution of the grafted cells. Future studies should carefully consider the number of transplanted chondrocytes, the use of periosteum, e.g. sutured correctly to allow expansion of growing tissue but deep enough to prevent articulation with opposing surface and the preservation of the cambium layer (Fitzsimmons and O'Driscoll, 1998).
An alternative therapeutic choice is the replacement of damaged cartilage with either small osteochondral plugs (mosaicplasty) obtained from a non-weight-bearing region of the knee or completely with an osteochondral transplant obtained from an unrelated donor. Of ten patients who received mosaicplasty and osteochondral autogenous grafts only four had mild pain in the knee (six and half years after operation) (Outerbridge et al., 1995). There have been no reports of major complications at donor site however patients must be carefully selected because of structural alterations created at the donor site. Osteochondral allografts have been demonstrated to be a "good option for symptomatic patients in which attempts to induce healing or regeneration of the articular surface are inappropriate or have failed" (O'Driscoll, 1998).

Cell and tissue transplantation can be either autogenous or allogenic with both techniques having advantages and disadvantages. Although allogenic transplantation of chondrocytes within rabbits has shown success (Wakitani et al., 1989) this was not the case for horses (Sams et al., 1995). It is possible that culturing might permit sequestering of cell-surface antigens by ECM production.

Several studies have also evaluated the potential of mesenchymal stem cells (MSCs) to generate cartilage when embedded in an appropriate carrier. Adult MSCs reside in bone marrow as supportive cells for haematopoiesis and act as a possible reservoir and regeneration pool for various mesenchymal tissues (Gerson, 1999).

1.15 STEM CELLS

Stem cells are defined as a cell that can retain a high capacity of self-renewal throughout life and have the ability to produce daughter cells that can undergo terminal differentiation (Lajtha, 1979a; Lajtha, 1979b). Stem cells are found within permanently renewing tissues whereby the terminally differentiated cells do not divide and have a short lifespan. Such examples include the haemopoietic system, the epidermis and the lining of the small intestine. Signals are presented to the daughter cell, which determine its fate: either to become a stem cell itself or to undergo terminal differentiation. Stem cells within the haemopoietic system are multipotent-giving rise to terminally differentiated cell types (Metcalf, 1989). The differentiated cells are generated by ‘committed progenitors’ which when compared to stem cells
have lower proliferative potential and a more restricted potential for differentiation than their parent stem cell. The progenitor cell allows amplification of each stem cell division, thus, only a small number of stem cell divisions' results in the production of a large number of terminally differentiated cells (Lajtha, 1979). The epidermis only has one terminally differentiated pathway. Basal layer cells attached to the basement membrane proliferate, with terminal differentiation occurring as the keratinocytes migrate upward before being shed from the skin surfaces as dead cornified squames (Watt, 1989). Although only a single differentiation pathway (producing the keratinocyte), there is evidence of heterogeneity within the proliferative compartment-the dividing cells that have high proliferative potential correspond to stem cells while those capable of self-renewal with a higher probability of undergoing terminal differentiation are known as transit amplifying cells which can be compared to committed progenitor cells of the hemopoietic tissue. Barrandon and Green (1987) showed that individual keratinocytes vary in their capacity for self-renewal; the clones are classified into 3 types. 1). Holoclones, having the highest reproductive capacity which are probably founded by stem cells. 2). Paraclones, all cells undergo terminal differentiation within a few generations founded by transit amplifying cells. 3). Meroclones, are intermediates between holoclones and paraclones and are possibly stem cells that generate transit-amplifying daughters at higher frequency than stem cells of holoclones. It has been hypothesised that stem cells reside in an optimal microenvironment or niche and that the fate of their progeny is determined by factors within its environment (Schofield, 1978; Trentin, 1978).

Indirect evidence of cell cycle kinetic analysis reveals that stem cells occupy specific locations within the basal layer, limbus of the cornea (Cotsarelis et al., 1989), outer root sheath or bulge region of hair follicles, or within tips of deep rete ridges in palm epidermis (Lavker and Sun, 1983) deep projections of tongue papillae (Hume and Potten, 1976).

Although stem cells are present throughout human developmental stages (embryo to adult) their versatility and number appear to diminish with age. Embryonic stem (ES) cells have the ability to produce any of the 200 specialised cells that form the human body. However, adult stem cells are ‘thought’ to be less versatile. When traumatised via damage or disease, many of the body’s specialised cells do not repair intrinsically.
In most cases, there is a potential to treat disorders with donated organs, however, a shortage of transplantation and the potential risks of xenotransplantation necessitates another source of tissue. An exciting prospect is the transplantation of the cultured specialised progeny that have derived from stem cells. Conflicting views remain between laboratory/research groups on whether to use embryonic stem cells or adult stem cells.

The rationale is that ES cells therapeutically are beneficial because of their longevity and capacity to produce a wide range of specialised cells in culture. Adult stem cells however, appear to have much shortened lifespans compared with ES cells, thus reducing their capabilities in forming new cell types. Current research is assessing the ability to obtain stem cells from aborted fetuses and the umbilical cord blood.

Kondo and Raff (2000) have re-programmed oligodendrocytes into multipotential central nervous system stem cells. They have also recognised the possibility of forcing specialised cells to replicate themselves (Tang et al., 2001). The rationale for the study (on embryonic stem cells) is to find out how stem cells from non-embryonic sources can be extracted, kept alive in the laboratory, multiplied for extended periods of time and directed to form specific types of specialised cells. The research on adult stem cells is at a very early stage, thus, there are many hurdles before therapeutic strategies can be considered viable.

Adult stem cells are only found in relatively small numbers replicating at a low rate due to their self-programming function to maintain the function of the adult organs and tissues. In contrast, embryonic and foetal stem cells are characteristically more prolific and versatile, and thus, have the potential to generate a larger amount of cells (followed by tissue) to repair or replace damaged areas of the body. To date, this field has a quarter of a century of experience in culturing and expanding these cells in the laboratory (Chan and Evans, 1991; Evans and Hunter, 2002; Evans, 2001; Evans and Kaufman, 1981).

Adult MSCs isolated from the bone marrow can be characterised by the ability to proliferate in culture and to differentiate into multiple lineages under defined culture conditions (fig 1.9) (Kadiyala et al., 1997). MSCs express a defined set of surface
markers. The frequency of MSCs in bone marrow can vary between $1 \times 10^4$ and $1 \times 10^6$ which also decreases with age of the donor. MSCs express the surface molecules CD44, CD71, CD90, CD106, CD120, CD124 but are negative for the haematopoietic lineage markers CD14, CD34 and CD45 (Campagnoli et al., 2001). The cells also secrete a distinct pattern of cytokines IL-6, IL-11, leukaemia inhibitory factor (LIF), granulocyte macrophage colony stimulating factor (GM-CSF) (Majumdar et al., 1998). Isolation of MSCs has been difficult due to scarcity of specific molecular markers.

**Figure 1.9.** Similarities and differences between embryonic and adult stem cells. Human embryonic and adult stem cells each have advantages and disadvantages regarding potential use for cell-based regenerative therapies. Of course, adult and embryonic stem cells differ in the number and type of differentiated cells types they can become. Embryonic stem cells can become all cell types of the body because they are pluripotent. Adult stem cells are generally limited to differentiating into different cell types of their tissue of origin. However, some evidence suggests that adult stem cell plasticity may exist, increasing the number of cell types a given adult stem cell can become (Adapted from Robby, 2004).

Many studies have utilised MSCs in an attempt to repair articular cartilage, during micro-fracture or following removal of bone marrow and their subsequent isolation and expansion for the use in engineered cartilage (Johnstone et al., 1998; Mackay et al., 1998; Yoo et al., 1998). Following microfracture, the fibrocartilage that results does not resemble the pre-existing cartilage biochemically or biomechanically and ultimately deteriorates leading to osteoarthritis.

MSCs have an extensive capacity for self-replication and give rise to daughter cells that can differentiate into multiple lineages. Although MSCs are a readily available
source, their removal is tedious involving bone marrow aspiration, fractionation, cell culturing and cell propagation, \textit{in vitro} conduction of chondrogenesis, preparation of the implants and finally implantation into articular defects.

The presence of a putative chondroprogenitor cell residing within the surface zone of articular cartilage opens up the possibility of using this population to engineer cartilage \textit{in vitro}. It will be interesting following further characterisation, if the chondroprogenitor cells have similar qualities of other well stem cells. For clinical use, the chondroprogenitor will have to be isolated from the articular cartilage, expanded in culture for extended periods of time and directed towards differentiation. Comparisons should be made to differentiated chondrocytes as to to the chondroprogenitors ability to retain a high capacity of self-renewal throughout life and to produce daughter cells that can undergo terminal differentiation.
AIMS

The aim of this study was to isolate the sub-populations of chondrocytes residing within 7-day bovine metacarpalphalangeal joint and to determine if superficial, middle or deep zone derived chondrocytes would differentially retain regional characteristics and regional markers. We also wanted to fabricate articular cartilage’s unique structure by stratifying surface zone derived chondrocytes atop deep zone derived cells.

The study also attempted to further characterise the articular cartilage progenitor cell residing within the surface of the articular cartilage of 7-day bovine metacarpalphalangeal joints.

The objectives of the study were:

- To isolate, clone and then extensively cultivate the chondroprogenitor cells.
- To detect whether both chondrogenic commitment and differentiation potential of the cell populations were preserved during their expansion in vitro by monitoring Sox9 expression.
- To characterise the effects of growth factors on chondroprogenitor expansion, by analysing both time to double and the expression of the transcription factor Sox9.
- To assess whether clonally isolated chondroprogenitor cells have the capability to differentiate towards the chondrogenic, osteogenic, adipogenic and neurogenic lineages in vitro.
- To assess the differentiation potential of clonally derived progenitor population in vivo by injecting them into the proximal limbs of stage 19-21 chick embryos.
- To assess the effects of introducing high density pellets containing the chondroprogenitor cell population to the wound site of an in vitro model of a partially wounded articular cartilage.
- To determine whether a population of chondroprogenitor cells resides within the surface of 18-month and over 30-month bovine articular cartilage.
CHAPTER 2

Characterisation of chondrocytes derived from the superficial, middle and deep zones of articular cartilage
2.1 INTRODUCTION

Damage to articular cartilage, by either trauma or disease, can permanently affect joint function within adults as the tissue has a minimal capacity for self-repair.

Although techniques are available to repair damaged cartilage (as discussed in section 1.15), e.g. mosaicplasty and autologous chondrocyte implantation, each has its limitations. Recent studies have addressed these problems and focussed on the tissue engineering of articular cartilage *in vitro* to use in repair strategies. This strategy could circumvent the minimal donor supply to be used in surgical procedure using minimum invasive procedures and allow for rapid patient remobilisation (Hunziker, 2002).

The tissue engineering of articular cartilage to date has utilised isolated chondrocytes that are seeded within biological or synthetic scaffolds (see section 1.15). Although these strategies develop cartilaginous like tissue containing the characteristic types of extracellular macromolecules (e.g. collagen type II and large sized proteoglycans), when they are compared to native cartilage, variable amounts of ECM are produced. The engineered tissue has reduced collagen content and provides only a fraction of the mechanical properties required for normal joint function (Martin et al., 2000).

The majority of studies typically obtain chondrocytes from the full-thickness of native cartilage (sometimes wrongly regarded as homogeneous where tissue engineering is concerned) which is a mixed population of chondrocytes. As discussed in section 1.5, articular cartilage is a heterogeneous tissue containing several distinct regions that are defined by both chondrocyte morphology and matrix structure. It is likely, therefore, that any attempt to replicate this structure using tissue engineering strategies will be difficult. Cartilage has been classically divided into several histologically distinct zones with relative depth from the surface according to changes in chondrocyte morphology and metabolic activity (Aydelotte et al., 1988; Aydelotte and Kuettner, 1988; Jacoby and Jayson, 1975). This stratification confers specialised functional properties to the superficial, middle and deep zones of cartilage (Maroudas, 1979).
The superficial zone, closest to the articulating surface, contains cells which are flattened and lie in a plane parallel to the surface. The cells residing in the superficial layer typically synthesise less ECM compared to deeper zones (Archer et al., 1990) composed primarily of collagen and low sulphated glycosaminoglycan and aggrecan content compared to deeper zones (Muir et al., 1970). The chondrocytes within the transitional or middle zone are larger and more rounded and appear randomly distributed. Deep zone chondrocytes, however, are arranged in vertical columns, perpendicular to the cartilage surface, and synthesise the greatest amount of ECM on a cell tissue volume basis (Wong et al., 1996). Biochemical and electron microscopy studies have also revealed that with cartilage depth collagen fibril size and orientation alter, which again is very important in cartilage function (see section 1.5).

Previous studies have shown that cells isolated from the different zones of articular cartilage retain their morphological and metabolic characteristics when maintained in homogeneous cell culture conditions such as monolayer and agarose culture (Archer et al., 1990; Aydelotte et al., 1988; Aydelotte and Kuettner, 1988; Lee and Bader, 1995; Lee et al., 1993; Siczkowski and Watt, 1990; Zanetti et al., 1985).

It is possible, therefore, that in tissue engineered cartilage both composition and mechanical properties may be modulated by using cells from the different zones of the native tissue. Recent studies compared the biochemical and mechanical properties of cartilaginous tissue formed from specific populations of chondrocytes to the tissue formed by cells obtained from the full thickness of the native tissue (Klein et al., 2003; Waldman et al., 2003). Few attempts have been made to fabricate articular cartilage with stratification using the various subpopulations, surface, middle and deep derived chondrocytes.

The objective of this chapter was to determine if subpopulations of chondrocytes from superficial, middle or deep zones would differentially retain regional characteristics and regional markers, e.g. SZP or type X collagen. Comparisons were made to full depth derived chondrocytes. Finally, stratification of surface zone derived chondrocytes atop deep zone derived cells attempted to fabricate articular cartilage's unique structure.
2.2. MATERIALS AND METHODS

2.2.1 Cell Isolation

Surface (SZ), middle (MZ), deep (DZ) and full depth (FD) explants were isolated from 7-day bovine metacarpal-phalangeal (MCP) joints by fine dissection (see figure 2.1a). Cartilage shavings were collected in a sterile 50ml tube and chondrocytes were released from surrounding matrix by sequential digestion in 3.17 units/ml pronase (Boehringer Mannheim) in Dulbecco’s Modified Eagle Medium (DMEM; GIBCO BRL, Life technologies Ltd., UK) and Ham’s F12 (F12; GIBCO) at a 1:1 ratio containing 5% foetal calf serum (FCS) supplemented with 5mg/ml glucose, 100μg/ml ascorbic acid and 50μg/ml Gentamycin (GIBCO) for 3 hours at 37°C on a roller. Pronase was removed and cartilage shavings incubated in 0.12 units/ml collagenase (type I (Sigma), in 5% DMEM/F12) overnight on a roller at 37°C. Following collagenase digestion, cells were filtered through a 40μm mesh ‘Nitex’ cell strainer (Falcon) and the cell suspension centrifuged at 2500 x g for 5 minutes. Aspiration of the supernatant was followed by resuspension of the cell pellet in 10ml of serum-free DMEM supplemented with 5mg/ml glucose, 50μl/ml Gentamycin and 100mg/ml ascorbic acid (DMEM-). Cell numbers were counted on a haemocytometer.

2.2.2. Pellet Culture

Following counting, SZ, MZ, DZ and FD cells were resuspended in DMEM/F12 containing 10% FCS supplemented with 5mg/ml glucose, 100μg/ml ascorbic acid and 50μg/ml Gentamycin DMEM/F12+ containing 1% HEPES buffer at 2x10⁶ cells/ml and 500 μl aliquots placed in 1.5ml polypropylene conical tubes. Tubes were spun at 2000 x g for 5 minutes to pellet the cells and the pellets maintained in DMEM+ in a humidified 37°C, 5% CO₂ air incubator for up to 21 days with medium changed twice weekly (see figure 2.1c).

2.2.3. Formation of stratified pellets

Cells were labelled by incubating for 45 minutes in PBS containing 10μm CellTracker™ (Molecular Probes) Green, 5-chloromethylfluorescein diacetate (CFDA) (SZ cells), Orange,5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethyl-rhodamine (CMTMR) (DZ cells) or PBS only (MZ cells) at 37°C on a roller. The CellTracker™ fluorescent probes are retained in living cells through several
generations, are inherited by daughter cells after cell division and are not transferred among adjacent cells in a population. The dyes contain a mildly thiol chloromethyl reactive group. CMFDA and CMTMR passively diffuse into cells but once inside the cell, their chloromethyl group reacts with intracellular thiols, transforming the probe into a cell-impermeant fluorescent dye-thioether adduct. CMFDA is colourless and nonfluorescent until cytosolic esterases cleave off its acetates, releasing a brightly fluorescent product. Table 2.1 summarises the spectral characteristics of the CellTracker™ probes.

<table>
<thead>
<tr>
<th>CellTracker Probe</th>
<th>Absorption (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CellTracker Green CMFDA</td>
<td>492</td>
<td>516</td>
</tr>
<tr>
<td>CellTracker Orange CMTMR</td>
<td>540</td>
<td>566</td>
</tr>
</tbody>
</table>

**Table 2.1. Spectral characteristics of the fluorescent CellTracker probes.**

Six types of pellets were formed (see figure 2.1). Homogeneous superficial (S), homogeneous middle (M), and homogeneous deep (D). Stratified; superficial layered atop deep (S/D), middle layered atop deep with superficial atop middle (S/M/D) and finally superficial and deep layers mixed (S+D). The stratified pellets were formed by firstly resuspending the SZ, MZ and DZ cells at 1x10⁶ cells/ml in DMEM+ containing 1% HEPES. S/D pellets were formed by centrifuging a 500μl aliquot of D at 2000 x g in a 1.5ml polypropylene conical tube for 5 min and then aliquoting 500μl of S cells atop and centrifuging at 2000 x g. S/M/D pellets were formed by individually aliquoting and centrifuging at 2000 x g, 333μl of D, followed by 333μl of M and finally 333μl S. Homogenous pellets were formed by simple aliquoting and centrifuging at 2000 x g 1ml of S,M and D. S+D mixed pellets were formed by mixing 500μl of both S and D together and centrifuging.

The stratified and homogeneous pellets were cultured for 7, 14 and 21 days with changes in media twice weekly.
Figure 2.1. Schematic illustration showing the overall strategy for chondrocyte isolation and pellet formation.
2.2.3. Cell death characterization

Following 21 days of culture, the SZ, MZ, DZ and FD derived pellets were cultured with 2mM ethidium homodimer (EtHD) (Molecular Probes, Cambridge) for 90 minutes in DMEM+ at 37°C, 5% CO₂ air incubator followed by washing in DMEM for 90 minutes. Subsequently media was removed and pellets were fixed in 10% NBFS overnight, wax embedded, sectioned at 10 μm and examined using a fluorescent microscope.

2.2.4. Histochemical and Immunohistochemical analysis

Pellets were cultured for 7, 14 and 21 days and fixed in 10% NBFS overnight and processed for routine histology. Pellet samples were dehydrated through a series of graded alcohols (70%, 95%, and 100% with two changes of 20 minute each), cleared in xylene (two changes of 20 minutes) and embedded in paraffin wax. The samples were sectioned using a Leitz 1512 microtome at 10μm, floated on water (45-50°C) and mounted on aminopropyltriethoxysaline (APES) coated slides and air dried for approximately 1 hour. The sections were then dried overnight in a 45°C oven. Slides were dewaxed and stained with Mayer's haematoxylin counterstained with safranin O to determine presence of sulphated glycosaminoglycans (GAGs) (appendix I). Stained sections were dehydrated and mounted in DPX (BDH, Poole, UK). Samples incubated with EtHD for cell death were dewaxed, washed in PBS and coverslips mounted with Vectashield (Vector Labs) and stored at 4°C for later observation. For immunohistochemical analysis pellets were rinsed in PBS, fixed in chilled 95% ethanol for 10 minutes followed by a PBS wash, frozen at -80°C in a cryoprotectant (OCT) and 10μm sections cut on a Cryostat (Bright). Sections were placed on slides coated with APES, air dried and stored at -20°C for subsequent immunofluorescence labelling. For intracellular labelling, matrix secretion was blocked by incubating pellets for 5-8 hours at 37°C in DMEM+ containing 5μM/ml monensin (Sigma, Poole, Dorset). Monensin blocks traffic through the Golgi apparatus (Griffiths et al., 1983) resulting in accumulation of freshly synthesised proteins within the cells. After the incubation, the Monensin treated media was removed and the pellets washed before being fixed, as above, in 95% chilled ethanol.
Full depth articular samples were dissected from the MCP joint of the 7-day bovine calf; tendon samples were also isolated in the same manner, washed in PBS and frozen in OCT at -80°C. Frozen samples were sectioned at 10μm on a cryostat and the sections collected onto APES-coated slides and stored at -20°C. These sections would function as positive controls during immunohistochemical analysis.

Indirect immunofluorescence labelling was performed in a light-proof, humidified chamber. Tissue sections were allowed to thaw at ambient temperature. Hydrophobic rings were drawn around tissue sections using a 'DAKO' pen (Dakopatts Ltd). Sections were then wetted with PBS containing 0.1% Tween 20 (poloxyethylenesorbitan monolaurate (Sigma, Dorset). All washes were in PBS containing 0.1% Tween 20, at 3 washes of 5 minutes each. Sections requiring enzyme pre-treatment (depending on primary antibody) were incubated with the relevant enzyme for 1 hour at 37°C. Sections that required no enzyme pre-treatment were kept in PBS during the 1-hour incubation. Enzyme was washed from sections with PBS/Tween 20 and sections were incubated with appropriate blocking serum for 30 minutes at room temperature. Blocking serum was blotted off and the sections were incubated for 1 hour at room temperature or overnight at 4°C with the primary antibody diluted in PBS/Tween 20 (see table 2.2 for a full list of antibodies used). Primary antibody was washed from slides using PBS. Slides were incubated with relevant fluorescent conjugated secondary antibodies diluted with PBS containing 20% bovine serum for 1 hour at room temperature and then washed with PBS. Sections were then mounted in Vectashield without propidium iodide. Secondary antibodies; Alexa fluor 488 donkey anti-goats or Alexa Fluor 488 goat anti-mouse used at 10μg/ml (Molecular Probes, Cambridge).

2.2.5. Controls for immunofluorescence

Negative Controls

a) Replaced primary antibody with isotype specific non-immune mouse (monoclonal) immunoglobulins (MIG) (Sigma, Dorset). Demonstrates that the secondary antibody is binding specifically to the primary antibody.

b) Replaced primary antibody with PBS. Demonstrates that the secondary antibody is not randomly binding (non-specifically) to the tissue.
Positive Controls

Positive controls were used to demonstrate that the panel of antibodies used (table 2.2) were reactive and were specific to their antigens.

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>P/M</th>
<th>RECOGNISES</th>
<th>OPTIMAL WORKING DILUTION/ Ig CONCN.</th>
<th>ENZYME PRETREATMENT</th>
<th>SECONDARY ANTIBODY</th>
<th>SOURCE OF ANTIBODY AND REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusterin</td>
<td>P (goat)</td>
<td>Clusterin</td>
<td>1:10</td>
<td>None</td>
<td>Alexa fluor 488 donkey anti-goat 10µg/ml</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Type X</td>
<td>P (mouse anti deer)</td>
<td>Type X collagen</td>
<td>1:100</td>
<td>1.5units/ml Hyaluronidase and 0.25units/ml Chondroitinase ABC mix</td>
<td>Alexa fluor 488 goat anti-mouse 10µg/ml or Alexa-Fluor 350 goat anti-mouse 10µg/ml</td>
<td>Gift from Prof. Gary Gibson</td>
</tr>
<tr>
<td>Anti-M</td>
<td>P (goat)</td>
<td>Type IX Collagen COL 2 domain</td>
<td>1:100</td>
<td>1.5units/ml Hyaluronidase and 0.25units/ml Chondroitinase ABC mix</td>
<td>Alexa fluor 488 donkey anti-goat 10µg/ml</td>
<td>Gift from Prof. Vic Duance</td>
</tr>
<tr>
<td>2B6</td>
<td>M</td>
<td>Chondroitin-4-sulphate and derman sulphate GAG stubs</td>
<td>1:500</td>
<td>0.25units/ml Chondroitinase ABC</td>
<td>Alexa fluor 488 goat anti-mouse 10µg/ml</td>
<td>Prof. Bruce Caterson Caterson et al., 1985</td>
</tr>
<tr>
<td>3B3 +</td>
<td>M</td>
<td>Chondroitin-6-sulphate stubs</td>
<td>1:500</td>
<td>0.25units/ml Chondroitinase ABC</td>
<td>Alexa fluor 488 goat anti-mouse 10µg/ml</td>
<td>Prof. Bruce Caterson Couchman et al., 1984)</td>
</tr>
<tr>
<td>3-A-4</td>
<td>M</td>
<td>SZP</td>
<td>1:400</td>
<td>N/A</td>
<td>Alexa fluor 488 goat anti-mouse 10µg/ml</td>
<td>Prof. Bruce Caterson</td>
</tr>
<tr>
<td>CIICl</td>
<td>M</td>
<td>Type II collagen</td>
<td>1:4</td>
<td>1.5units/ml Hyaluronidase and 0.25units/ml Chondroitinase ABC mix</td>
<td>Alexa fluor 488 goat anti-mouse 10µg/ml</td>
<td>DHSB* Holmdahl et al., 1986</td>
</tr>
<tr>
<td>Link protein</td>
<td>P</td>
<td>Link protein</td>
<td>1:30</td>
<td>0.25units/ml Chondroitinase AC and 0.25units/ml Chondroitinase ABC mix</td>
<td>Alexa fluor 488 donkey anti-goat 10µg/ml</td>
<td>DSHB</td>
</tr>
</tbody>
</table>

M, monoclonal, P, Polyclonal, *ABC, Chondroitinase ABC, +H, Hyaluronidase, #AC, Chondroitinase ACII; DSHB Developmental Studies hybridoma bank (University of Iowa, USA)

*Table 2.2. Antibodies towards extracellular matrix components used during immunohistochemistry*
2.2.6. Microscopic Characterisation

Fluorescence was examined using a Leitz Laborlux 12 fluorescent microscope. Images were captured using a RS Photometrics Coolsnap camera and Coolsnap image software and formatted with Adobe Photoshop (version 5.5). Histochemical staining were viewed with a Leica (Leitz DMRB) light microscope and images taken using Image grabber software and formatted using Adobe photoshop (version 5.5).

2.2.7. SEM

S, M, D, FD pellets and full depth excised explants were fixed for 1 hour at room temperature in 2.5% glutaraldehyde buffered at pH 7.4 with 0.1M sodium cacodylate-HCL. The fixed samples were then briefly rinsed in the same buffer and post-fixed at 4°C for 1 hour in 1% osmium tetroxide. Following this post-fixation the tissue samples were dehydrated in an ascending series of ethanols. The samples were finally rinsed in two washes of absolute alcohol (2 x 10 minutes) and transferred into a critical point dryer (SAMDRI 780 CPD, Maryland, USA) for 1 hour.

After drying, the pellets and full depth excised tissue explants were attached onto specimen carrying stubs using carbon double-sided tape and coated with a thin layer of gold in a sputtering coating unit. The specimens were examined using a Philips scanning electron microscope (SEM XL20) operated at 20kV accelerating voltage.

2.2.8. Dissecting microscope

The gross morphology of SZ, MZ, DZ and full depth pellets were analysed at 7, 14 and 21 days using an Olympus SZX12 dissecting microscope. Images were taken on Kodak 64T film and scanned with a CanoScan 2700F slide scanner (Canon) and formatted with Adobe Photoshop (version 5.5).

2.2.9. Biochemical analysis of cell pellets

DMMB Assay

Pellets (n=8 from each experimental group) were incubated in 1ml Papain Digestion Buffer (20mM sodium acetate (NaAc) pH 6.8, 1mM EDTA, 2mM dithiothreitol and
300 μg/ml papain (Sigma) at 65°C for 60min and then stored at -20°C until analysis. Aliquots of the digest were assayed separately for GAG, collagen and DNA content.

Dimethylmethylene blue is a strongly metachromatic dye for the histochemical detection of sulphated glycosaminoglycans ((Chandrasekhar et al., 1987; Farndale et al., 1986). Appropriate dilutions of the sample were mixed with 200μl of DMMB reagent (16mg DMMB (Serva, Feinbiochemic, GMBH) was dissolved in 1L of water containing 3g Polyvinyl alcohol (30-70,000 Da) 3.04g glycine, 2.37g NaCL and 95ml 0.1M HCL. The final pH of the solution was 3.0 and the absorbance of the solution, read at 525 nm, should approximate 0.31 in a microtitre plate (Groves et al., 1997). The absorbance was determined in a microtitre plate reader at a wavelength of 525 nm. Standard concentrations within the linear range of measurement, 1-40 μg/ml of shark chondroitin-6-sulphate (Sigma), were used to generate a standard curve and quantify unknown values.

**DNA Content**

A 50μl aliquot from each papain digest of pellets was placed in a plastic 2ml cuvette and 950μl of 20mM Na Ac pH 6.8, 1 mM EDTA and 100ng/ml Hoescht 33258 dye (Sigma). Duplicate samples were placed in a fluorescence spectrophotometer (Cary Eclipse, Varian, USA) set to excitation and emission wavelengths of 350 nm and 450 nm, respectively. A standard curve was produced using 1-20μg/ml salmon sperm DNA.

**Hydroxyproline Assay for Collagens**

Collagen content of samples was quantified by the determination of the hydroxyproline content. Hydroxyproline occurs at high levels in collagen proteins and is therefore used as an estimator of collagen content.

Five hundred microliters each of papain digested samples were hydrolysed in 6N HCL for 24 hours at 110°C. After hydrolysis, hydrolysates were freeze-dried to remove the acid. The hydrolysates were then reconstituted in double distilled water and centrifuged for 10 minutes in a microcentrifuge to remove any insoluble, particulate matter. Thirty microliters of sample and standard concentrations of
hydroxyproline diluted in distilled water were pipetted into duplicate wells on a 96 well plate, 70μl of diluent (2:1 propan-2-ol and distilled water) and 50μl of oxidant (0.7g chloramine-T, 10ml water, 50ml stock buffer (500ml; 28.5g sodium acetate trihydrate, 18.75g tri-sodium citrate dehydrate, 2.75g citric acid, 200ml propan-2-ol). One hundred and twenty-five microliters of the colour reagent (7.5g dimethylamino benzaldehyde, 11.25ml perchloric acid (60%) and 62.5ml propan-2-ol) was added using a multi-channel pipette. The plate covered with plastic film and incubated in an oven at 70°C for 10-20 minutes. When samples turned a peach colour the absorbance at 540nm was recorded (Creemers et al., 1997). The standard curve for this analysis was generated using L-hydroxyproline (Sigma) and for conversion of hydroxyproline to collagen values we estimated 7.25g collagen is equivalent to 1g hydroxyproline (Pal et al., 1981).

2.2.10. Statistical Analyses

Data are expressed as mean +/- standard error of the mean (S.E.M) and effects of culture duration were assessed using one-way ANOVA (Analyse-it for Microsoft Excel 2003 software supplier). All pairwise comparisons between groups were conducted using the Fisher's LSD post-hoc test. Significance was assigned at p-values less then 0.0001.
2.3 RESULTS

The chondrocytes formed coherent pellets that retained their structure when removed for analyses.

The use of a dissecting microscope demonstrated a glassy hyaline blue/white appearance of the pellets formed during cultivation (fig 2.2). Every pellet developed outgrowths from the surface. It was difficult to identify any differences in size between the pellets.

The surface of a normal bovine articular cartilage explant appeared smooth when visualised using scanning electron microscopy (SEM) (fig 2.3A). At higher power it was possible to see surface zone chondrocytes indenting the surface (arrows) (fig 2.3B). At low power it was possible to assess the differences in size between the pellets, surface derived (SZ) appearing the smallest (fig 2.3C) followed by full depth (FD) (fig 2.3I) with middle (MZ) (fig 2.3E) and deep (DZ) (fig 2.3G) derived, roughly equal in size. The surfaces of the SZ derived pellets were highly cellular, with clusters of rounded cells wrapped in matrix (arrows)(fig 2.3D). This was also true for the FD derived pellet (arrows)(fig 2.3J). The representative surface areas of MZ (fig 2.3F) and DZ (fig 2.3H) derived pellets were covered in matrix with occasional, scattered cells (rounded) attached (arrows).

The ECM present in pellets was visualised by Safranin O staining after 7, 14 and 21 days of culture. Safranin O staining showed areas of GAG deposition in all pellets from each tissue region and at all time points.

At day 7, all pellets were similar in appearance when stained with Safranin O. Weak stain was apparent between the tightly packed cells of SZ derived pellets (fig 2.4A). MZ, DZ and FD derived pellets were also highly cellular following 7 days of culture but stained more intensely for matrix (fig 2.4 D, G, J). By 14 days, distinct differences in pellet morphology were observed. SZ derived pellets (fig 2.4B) consisted of closely packed rounded cells with small amounts of matrix between them. Pellets derived from DZ and FD contained larger, vacuolated cells separated by more matrix (fig 2.4H &K). MZ derived pellets comprised cells intermediate in size to those found in
SZ and DZ/FD derived pellets. At 21 days SZ derived pellets consisted of a mix of closely packed cells and larger vacuolated cells (fig 2.4C). The MZ derived pellets at 21 days (fig 2.4F) were similar in appearance to those at 14 days. At 21 days the chondrocytes within DZ and FD derived pellets were again very large and vacuolated (fig 2.4I & L). In all cases the periphery of the pellets were occupied by flattened chondrocytes (fig 2.4L).

Pellets labelled with a panel of antibodies to ECM components demonstrated that the pellets expressed molecules typical of articular cartilage at day 7, 14 and 21.

All pellets labelled for collagen type II (fig 2.5), type IX (fig 2.6), link protein (fig 2.7) and 2B6 (fig 2.8) within their ECM at all time points. Type I collagen was found at the periphery, following 21 days in culture within SZ, MZ, DZ and FD derived chondrocytes (fig 2.9 A, B, C, D).

Only pellets derived from the SZ labelled continuously for SZP (fig 2.10 A, B, C) and only occasional labelling for SZP was visualised within FD derived pellets (fig 2.10 D, E, F). Only a few cells labelled for clusterin within pellets derived from SZ (fig 2.11).

Labelling for type X collagen was found within SZ derived pellets at days 7, 14 (fig 2.11 A, B) and within DZ (fig 2.11 G, H, I) and FD (fig 2.11 J, K, L) derived pellets at all time points. No labelling was found within MZ derived pellets (fig 2.11 E, D, E, F).

Following incubation with ethidium homodimer (EtHD) to assess for cell death there appeared to be a higher proportion of cells labelling within the SZ than MZ, DZ and FD derived pellets (fig 2.13 A, B, C, D). However, these pellets have less matrix and therefore are more cellular at a unit volume or area of micrograph. Overall, culturing within a pellet culture system maintained the cells viability.

Cartilage matrix constituents, glycosaminoglycans (GAGs) and collagen were deposited at different rates by the various pellets derived from surface, middle, deep and full depth chondrocytes.
The cellularity of each pellet was determined by the DNA content. With time in culture, the DNA content significantly increased within SZ, MZ and DZ derived pellets (p<0.0001) (fig 2.14). Within full depth cultures there was a significant decrease (almost 50%) between 7 and 14 days, followed by a significant increase (doubling) between 14 and 21 days.

When analysing GAG deposition an interesting trend was apparent. Although GAG deposition increased with time in culture between 7 and 14 days this was only significant within full depth pellets (fig 2.14). There was, however, a significant decrease in (or loss of GAG) between 14 and 21 days in all pellet cultures (p<0.0001).

With time in culture, collagen deposition significantly increased, almost doubling (p<0.0001) (fig 2.16) within MZ, DZ and FD derived pellets between 7 and 21 days. SZ derived pellets followed a similar trend, but, the increases were not significant.

When comparing the deposition of GAG between pellets following 21 days in culture (fig 2.15 & 2.17) MZ, DZ and FD derived pellets all had significantly greater (p<0.0001) amounts of GAG than SZ derived pellets. FD derived pellet also contained a significantly greater amount of GAG compared to MZ and DZ derived pellets. There were no significant differences in GAG concentration between MZ and DZ derived pellets following 21 days in culture. The GAG content of the native tissue significantly increased with distance from the surface, with deep zone derived native tissue (NTDZ) containing the greatest amount of GAG (fig 2.17).

The difference in GAG content between 21 day pellets and native tissue was significant in all cases (p<0.0001). The difference between 21 day pellet and the native tissue was 1:9, 1:5, 1:6, and 1:3 for SZ, MZ, DZ and FD respectively (fig 2.17).

When comparing the deposition of collagen between pellets following 21 days in culture (fig 2.16 & 2.18) MZ, DZ and FD derived pellets all had significantly greater (p<0.0001) amounts of collagen than SZ derived pellets. Both MZ and FD derived pellets contained significantly more collagen than DZ derived pellets.
The collagen content of the native tissue was also analysed. NTSZ and NTMZ were found to have significantly greater (p<0.0001) amounts of collagen than NTDZ. NTSZ and NTMZ also contained greater amounts of collagen than NTFD however, only NTMZ was significantly greater. NTFD also had a significantly greater amount of collagen than NTDZ (p<0.0001).

The difference in collagen content between 21 day pellets and native tissue was significant in all cases. The difference between 21 day pellet and the native tissue was 1:14, 1:5, 1:4, and 1:3 for SZ, MZ, DZ and FD respectively.

Within S/D and S/M/D stratified pellets, the tissue layers with cells of distinct origin were created and maintained following 21 days in culture (fig 2.19 A-F and fig 2.21 A-D). This was demonstrated by localisation of fluorescently labelled SZ derived chondrocyte labelled with CFDA (green), MZ unlabelled and visualised using phase contrast and DZ derived chondrocytes labelled with CMTMR (red). The ECM present in stratified (S/D) pellets was visualised by Safranin O staining after 7, 14 and 21 days of culture (fig 2.20 A-L). Safranin O staining showed focal areas of GAG deposition in all pellets analysed all time points. At 7 days the pellet (fig 2.20 A-D) contained focal areas of tightly packed cells with intensely stained matrix between them. By 14 days the cells were further separated by matrix (fig 2.20 E-H) and within 21 day pellets there were larger areas of matrix deposition (although weakly stained) (fig 2.20 K & L).

Following 21 days of culture the ECM within S/M/D pellets was visualised by Safranin O staining (fig 2.22). The pellets contained a mix of both small rounded and larger vacuolated chondrocytes separated by intensely stained matrix. No zonal organisation was observed.

Following 21 days of culture the S&D mixed pellets remained heterogeneous with no apparent evidence of cell sorting (fig 2.23 E&F). SZ and DZ had similar characteristics as the pellets cultured without cell tracker. Again Safranin O staining revealed areas of GAG deposition in all pellets following 21 days in culture (fig 2.24).
No SZP labelling was detected in stratified or mixed pellets. Type X collagen labelling was visualised in S/D stratified pellets only (fig 2.2).
Figure 2.2. Photomicrographs of the gross morphology of pellet. Surface zone (SZ) (A,B,C), middle zone (MZ) (D,E,F), deep zone (DZ) (G,H,I) and full depth derived chondrocytes (FD) (J,K,L) were cultured for 7 days (A, D, G, J), 14 days (B, E, H, K) and 21 days (C, F, I, L) in the form of pellets. Scale bar 1mm.

The pellets had a glassy blue/white appearance during cultivation and developed spheroidal cellular outgrowths.
Figure 2.3. The following scanning electron micrographs (SEM) were prepared from normal articular cartilage excised from a 7-day bovine metacarpal phalangeal (MCP) joint (A,B) and surface zone (SZ) (C,D), middle zone (MZ) (E,F), deep zone (DZ) (G,H) and full depth derived chondrocytes (FD) (I,J) cultured for 21 days in the form of pellets.

The surface of a normal bovine articular cartilage explant appeared smooth when visualised using scanning electron microscopy (SEM) (A). At higher power it was possible to see surface zone chondrocytes indenting the surface, arrows (B). At low power it was possible to assess the differences in size between the pellets. The surface of the SZ derived and FD derived pellets were highly cellular (D&J) - arrows. The surface areas of MZ (F) and DZ (H) derived pellets were covered in matrix, with occasional scattered cells - arrows.
Figure 2.4. Photomicrographs of pellets stained with Safranin O for glycosaminoglycans and counterstained with haematoxylin. Surface zone (SZ) (A,B,C), middle zone (MZ) (D,E,F), deep zone (DZ) (G,H,I) and full depth derived chondrocytes (FD) (J,K,L) were cultured for 7 days (A, D, G, J), 14 days (B, E, H, K) and 21 days (C, F, I, L) in the form of pellets. Scale bar = 100\(\mu\)m.

Inset: photomicrographs showing the cross section morphology of the pellet. Scale bar = 500\(\mu\)m.

Following 7 days of culture all pellets were similar in appearance when stained with Safranin O; however, weak stain was apparent between the tightly packed SZ cells (A). At day 14, distinct differences in morphology were observed: cells within the SZ pellet were rounded and closely packed with only small amounts of matrix between them (B). DZ and FD pellets contained larger vacuolated cells separated by more matrix (H&K). SZ pellets following 21 days in culture (C) contained densely packed cells separated by weakly stained ECM. MZ (D,E,F) DZ (G,H,I) and FD (J,K,L) derived pellets consisted of large vacuolated cells separated by an intensely stained matrix. Arrow indicates flattened chondrocytes at the edge.
**Figure 2.5.** Photomicrographs of pellets immunolabelled for type II collagen using ClICl antibody. Surface zone (SZ) (A,B,C), middle zone (MZ) (D,E,F), deep zone (DZ) (G,H,I) and full depth derived chondrocytes (FD) (J,K,L) were cultured for 7 days (A, D, G, J), 14 days (B, E, H, K) and 21 days (C, F, I, L) in the form of pellets. Scale bar = 100μm.

All pellets labelled for collagen type II. Labelling within SZ, DZ and FD and derived pellets was variable at all time points (A-C, G-I & J-L respectively). Labelling for collagen type II was weak within MZ derived pellets (D-F) and appeared cellular at 14 days.
Figure 2.6. Photomicrographs of pellets labelled for type IX collagen. Surface zone (SZ) (A,B,C), middle zone (MZ) (D,E,F), deep zone (DZ) (G,H,I) and full depth derived chondrocytes (FD) (J,K,L) were cultured for 7 days (A, D, G, J), 14 days (B, E, H, K) and 21 days (C, F, I, L) in the form of pellets. Scale bar = 100μm.

All pellets labelled for collagen type IX continuously however, labelling within 7-day SZ derived pellets appeared patchy (A) and sparse within 14-day MZ derived pellets (E).
Figure 2.7. Photomicrographs of pellets labelled for link protein. Surface zone (SZ) (A,B,C), middle zone (MZ) (D,E,F), deep zone (DZ) (G,H,I) and full depth derived chondrocytes (FD) (J,K,L) were cultured for 7 days (A, D, G, J), 14 days (B, E, H, K) and 21 days (C, F, I, L) in the form of pellets. Scale bar = 100 μm.

All pellets labelled for link protein antibody.
Figure 2.8. Photomicrographs of pellets labelled with 2B6 (chondroitin-4-sulphate and dermatan sulphate chains). Surface zone (SZ) (A,B,C), middle zone (MZ) (D,E,F), deep zone (DZ) (G,H,I) and full depth derived chondrocytes (FD) (J,K,L) were cultured for 7 days (A, D, G, J), 14 days (B, E, H, K) and 21 days (C, F, I, L) in the form of pellets. Scale bar = 100μm.

All pellets labelled with 2B6, however, labelling was variable within 14 day SZ derived pellets (B) and patchy following 21 days of culture (C). Labelling was also patchy within MZ derived pellets following 14 days of culture (E).
Figure 2.9. Photomicrographs of pellets labelled for type I collagen. Surface zone (SZ) (A), middle zone (MZ) (B), deep zone (DZ) (C) and full depth derived chondrocytes (FD) (D) following 21 days of culture in the form of pellets. Scale bar = 100μm.

All pellets labelled for collagen type I following 21 days in culture and this labelling was restricted to the periphery.
**Figure 2.10.** Photomicrographs of pellets labelled for SZP. Surface zone (SZ) (A, B, C) and full depth derived chondrocytes (FD) (D, E, F) were cultured for 7 days (A, D), 14 days (B, E) and 21 days (C, F) in the form of pellets. Scale bar = 100μm.

Only pellets derived from the SZ labelled continuously for SZP (A, B, C) and only occasional labelling for SZP was visualised within FD derived pellets (D, E, F).
Figure 2.11. Photomicrographs of pellets labelled for clusterin. Surface zone (SZ) derived chondrocytes (A, B, C) were cultured for 7 days (A), 14 days (B) and 21 days (C) in the form of pellets. Scale bar = 100μm.

Only a few cells labelled for clusterin within pellets derived from SZ.
Figure 2.12. Photomicrographs of pellets labelled for type X collagen. Surface zone (SZ) (A,B,C), middle zone (MZ) (D,E,F), deep zone (DZ) (G,H,I) and full depth derived chondrocytes (FD) (J,K,L) were cultured for 7 days (A, D, G, J), 14 days (B, E, H, K) and 21 days (C, F, I, L) in the form of pellets. Scale bar = 100μm.

Labelling for type X collagen was found within SZ derived pellets at days 7, 14 (A, B) and within DZ (G, H, I) and FD (J,K,L) derived pellets at all time points. No labelling was found within MZ derived pellets (D, E, F).
Figure 2.13. Photomicrographs of pellets labelled with ethidium homodimer (EtHD) to assess cell death. Surface zone (SZ) (A,B), middle zone (MZ) (C,D), deep zone (DZ) (E,F) and full depth (FD) (GH) derived chondrocytes were cultured for 21 days in the form of pellets. Corresponding phase images (B, D, F, H). Scale bar 100μm.

All pellets are viable following 21 days in culture however there appeared to be a higher proportion of cells labelling with EtHD within the SZ (A) than MZ (B), DZ (C) and FD (D) pellets. However, these pellets have less matrix and therefore are more cellular at a unit volume or area of micrograph.
Figure 2.14. Graph representing the DNA content (mean +/- standard deviation) in surface, middle, deep and full depth derived pellets following 7, 14 and 21 days in culture (n=8 from each experimental group). Statistically significant differences with time in culture and between pellets (p < 0.0001) are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>s7d v s14d</td>
<td>-0.387</td>
<td>-1.115 to 0.340</td>
<td></td>
</tr>
<tr>
<td>s7d v s21d</td>
<td>-0.819</td>
<td>-1.547 to -0.092</td>
<td>(significant)</td>
</tr>
<tr>
<td>s7d v m7d</td>
<td>-1.821</td>
<td>-2.574 to -1.069</td>
<td>(significant)</td>
</tr>
<tr>
<td>s7d v d7d</td>
<td>-1.613</td>
<td>-2.340 to -0.885</td>
<td>(significant)</td>
</tr>
<tr>
<td>s7d v fd7d</td>
<td>-1.900</td>
<td>-2.686 to -1.114</td>
<td>(significant)</td>
</tr>
<tr>
<td>s14d v s21d</td>
<td>-0.432</td>
<td>-1.160 to 0.295</td>
<td></td>
</tr>
<tr>
<td>s14d v m14d</td>
<td>-2.342</td>
<td>-3.069 to -1.614</td>
<td>(significant)</td>
</tr>
<tr>
<td>s14d v d14d</td>
<td>-2.834</td>
<td>-3.561 to -2.106</td>
<td>(significant)</td>
</tr>
<tr>
<td>s14d v fd14d</td>
<td>0.455</td>
<td>-0.273 to 1.182</td>
<td></td>
</tr>
<tr>
<td>s21d v m21d</td>
<td>-4.768</td>
<td>-5.495 to -4.040</td>
<td>(significant)</td>
</tr>
<tr>
<td>s21d v d21d</td>
<td>-4.830</td>
<td>-5.558 to -4.103</td>
<td>(significant)</td>
</tr>
<tr>
<td>s21d v fd21d</td>
<td>-1.104</td>
<td>-1.832 to -0.377</td>
<td>(significant)</td>
</tr>
<tr>
<td>m7d v m14d</td>
<td>-0.908</td>
<td>-1.660 to -0.155</td>
<td>(significant)</td>
</tr>
<tr>
<td>m7d v m21d</td>
<td>-3.766</td>
<td>-4.519 to -3.013</td>
<td>(significant)</td>
</tr>
<tr>
<td>m7d v d7d</td>
<td>0.209</td>
<td>-0.544 to 0.962</td>
<td></td>
</tr>
<tr>
<td>m7d v fd7d</td>
<td>-0.079</td>
<td>-0.888 to 0.731</td>
<td></td>
</tr>
<tr>
<td>m14d v m21d</td>
<td>-2.858</td>
<td>-3.585 to -2.131</td>
<td>(significant)</td>
</tr>
<tr>
<td>m14d v d14d</td>
<td>-0.492</td>
<td>-1.219 to 0.236</td>
<td></td>
</tr>
<tr>
<td>m14d v fd14d</td>
<td>2.797</td>
<td>2.069 to 3.524</td>
<td>(significant)</td>
</tr>
<tr>
<td>m21d v d21d</td>
<td>-0.063</td>
<td>-0.790 to 0.665</td>
<td></td>
</tr>
<tr>
<td>m21d v fd21d</td>
<td>3.663</td>
<td>2.936 to 4.391</td>
<td>(significant)</td>
</tr>
<tr>
<td>d7d v d14d</td>
<td>-1.608</td>
<td>-2.336 to -0.881</td>
<td>(significant)</td>
</tr>
<tr>
<td>d7d v d21d</td>
<td>-4.037</td>
<td>-4.764 to -3.310</td>
<td>(significant)</td>
</tr>
<tr>
<td>d7d v fd7d</td>
<td>-0.288</td>
<td>-1.073 to 0.498</td>
<td></td>
</tr>
<tr>
<td>d14d v d21d</td>
<td>-2.429</td>
<td>-3.156 to -1.701</td>
<td>(significant)</td>
</tr>
<tr>
<td>d14d v fd14d</td>
<td>3.288</td>
<td>2.561 to 4.016</td>
<td>(significant)</td>
</tr>
<tr>
<td>d21d v fd21d</td>
<td>3.726</td>
<td>2.999 to 4.453</td>
<td>(significant)</td>
</tr>
<tr>
<td>fd7d v fd14d</td>
<td>1.968</td>
<td>1.182 to 2.753</td>
<td>(significant)</td>
</tr>
<tr>
<td>fd7d v fd21d</td>
<td>-0.024</td>
<td>-0.809 to 0.762</td>
<td></td>
</tr>
<tr>
<td>fd14d v fd21d</td>
<td>-1.991</td>
<td>-2.719 to -1.264</td>
<td>(significant)</td>
</tr>
</tbody>
</table>
Figure 2.15. Graph representing the GAG content (mean +/- standard deviation) in surface, middle, deep and full depth derived pellets following 7, 14 and 21 days in culture (n=8 from each experimental group). Statistically significant differences with culture and between pellets (p < 0.0001) are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>s7d v s14d</td>
<td>-2.072</td>
<td>-7.775 to 3.631</td>
</tr>
<tr>
<td>s7d v s21d</td>
<td>5.942</td>
<td>0.239 to 11.644</td>
</tr>
<tr>
<td>s7d v m7d</td>
<td>-18.862</td>
<td>-24.564 to -13.159</td>
</tr>
<tr>
<td>s7d v d7d</td>
<td>-10.107</td>
<td>-15.810 to -4.404</td>
</tr>
<tr>
<td>s7d v fd7d</td>
<td>-12.078</td>
<td>-18.237 to -5.918</td>
</tr>
<tr>
<td>s14d v s21d</td>
<td>8.014</td>
<td>2.311 to 13.716</td>
</tr>
<tr>
<td>s14d v m14d</td>
<td>-20.868</td>
<td>-26.571 to -15.165</td>
</tr>
<tr>
<td>s14d v d14d</td>
<td>-13.046</td>
<td>-18.749 to -7.344</td>
</tr>
<tr>
<td>s14d v fd14d</td>
<td>-46.025</td>
<td>-51.727 to -40.322</td>
</tr>
<tr>
<td>s21d v m21d</td>
<td>-14.796</td>
<td>-20.498 to -9.093</td>
</tr>
<tr>
<td>s21d v d21d</td>
<td>-14.245</td>
<td>-19.948 to -8.543</td>
</tr>
<tr>
<td>s21d v fd21d</td>
<td>-30.935</td>
<td>-36.638 to -25.233</td>
</tr>
<tr>
<td>m7d v m14d</td>
<td>-4.078</td>
<td>-9.781 to 1.625</td>
</tr>
<tr>
<td>m7d v m21d</td>
<td>10.008</td>
<td>4.305 to 15.710</td>
</tr>
<tr>
<td>m7d v d7d</td>
<td>8.754</td>
<td>3.052 to 14.457</td>
</tr>
<tr>
<td>m7d v fd7d</td>
<td>6.784</td>
<td>0.624 to 12.943</td>
</tr>
<tr>
<td>m14d v m21d</td>
<td>14.086</td>
<td>8.383 to 19.789</td>
</tr>
<tr>
<td>m14d v d14d</td>
<td>7.821</td>
<td>2.119 to 13.524</td>
</tr>
<tr>
<td>m14d v fd14d</td>
<td>-25.157</td>
<td>-30.860 to -19.454</td>
</tr>
<tr>
<td>m21d v d21d</td>
<td>0.550</td>
<td>-5.152 to 6.253</td>
</tr>
<tr>
<td>m21d v fd21d</td>
<td>-16.140</td>
<td>-21.843 to -10.437</td>
</tr>
<tr>
<td>d7d v d14d</td>
<td>-5.011</td>
<td>-10.714 to 0.691</td>
</tr>
<tr>
<td>d7d v d21d</td>
<td>1.803</td>
<td>-3.899 to 7.506</td>
</tr>
<tr>
<td>d7d v fd7d</td>
<td>-1.971</td>
<td>-8.130 to 4.189</td>
</tr>
<tr>
<td>d14d v d21d</td>
<td>6.815</td>
<td>1.112 to 12.517</td>
</tr>
<tr>
<td>d14d v fd14d</td>
<td>-32.978</td>
<td>-38.681 to -27.276</td>
</tr>
<tr>
<td>d21d v fd21d</td>
<td>-16.690</td>
<td>-22.393 to -10.987</td>
</tr>
<tr>
<td>fd7d v fd14d</td>
<td>-36.019</td>
<td>-42.178 to -29.859</td>
</tr>
<tr>
<td>fd7d v fd21d</td>
<td>-12.916</td>
<td>-19.076 to -6.756</td>
</tr>
<tr>
<td>fd14d v fd21d</td>
<td>23.103</td>
<td>17.400 to 28.806</td>
</tr>
</tbody>
</table>

(significant)
The graph shows the distribution of uGAG/uDNase across different depths and time points. The x-axis represents different pellets (surface, middle, deep, full depth), while the y-axis represents the concentration of uGAG/uDNase. Three time points are indicated: 7 days, 14 days, and 21 days. The error bars indicate the variability or uncertainty in the measurements.
Figure 2.16. Graph representing the collagen content (mean +/- standard deviation) in surface, middle, deep and full depth derived pellets following 7, 14 and 21 days in culture (n=8 from each experimental group). Statistically significant differences with culture and between pellets (p < 0.0001) are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>S7d v S14d</td>
<td>-1.535</td>
<td>-9.047 to 5.977</td>
</tr>
<tr>
<td>S7d v S21d</td>
<td>-2.805</td>
<td>-10.317 to 4.707</td>
</tr>
<tr>
<td>S7d v M7d</td>
<td>-15.414</td>
<td>-22.926 to -7.903</td>
</tr>
<tr>
<td>S7d v D7d</td>
<td>-10.097</td>
<td>-17.855 to -2.339</td>
</tr>
<tr>
<td>S7d v FD7d</td>
<td>-11.193</td>
<td>-19.691 to -2.694</td>
</tr>
<tr>
<td>S14d v S21d</td>
<td>-1.270</td>
<td>-8.527 to 5.987</td>
</tr>
<tr>
<td>S14d v M14d</td>
<td>-22.114</td>
<td>-29.371 to -14.857</td>
</tr>
<tr>
<td>S14d v D14d</td>
<td>-11.561</td>
<td>-18.818 to -4.304</td>
</tr>
<tr>
<td>S14d v FD14d</td>
<td>-34.151</td>
<td>-41.408 to -26.894</td>
</tr>
<tr>
<td>S21d v M21d</td>
<td>-29.065</td>
<td>-36.322 to -21.808</td>
</tr>
<tr>
<td>S21d v D21d</td>
<td>-19.407</td>
<td>-26.664 to -12.150</td>
</tr>
<tr>
<td>S21d v FD21d</td>
<td>-34.787</td>
<td>-42.044 to -27.530</td>
</tr>
<tr>
<td>M7d v M14d</td>
<td>-8.235</td>
<td>-15.492 to -0.978</td>
</tr>
<tr>
<td>M7d v M21d</td>
<td>-16.455</td>
<td>-23.712 to -9.198</td>
</tr>
<tr>
<td>M7d v D7d</td>
<td>5.318</td>
<td>-2.194 to 12.829</td>
</tr>
<tr>
<td>M7d v FD7d</td>
<td>4.222</td>
<td>-4.053 to 12.496</td>
</tr>
<tr>
<td>M14d v M21d</td>
<td>-8.220</td>
<td>-15.478 to -0.963</td>
</tr>
<tr>
<td>M14d v D14d</td>
<td>10.553</td>
<td>3.296 to 17.810</td>
</tr>
<tr>
<td>M14d v FD14d</td>
<td>-12.037</td>
<td>-19.294 to -4.780</td>
</tr>
<tr>
<td>M21d v D21d</td>
<td>9.658</td>
<td>2.401 to 16.915</td>
</tr>
<tr>
<td>M21d v FD21d</td>
<td>-5.722</td>
<td>-12.979 to 1.535</td>
</tr>
<tr>
<td>D7d v D14d</td>
<td>-3.000</td>
<td>-10.511 to 4.512</td>
</tr>
<tr>
<td>D7d v D21d</td>
<td>-12.115</td>
<td>-19.627 to -4.603</td>
</tr>
<tr>
<td>D7d v FD7d</td>
<td>-1.096</td>
<td>-9.595 to 7.403</td>
</tr>
<tr>
<td>D14d v D21d</td>
<td>-9.115</td>
<td>-16.373 to -1.858</td>
</tr>
<tr>
<td>D14d v FD14d</td>
<td>-22.589</td>
<td>-29.847 to -15.332</td>
</tr>
<tr>
<td>FD7d v FD14d</td>
<td>-24.493</td>
<td>-32.768 to -16.219</td>
</tr>
<tr>
<td>FD7d v FD21d</td>
<td>-26.399</td>
<td>-34.673 to -18.124</td>
</tr>
<tr>
<td>FD14d v FD21d</td>
<td>-1.906</td>
<td>-9.163 to 5.351</td>
</tr>
</tbody>
</table>
Figure 2.17. Graph representing the GAG content (mean +/- standard deviation) in surface, middle, deep and full depth derived pellets following 7, 14 and 21 days in culture and within representative native tissue. Statistically significant differences with culture and between pellets (p < 0.0001) are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
<th>95% CI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>S3 v NTSZ</td>
<td>-76.249</td>
<td>-98.640</td>
<td>to -53.857</td>
<td>(significant)</td>
</tr>
<tr>
<td>S3 v M3</td>
<td>-14.948</td>
<td>-38.073</td>
<td>to 8.178</td>
<td></td>
</tr>
<tr>
<td>S3 v D3</td>
<td>-14.470</td>
<td>-37.595</td>
<td>to 8.656</td>
<td></td>
</tr>
<tr>
<td>S3 v FD3</td>
<td>-31.232</td>
<td>-54.358</td>
<td>to -8.107</td>
<td>(significant)</td>
</tr>
<tr>
<td>NTSZ v NTMZ</td>
<td>-36.222</td>
<td>-57.854</td>
<td>to -14.590</td>
<td>(significant)</td>
</tr>
<tr>
<td>NTSZ v NTDZ</td>
<td>-60.686</td>
<td>-82.318</td>
<td>to -39.055</td>
<td>(significant)</td>
</tr>
<tr>
<td>NTSZ v NTFD</td>
<td>-45.486</td>
<td>-67.118</td>
<td>to -23.854</td>
<td>(significant)</td>
</tr>
<tr>
<td>M3 v NTMZ</td>
<td>-97.523</td>
<td>-119.914</td>
<td>to -75.132</td>
<td>(significant)</td>
</tr>
<tr>
<td>M3 v D3</td>
<td>0.478</td>
<td>-22.647</td>
<td>to 23.604</td>
<td></td>
</tr>
<tr>
<td>M3 v FD3</td>
<td>-16.285</td>
<td>-39.410</td>
<td>to 6.841</td>
<td></td>
</tr>
<tr>
<td>NTMZ v NTDZ</td>
<td>-24.465</td>
<td>-46.096</td>
<td>to -2.833</td>
<td>(significant)</td>
</tr>
<tr>
<td>NTMZ v NTFD</td>
<td>-9.264</td>
<td>-30.896</td>
<td>to 12.368</td>
<td></td>
</tr>
<tr>
<td>D3 v NTDZ</td>
<td>-122.485</td>
<td>-144.857</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3 v FD3</td>
<td>-16.763</td>
<td>-39.888</td>
<td>to 6.363</td>
<td></td>
</tr>
<tr>
<td>NTDZ v NTFD</td>
<td>15.201</td>
<td>-6.431</td>
<td>to 36.833</td>
<td></td>
</tr>
<tr>
<td>FD3 v NTFD</td>
<td>-90.502</td>
<td>-112.893</td>
<td>to -68.111</td>
<td>(significant)</td>
</tr>
</tbody>
</table>
Figure 2.18. Graph representing the collagen content (mean +/- standard deviation) in surface, middle, deep and full depth derived pellets following 7, 14 and 21 days in culture and within representative native tissue. Statistically significant differences with culture and between pellets (p < 0.0001) are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>95% CI</td>
</tr>
<tr>
<td>NTSZ v S3</td>
<td>1074.624</td>
<td>891.842</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>NTSZ v NTMZ</td>
<td>-343.903</td>
<td>-526.685</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>NTSZ v NTDZ</td>
<td>268.834</td>
<td>92.250 to 445.419</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>NTSZ v NTFD</td>
<td>136.250</td>
<td>-40.334 to 312.835</td>
</tr>
<tr>
<td>S3 v M3</td>
<td>-222.893</td>
<td>-411.670 to -34.117</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>S3 v D3</td>
<td>-147.866</td>
<td>-336.642 to 40.911</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>S3 v FD3</td>
<td>-250.062</td>
<td>-438.838 to -61.285</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>NTMZ v M3</td>
<td>1195.634</td>
<td>1006.857</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>NTMZ v NTDZ</td>
<td>612.737</td>
<td>429.954 to 795.519</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>NTMZ v NTFD</td>
<td>480.153</td>
<td>297.370 to 662.935</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>M3 v D3</td>
<td>75.028</td>
<td>-113.749 to 263.805</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>M3 v FD3</td>
<td>-27.168</td>
<td>-215.945 to 161.609</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>NTDZ v D3</td>
<td>657.925</td>
<td>475.142 to 840.707</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>NTDZ v NTFD</td>
<td>-132.584</td>
<td>-309.169 to 44.000</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>D3 v FD3</td>
<td>-102.196</td>
<td>-290.973 to 86.581</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
<tr>
<td>NTFD v FD3</td>
<td>688.313</td>
<td>505.530 to 871.095</td>
</tr>
<tr>
<td></td>
<td>(significant)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.19: Photomicrographs of representative sections of skeletal pellets. Superficial zone derived chondrocyte and deep zone derived chondrocyte (SZD) following 7 days (A, B), 14 days (C, D) and 21 days (E, F) culture at low magnification (A, C, E) and at high power magnification (B, D, F). SZ derived chondrocytes are labelled with CFDA (green) and SZ derived chondrocytes with CMFDA (red). Scale bar = 100µm.
Figure 2.19 Photomicrograph of representative sections of stratified pellets; Superficial zone derived chondrocytes atop deep zone derived chondrocytes (S/D) following 7 days (A, B), 14 days (C, D) and 21 days (E, F) culture at low magnification (A, C, E) and at high power magnification (B, D, F). SZ derived chondrocytes are labelled with CFDA (green) and DZ derived chondrocytes with CMTMR (red). Scale bar = 100μm.
Figure 2.20. Photomicrograph of representative sections of stratified pellets stained with Safranin O and counterstained with haematoxylin; Superficial zone derived chondrocytes atop deep zone derived chondrocytes (S/D) following 7 days (A, B, C, D), 14 days (E, F, G, H) and 21 days (I, J, K, L) at low magnification (A, E, J) and at high power magnification (other). SZ derived chondrocytes were labelled with CFDA and DZ derived chondrocytes with CMTMR. Scale bar= 200μm: A, E, I & 100μm (B,C,D,F, G, H, J, K, L).
Figure 2.21 Photomicrograph of representative sections of stratified pellets; middle (MZ) layer atop deep (DZ) with superficial (SZ) atop middle (S/M/D) following 7 days (A, B) and 21 days (C, D) at low magnification (A, C) and at high power magnification (B, D). SZ derived chondrocytes are labelled with CFDA green, MZ are not labelled and DZ derived chondrocytes are labelled with CMTMR (red). Scale bar = 100μm.
Figure 2.22. Photomicrograph of representative sections of stratified pellets stained with Safranin O and counterstained with haematoxylin; Superficial zone derived chondrocytes atop middle zone derived chondrocytes atop deep zone derived chondrocytes (S/M/D) following 21 days at low magnification (A) and at high power magnification (B, C &D). SZ derived chondrocytes were labelled with CFDA and DZ derived chondrocytes with CMTMR. Scale bar = 200μm: A & 100μm (B, C & D).
Figure 2.23. Photomicrograph of representative sections of pellets; Superficial zone (SZ) derived chondrocytes (A,B), deep zone (DZ) derived chondrocytes (C,D) and SZ and DZ derived chondrocytes mixed (S&D) and following 21 days in culture at low magnification (A, C, E) and at high power magnification (B, D, F). SZ derived chondrocytes are labelled with CFDA and DZ derived chondrocytes with CMTMR. Scale bar = 100µm.
Figure 2.24. Photomicrograph of representative sections of pellets stained with Safranin O and counterstained with haematoxylin; Superficial zone (SZ) derived chondrocytes (A, B, C, D), deep zone (DZ) derived chondrocytes (E, F, G) and SZ and DZ derived chondrocytes mixed (S&D) (H, I, J, K) 21 days in culture at low magnification (A, E, J) and at high power magnification (B, C, D, F, G, H, J, K). SZ derived chondrocytes were labelled with CFDA and DZ derived chondrocytes with CMTMR. Scale bar= 200μm: A, E, I & 100μm (B, C, D, F, G, H, J, K).
Figure 2.25. Photomicrograph of representative sections of stratified pellets labelled for type X collagen; superficial zone derived chondrocytes atop deep zone derived chondrocytes (S/D) following 21 days in culture at low magnification (A) and at high power magnification (B,C). SZ derived chondrocytes are labelled with CFDA and DZ derived chondrocytes with CMTMR. Scale bar = 100μm.
Figure 2.26. Photomicrograph of representative positive control sections of primary antibodies used. 7-day bovine tendon labelled with collagen type I (A). 7-day bovine articular cartilage labelled for 2B6 (B), collagen type IX (C) and link protein (D). The articular surface of 7-day bovine cartilage labelled for SZP (E) and the deeper zones labelled for collagen type X (F). Negative controls included excluding primary antibody (G) and by using MIG (H). Scale bar = 100mm.
2.4 DISCUSSION

We isolated surface zone (SZ), middle zone (MZ), deep zone (DZ) and full depth (FD) chondrocytes and cultured them as high-density pellets (1 x10/ml) for up to 21 days. The pellet cultures initially contained zero ECM, and, thus, the effects of time in culture upon synthesis of GAG and other matrix components and deposition could be analysed as well as the release and degradation of matrix macromolecules in any one culture well. In this study, we analysed the morphology of the pellet and the accumulation of matrix within the pellet alone.

The use of the dissecting microscope demonstrated the glassy blue/white appearance of the pellets formed during cultivation, which resemble the appearance of native cartilage (example found in 2.1a). All cells developed outgrowths from their surface, which may be due to an increased proliferation in that area. The reasons for such an outgrowth are unclear. In comparing differences in size, the results of the SEM may be artefactual due to the extensive fixation process carried out, and the effect this may have on the matrix components. It was difficult to ascertain which pellets were bigger using the dissecting microscope. At 14 and 21 days the SZ derived pellets appeared smaller than the rest. Future studies would involve quantification of size for example surface area and more importantly depth since surface area is dictated by the diameter of the culture tube.

The surface of normal bovine articular cartilage explants appeared smooth when viewed using scanning electron microscopy. At higher power it was possible to see surface zone chondrocytes indenting the surface. SEM analysis was used to visualise chondrocytes and the 3D structure of the synthesised ECM. Chondrocytes that were visualised on the surface of the pellet retained a spherical shape, indicative of a well-differentiated phenotype. Using the SEM at low power was useful to assess any differences in size of the pellets analysed. SZ derived pellets were the smallest, followed by full depth, with middle and deep roughly equal in size, reflecting the differences in matrix production and proliferation. The surface of the SZ and FD derived pellets were highly cellular, with clusters of rounded cells wrapped in matrix. The surface areas of MZ and DZ derived pellets were covered in matrix and looked similar to the native cartilage. Clusters of cells were attached to the surface.
Studies by Riesle et al., (1998) addressed the 3D structure of collagen in engineered constructs by firstly removing proteoglycan from tissue samples (using the enzymes hyaluronidase and α-chymotrypsin). Such a treatment or perhaps simply fracturing the pellets would have benefited our study as it would have allowed the infrastructure of the pellets to be analysed. Ultrastructural analysis of the pericellular microenvironment in adult human articular cartilage using SEM and TEM has previously revealed that each chondrocyte is surrounded by a filamentous matrix which in the middle and deep layers is separated from the adjacent territorial matrix by a felt like structure composed of fine fibrillar materials. No distinct capsule, however, was found around the superficial layer of chondrocytes (Poole et al., 1987). Clearly these findings reflect the importance of the development of an adjacent territorial matrix (or not in the case of the superficial zone), to the function of the specific zonal layer and thus overall function of articular cartilage. Eyre et al, (1987) using SEM also demonstrated that the collagen network organisation in engineered constructs were indistinguishable from that in natural cartilage correlating well with the finding of fraction of collagen IX which is pivotal for the development of a stable interconnected 3D collagen mesh.

Although pellets from SZ, MZ, DZ and FD initially contained closely packed cells recapitulating certain features of immature tissue such as high cellularity and little ECM, over the 21-day culture period distinct differences in pellet morphology were observed. SZ pellets remained tightly packed and were separated by a sparse ECM. DZ chondrocytes within the pellet become increasingly large and vacuolated, suggestive of hypertrophic chondrocytes as they also labelled for type X collagen. MZ and FD pellets contained a mixed population of both small rounded cells and larger vacuolated cells.

All pellets labelled for collagen types II and IX, link protein and 2B6 (indicative of the presence of chondroitin-4-sulphate and dermatan sulphate chains) throughout their ECM at all time points demonstrating that the pellets expressed molecules typical of articular cartilage. Collagen type II is described as a marker of differentiated chondrocytes but also a prerequisite for a mechanically functional collagen network (Benya et al., 1977). Collagen type IX is the only specific marker for differentiated
chondrocytes (Morrison et al., 1996). The widespread labelling for link protein indicated the presence of the large aggregating molecule –aggrecan.

It is conclusive that within the pellet culture systems the subpopulations of chondrocytes retained their characteristic morphologies and synthesis of cartilage specific molecules. However, distinct differences in pellet morphology required further investigation to identify SZ, MZ and DZ markers within the pellets, e.g. superficial zone protein (SZP) (Schumacher et al., 1994) or clusterin (Khan et al., 2001) and collagen type X (Schmid and Linsenmayer, 1987) respectively. Human adult mesenchymal stem cells derived from bone marrow aspirates have the ability to differentiate into chondrocytes under specific culture conditions by growth factors and express cartilage specific collagens; type II and X as well as proteoglycans (Naumann et al., 2002). Pellets derived from the SZ labelled continuously for SZP and only punctate labelling for SZP was visualised within FD derived pellets. Clusterin, a secreted glycoprotein, is expressed at high levels by chondrocytes residing in the surface zone of articular cartilage (Khan et al., 2001). It has been demonstrated within our own laboratory that chondrocytes from the superficial zone can also be expanded and maintain expression of SZP following redifferentiation in high density culture and in monolayer. Synovial cells have also been shown to express SZP and, thus, could also be used in attempts to engineer cartilage (Schumacher et al., 1999).

Within this study there was punctate clusterin labelling within SZ derived pellets only. The biochemical function of clusterin within cartilage has yet to be conclusively demonstrated.

Labelling for type X collagen was found within SZ derived pellets at days 7 and 14 and within DZ and FD derived pellets at all time points. No labelling was found within MZ derived pellets. Type X collagen is found exclusively within cartilage, its synthesis is restricted to the calcified cartilage zone, hence it is often used as a marker for terminal differentiation (Gannon et al., 1991). Type X is also expressed in the surface during development (Morrison et al., 1996) but can be re-expressed by osteoarthritic chondrocytes (von der Mark and Gluckert, 1990). Archer et al., (1990) proposed that this mis-expression by the chondrocytes in the diseased joint may indicate an attempt to return to an embryonic phenotype aiding repair and initiating
growth (see section 1.14.1) which may indicate a reason for its presence within SZ pellets.

Type I collagen was found following 21 days in culture within SZ, MZ, DZ and FD derived chondrocytes at the periphery. Collagen type I has been found in the superficial and calcifying regions of cartilage (Wardale and Duance, 1993). Fibrocartilage consists of dedifferentiated chondrocytes and mainly collagen type I (Eyre and Wu, 1983; Minas and Nehrer, 1997) and is insufficient as a weight bearing tissue and, therefore, it is encouraging that type I collagen was only found within the periphery of our pellets. Fibrocartilage cells can account for a whole range of tissue types from near hyaline to near fibrous. This may reflect the shape of the cell recapitulating the surface zone, and related to cell shape in the same way as the perichondrium.

The assessment of cell death using ethidium homodimer revealed that overall culturing within a pellet culture system maintained the cells' viability. There appeared to be a higher proportion of cells labelling within SZ than MZ, DZ and FD derived pellets. This may also reflect the results of the DNA assay, as all pellet cultures began the experiment with the same amount of cells (1x10^6). SZ derived pellets had a significantly lower amount of DNA compared to all other pellets at all time points. This may reflect a lower proliferative capability or a higher rate of cell death due to the amount of positive ethidium homodimer labelling found within the SZ pellet compared to other pellets. Proliferation within high density cultures is low compared to a rapidly dividing monolayer culture (Mitrovic et al., 1979). With time in culture the DNA content significantly increased (p<0.0001) indicative of cell proliferation. We did not, however, measure the DNA content at time 0. Within full depth cultures, there was a significant decrease (almost 50%) between 7-14 days followed by a significant increase (doubling) between 14-21 days. It is unclear why this decrease occurred. Perhaps it was due to the presence of a heterogeneous population. Although this explanation appears to be verified by the GAG analysis it must be remembered that these figures are generated by the ratio of GAG/DNA, reflecting increased production of ECM macromolecules per cell. Therefore, the lower DNA allows for an artificial 'jump' in GAG synthesis. When re-analysing the raw data there is no sudden jump in GAG synthesis.
Following 21 days of culture, MZ and DZ derived pellets had similar quantities of DNA, whereas SZ and FD were significantly lower. Waldman et al. (2003) reflected that the cellularity of their in vitro formed tissue was also a function of the chondrocyte population used. They showed that the tissue produced by the deep chondrocytes was significantly more cellular than middle & deep combined tissue and the full depth derived tissue. We cannot compare our results with their middle & deep tissue but our results are similar in that of cellularity. FD derived pellets were significantly lower (in fact 50% in both cases). Direct comparisons cannot be made however since as the group used 20% FCS compared to our 10% FCS/media and their study was carried out over 8 weeks using a porous CPP substrate.

It is difficult to compare our results with other studies comparing sub-populations as many groups use different methods of quantification. For example Klein et al., (2003) used mg/cm², we could not analyse wet weight as the pellets are so small and so we referred all our data back to µg/µg DNA i.e. on a cellular basis.

When analysing GAG deposition an interesting trend was apparent. Although GAG deposition increased with time in culture between 7 and 14 days this was only significant within full depth pellets. There was however a significant decrease in (or loss of GAG) between 14 and 21 days in all pellet cultures (p<0.0001). It was difficult for us to weigh the pellets and therefore we were unable to calculate GAG content as µg/mg dry weight in this study. The reason for this decrease is unclear. However, it is important to realise that we did not as analyse “total” GAG deposition as we did not analyse the media. We were only concerned with what was retained within the pellet. It is possible therefore that following 14 days in culture that any further GAG synthesised would be released into the media, due to an inadequately formed collagen network.

When comparing the deposition of GAG between pellets following 21 days in culture, MZ, DZ and FD derived pellets all had significantly greater amounts of GAG than SZ derived pellets. The GAG content of the native tissue significantly increased with distance from the surface, with deep zone derived native tissue (NTDZ) containing the greatest amount of GAG. Previous studies which examined native cartilage also reported increasing rates of proteoglycan synthesis with increasing depth of articular
surface (Aydelotte et al., 1988; Jacoby and Jayson, 1975; Korver et al., 1990; Marles et al., 1991; Muir et al., 1970; Parkkainen et al., 1990; Wong et al., 1996). The groups demonstrated the highest rate of synthesis either in the intermediate or deep zones of the tissue, which correlates with our results. Unfortunately, within this study we did not look at the rates of synthesis or determine the monomeric size of the proteoglycan produced. The dimethylmethylen blue-dye binding assay used to quantify GAG would, therefore, interpret the presence of larger-sized proteoglycans as a greater accumulation of GAG. It has been previously shown that middle zone chondrocytes synthesise larger proteoglycan monomers (Korver et al., 1990).

FD derived pellet contained a significantly greater amount of GAG compared to MZ and DZ derived pellets. These results are difficult to interpret as previous studies have shown that tissue formed by full depth cells in their study produced the lowest proportion of proteoglycans (Waldman et al., 2003) which corresponds to the results of the native tissue. They hypothesised that this was due to the presence of SZ derived chondrocytes within the cultures.

The difference in GAG content between 21 day pellets and native tissue was significant in all cases. The difference between 21 day pellets and the native tissue was 1:9, 1:5, 1:6, and 1:3 for SZ, MZ, DZ and FD respectively. This information is important if we want to regenerate cartilage with similar characteristics as the native tissue. Our study was only over 3 weeks and therefore relatively short term, and, thus, an important consideration during the tissue engineering of cartilage is the degree of in vitro maturation during culture. However, it is interesting to note that the closest ratio to native was with FD cultures.

Lee et al., (1993) demonstrated using agarose cultures that GAG synthesis and \(^{3}\)H (tritiated) thymidine incorporation rates were greater in deep cells than surface cells, which complimented studies using isolated chondrocytes and intact cartilage (Aydelotte and Kuettner, 1988; Lee et al., 1993; Marles et al., 1991; Wong et al., 1996).

It must be remembered that this was only a 3 week period and that the native tissue has developed during gestation; a period of 279 days on average (Terri, 2004)
followed by 7 days post-gestation. It is possible that an extended culture duration with stimulation with growth factors and the use of a bioreactor culture (involving movement and mechanical influences) (Martin et al., 2000) would improve matrix production. Still to be elucidated, however, are the mechanisms of regulation of chondrocyte and tissue growth during extensive in vitro culture conditions.

As well as containing the largest proportion of GAG, FD derived pellets contained the largest proportion of collagen in comparison to SZ, MZ and DZ derived pellets. MZ, DZ and FD derived pellets following 21 days of culture all had significantly greater amounts of collagen than SZ derived pellets. Both MZ and FD derived pellets contained significantly more collagen than DZ derived pellets.

Of interest is that in all pellets analysed, a higher ratio of collagen was synthesised compared to GAG (μg DNA).

The collagen content of the native tissue was also analysed. NTSZ and NTMZ were found to have significantly greater amounts of collagen than NTDZ. NTMZ contained greater amounts of collagen than NTFD. NTFD also had a significantly greater amount of collagen than NTDZ. The difference in collagen content between 21-day pellets and native tissue was significant in all cases. The difference between 21 day pellets and the native tissue was 1:14, 1:5, 1:4, and 1:3 for SZ, MZ, DZ and FD respectively. This demonstrates again that the pellets do not produce the same amount of collagen as their native zonal chondrocytes. The SZ derived pellet produced the lowest amount of collagen and had the greatest difference in ratio whilst again the FD pellets had the closest.

Although (Riesle et al., 1998) demonstrated that the wet weight fraction of total collagen in engineered cartilage after 6 weeks of cultivation was significantly lower than bovine calf articular cartilage, interestingly, comparable fractions of collagens type II and IX were found in the engineered cartilage to that of natural cartilage. Constructs cultured for 4 weeks, however, only had 30% as many pyridinium crosslinks as compared with those in natural cartilage.
It would have benefited this study to have analysed the crosslinks found within the pellets as tensile strength of the collagen fibril depends on the intermolecular crosslinks between collagen molecules within the triple helix and on fibril diameter. A considerable zonal variation has been observed with regards to crosslinks (Bank et al., 1998). This information would benefit future attempts at repair as previous attempts at regenerating cartilage have observed a reduced crosslink content as well as lower collagen cross-link density relative to native cartilage (Riesle et al., 1998; Waldman et al., 2003).

These results are consistent with studies by Aydelotte et al. (1988a) who demonstrated that when articular cartilage sub-populations were isolated and cultured in suspension, that the chondrocytes retained their zonal characteristics and continued to perform their role as matrix producers. DZ cells also produced more GAG than SZ chondrocytes in this study.

Pellet cultures such as a 3D cell culture system have the advantage of allowing anchorage independent cell growth allowing cell motility, the synthesis of a specific pericellular and/or intracellular matrix as well as the physiological release and storage (binding) of bioactive molecules such as cytokines and morphogenetic factors.

Previous pellet culture studies have utilised rabbit epiphyseal chondrocytes (Kato et al., 1988). They were able to study the differentiation of epiphyseal chondrocytes and revealed that with time, the initial cartilage like matrix synthesised reorganised itself and, chondrocytes were able to terminally differentiate. They demonstrated that blocking interactions of the growth plate chondrocytes’ integrins with RGD-containing ECM proteins suppressed accomplishment of terminal differentiation as well as the accompanied matrix mineralisation. Maintenance of the chondrogenic phenotype is primarily achieved by cell interaction with the ECM (possibly via integrins) triggering actin polymerisation, which results in focal contact formation and the stabilisation of cell shape. (Daniels and Sollursh, 1991).

Full depth bovine chondrocytes have been studied using 3D cell pellet culture systems (Xu et al., 1996, Croucher et al., 2000). However, we believe we are the first to look at the effects of high-density cell culture on subpopulations within the pellet system.
Various groups have studied the effects on these subpopulations denuded from original matrix within suspension cultures (Archer et al., 1990; Aydelotte et al., 1988; Aydelotte and Kuettner, 1988) and concluded that they retain their metabolic features characteristic of their layer of origin in terms of quantitative synthesis. Recent studies have studied the effects of various growth factors on high-density full depth pellet cultures. Their studies revealed that chondrocytes increase synthesis/deposition of proteoglycan within the matrix with addition of insulin-like growth factor-1 (IGF-1) and foetal calf serum (FCS) (Curtis et al., 1992).

IGF-1 and TGF-β promote matrix synthesis and cartilage repair respectively. IGF-I is a potent stimulator of proteoglycan synthesis on chondrocytes and may inhibit proteoglycan degradation by inhibiting the synthesis of proteolytic enzymes (Tyler 1989). It would be interesting to investigate the effects of IGF-1 on type II collagen synthesis within the pellet cultures as contradictory results prevail; some investigators have shown no effect (Horton, 1989) while others demonstrated an increase in synthesis (Curtis, 1992). There are again contradictory results regarding the effects of TGFβ. Hiraki (1988) demonstrated a stimulatory effect on cartilage matrix synthesis while others demonstrated an inhibitory effect (Horton, 1989). Xu (1996) demonstrated that TGFβ increases synthesis of proteoglycan within pellet cultures, but there was a decrease in deposition of the molecule into the ECM due to increased release of GAG into the medium - possibly due to direct inhibition of aggrecan binding into the matrix by a decrease in synthesis of link protein. However, in our study link protein appeared prevalent in the pellet matrix although demonstrated by immunohistochemistry that is not quantitative. TGFβ also causes a biphasic response dependent upon concentration.

In a recent study, it was demonstrated that adenosine triphosphosphate (ATP) and uridine triphosphate in the presence of serum increased sulphated GAG and collagen deposition above control levels in full depth bovine articular chondrocyte pellet cultures (Croucher et al., 2000). This study suggests that therapeutic agents aimed at activating receptors involved in the anabolic response (above) may be beneficial in the treatment of degenerative diseases such as OA and RA. Xu et al., (1996) demonstrated that ATP inhibited and IL-1 stimulated proteoglycan breakdown in
articular cartilage explants. Thus, it is unknown whether the increased accumulation of matrix is in fact due to an inhibition of breakdown or an increase in synthesis and successful deposition or a combination of both. Future studies could assess the affects of existing therapeutic agents on pellet cultures.

Within S/D and S/M/D stratified pellets, the tissue layers with cells of distinct origin were created and maintained following 21 days in culture. This maintenance was demonstrated by localisation of fluorescently labelled SZ derived chondrocytes labelled with CFDA (green), MZ cells by using phase contrast and DZ derived chondrocytes labelled with CMTMR (orange). Klein et al., (2003) also used cell trackers to track the cells and stated that surface atop middle derived chondrocytes (S/M) constructs “were created and maintained as demonstrated by localisation of fluorescent- labelled surface and middle zone cells”. They did not demonstrate this within their paper with any photographic evidence.

The ECM present in stratified (S/D) pellets was visualised by Safranin O staining after, 7, 14 and 21 days of culture. Safranin O staining showed focal areas of GAG deposition in all pellets analysed all time points. At 7 days, the pellet contained focal areas of tightly packed cells with intensely stained matrix between them. By 14 days the cells were further separated by matrix and within 21 day pellets there were larger areas of matrix deposition (although weakly stained). Following 21 days of culture the S&D mixed pellets remained heterogeneous with no apparent evidence of cell sorting. SZ and DZ had similar characteristics as the pellets cultured without cell tracker indicating no adverse affects by using them. Again Safranin O staining revealed areas of GAG deposition in all pellets following 21 days in culture. Following 21 days of culture the ECM within S/M/D pellets was also visualised by Safranin O staining. The pellets contained a mix of both small rounded and larger vacuolated chondrocytes separated by intensely stained matrix. No zonal organisation was observed. However, the pellets were only cultured for 21 days and, thus, further cultivation may have induced or allowed cellular organisation.

It would be of interest to analyse the amount of matrix accumulated within the stratified pellets, as Waldman et al., (2003) showed that constructs (calcium polyphosphate substrate in the form of 4mm diameter cylinders) generated using the
combined populations of intermediate and deep zone derived chondrocytes accumulated the highest proportion of proteoglycans and formed tissue with substantially superior compressive mechanical properties when it was compared to tissues produced by the other chondrocyte populations. Klein et al., (2003) compared stratified constructs; surface derived chondrocytes atop middle derived chondrocytes (S/M) with S and M alone, prepared by seeding the cells onto cell inserts with a 0.4μm pore size polyester membrane (the chondrocytes following their isolation from bovine knees were first cultured within alginate cultures to generate “an associated matrix”). The study demonstrated that middle (M) constructs were thicker than (S/M) which were also thicker than surface (S) alone which is in agreement with our study (comparing S and M derived chondrocyte pellets) which correlated with their GAG content. Although there was an observed increase in DNA with time, no differences were noted between the cultures. An increase in the compressive modulus with time was noted being greater in M than S or in S/M. The group used Alcian blue dye with conditions specific for chondroitin and keratan sulphate, which was visualised throughout the constructs, appearing “heavier” at the bottom. It was difficult to agree with this statement as the presenting image did not reflect this. The study revealed collagen II labelling throughout the construct as important. We did not immunolabel for collagen type II within the stratified pellets.

In agreement to our initial experiment within this chapter, Klein et al., (2003) demonstrated a high proportion of SZP in S constructs, with a lower frequency detected within M and S/M constructs which is interesting as no SZP was detected in middle derived pellets within our study. It is possible that contamination of the surface layer could explain this particular groups’ results. Noteworthy, however, was the revelation that cells at the top (free surface of the constructs) and bottom (membrane) surfaces of S constructs labelled more frequently than the interior, while cells at the free surface labelled more frequently than other cells for SZP. This could be explained by the cells adopting positional information due to phenotypic dependence on nutrient levels, chemical gradients in the tissue cell- cell, cell-matrix interactions or simply but less likely by contamination during isolation. Work by Tew et al., (2000) demonstrated that when a plug of cartilage was removed from a bovine explant, upturned and then replaced the deep cells that were now at the surface of the explant began to synthesise 3B3- epitope a characteristic of hypertrophic
chondrocytes. Klein et al., (2003) concluded that chondrocyte subpopulations can play an important role in determining the structure, composition, metabolism and mechanical function of tissue engineered constructs.

Future attempts of cartilage repair should use multiple chondrocyte populations to form cartilage with stratification features typical of normal articular cartilage. Previous studies using full depth chondrocytes lose the functional properties of each zone, as demonstrated in my study with only a few cells labelling with SZP within full depth pellets e.g. SZP appears to have important mechanical properties, being closely related to the lubricin molecule (Swann et al., 1985), imparting lubricating properties to the articular surface (Jay et al., 2001) and the higher resistance to compression in deeper layers (Schinagl et al., 1997).

Pellet formation of stratification could be modified by mimicking the methods used by Klein et al. (2003) using alginate, as it has been shown to facilitate cartilage formation. This could be further developed by using substrate scaffolds such as those described by Waldman et al. (2003) and Freed et al. (1993). Introducing a lag phase between layering of the chondrocyte population may aid the development of a stratified construct with properties resembling native cartilage. Future studies may benefit from culturing the isolated sub-populations individually for a period of time and then stratify them following establishment of their unique characteristics, although integration of the respective matrices may be problematic.

Intrinsic differences in the response of cells from surface and deep zones to mechanical compression have been shown to exist in terms of GAG synthesis (Lee et al., 1998). The study demonstrated marked differences in GAG synthesis by surface and deep zone derived cells. Deep zone cells exhibited a frequency dependent response to dynamic strains, with 0.3HZ producing a significant inhibition in GAG synthesis and 1HZ inducing a 50% stimulation of synthesis. In contrast, surface zone cells exhibited a generalised reduction in GAG synthesis in response to strain. The group proposed that distinct mechanotransduction pathways may be induced that elicit variations in local responses, which may be important in maintaining local variations in ECM composition, mechanical properties and overall tissue integrity. However, Knight et al. (1998) showed that the effects of mechanical loading on GAG content
were greatest in the superficial zone of intact cartilage, but this has been proposed to be due to intrinsic differences being masked when intact tissue is used. Perhaps, qPCR of proteoglycan core proteins may help resolve this issue.

It was recently demonstrated that hydrostatic pressure enhanced the cartilaginous matrix formation of mesenchymal progenitor cells' differentiation in vitro, revealing a quantitative difference in ECM production between non-loaded and cyclically loaded aggregated cells undergoing chondrogenic differentiation. (Angele et al., 2003). The physiological range is 3 – 10 MPa (Mow et al., 1992). This result highlights that again mechanical forces play an important role in cartilage repair and regeneration in vivo. It has also been shown that chondrocytes react in the same manner, increasing their ECM production when intermittent pressure (2.96-9.86 MPa) is applied to them in vitro (Buschmann et al., 1995; Carver and Heath, 1999; Hall et al., 1991; Suh et al., 1999). The importance of physiologic load during skeletal tissue regeneration is demonstrated during the chondrogenic differentiation of mesenchymal stem cells in secondary fracture repair (Bolander, 1992), early articular cartilage regeneration, when subchondral bone is penetrated and marrow fills the defect (Shapiro et al., 1993), during development of the limb (Fell and Canti 1925) and during secondary cartilage induction.

Future attempts at cartilage regeneration and repair should address chondrocytes as separate cell populations rather than the preconceived single cell type, as it has been clearly shown by this study that SZ, MZ, DZ and FD derived chondrocytes behave very differently both in vivo and in vitro. Important considerations are that chondrocyte metabolism in vitro is not only influenced by the mechanical environment through mechanotransduction pathways involving hydrostatic pressure, cell deformation, streaming potentials, variations in osmotic and pH environment and fluid flow (Gray et al., 1988; Guilak et al., 1994; Urban, 1994) but cell density, type of construct used, medium additives and time in culture.

The limiting factor that is not highlighted in our study is that bovine cartilage is thick, and, therefore, contains a substantial amount of chondrocytes; a quantity that would never be extracted from an autologous biopsy from human cartilage. We have a
limited supply of chondrocytes and, thus, limited supply of chondrocytes from the various zones when using and attempting to regenerate human articular cartilage.
CHAPTER 3
Isolation and characterisation of articular cartilage progenitor cells
3.1 INTRODUCTION

Chondrocytes when enzymatically released and grown in monolayer culture will undergo rapid proliferation. This is dependent on factors such as initial cell density on plating and, the duration of culture. When cultured at low density, chondrocytes will undergo ‘phenotypic modulation’ a process by which the synthesis of cartilage specific macromolecules (type II collagen and the large aggregating proteoglycans) are downregulated and begin synthesising type I collagen and small non-aggregating proteoglycans concomitant with a change in phenotype (fibroblast-like morphology). Thus, it is proposed that the role of cell shape and cytoskeletal microfilament organisation influences the matrix produced, as modulated cells when resuspended in agarose gels (which promotes a rounded configuration) reexpress type II collagen, a process termed ‘redifferentiation’ (Benya and Shaffer, 1982, Brown PD, Benya PD, 1988). The precise mechanism for this reversal or re-establishment of the original phenotype is unknown but is related to the status of the actin cytoskeleton. Morphological changes caused by suspension culture are accompanied by a simultaneous downregulation of genes that are characteristic of proliferating fibroblasts and an upregulation of cartilage specific genes (Binette et al., 1998; Yaeger et al., 1997). Recent studies by Vinall and colleagues (2002) demonstrated that environmental cues such as BMP7 (and other factors that promote chondrocyte phenotype) resulted in increased expression of focal adhesion proteins (tensin, talin, paxillin and FAK proteins which link the ECM to the cell) and concomitant increase in type II collagen. Exposure to factors that promote loss of chondrocyte phenotype (i.e. flattened configuration or monolayer culture) and addition of the catabolic cytokine interleukin-1 (II-1) resulted in decreased expression of focal adhesion proteins and concomitant decrease in type II collagen expression. It was also shown in this study that these external cues influenced the distribution of the focal adhesion proteins within the chondrocyte.

In order to retain inherent zonal specificity the chondrocytes must be cultured either in high-density monolayers or in suspension cultures (agarose gel, alginate or in medium over agarose) which prevents dedifferentiation (Aydelotte and Kuettner, 1988; Aydelotte et al., 1988, Archer et al., 1990). These studies examined articular chondrocytes isolated from bovine (Aydelotte and Kuettner, 1988; Aydelotte et al.,
1988), human \cite{Archer1990} and porcine sources \cite{Zanetti1985} and Siczhowski and Watt, 1990).

The studies studied cell proliferation, proteoglycan synthesis, collagen synthesis and the effect of long term culture and the variation in results could be due to both species analysed and culture condition used. Archer et al., \cite{Archer1990} in their study on isolated human chondrocytes observed that surface and deep zone cells were distinguishable initially in monolayer but with time became fibroblastic and confirmed that distinct morphological differences were retained when cultured over agarose. They identified two types of clusters produced by the surface cells, one that synthesised little matrix (whorled cluster) compared to deeper zone and another that produced large amounts of fibrillar matrix that lacked proteoglycan. This sub-population has been described as occurring due to cell sorting or possibly due to different maturation stages. The deep zone chondrocytes produce a single cluster with rounded cells, which are separated by a ‘cartilage like’ matrix.

The studies correlated with biochemical and histochemical differences in native matrix. The authors were not able to conclude whether superficial and deep are ‘distinctly different types of chondrocytes or whether they represent the two extremes of a continuum of chondrocytes which vary both morphologically and metabolically’.

\textit{In vivo}, the chondrocyte lineage is characterised by the expression of Sox9, a high mobility group (HMG) domain transcription factor present in all chondroprogenitor cells, whose expression is necessarily required for cartilage expression but not synthesis \cite{Bi1999}. Sox9 binds to specific target enhancers on cartilage marker genes type II, IX and XI collagens, and aggregan, inducing the expression of the chondrocyte phenotype at the early stage of condensation of mesenchymal precursors that are fundamental for the onset of chondrogenesis. Sox9 bears structural homology to the product of the SRY gene (sex determining region Y chromosome (SRY)), by virtue of a conserved HMG-box DNA binding domain \cite{Bridgewater1998, Lefebvre1997, Ng1997}.

Sox9 is required in several successive steps of the chondrocyte differentiation pathway during endochondral bone formation \textit{in vivo} and for the proper progression
of cells through the sequential steps of this process. Sox9 is needed for the commitment of undifferentiated mesenchymal cells to a cell type that is both a chondroprogenitor and an osteoprogenitor but is normally upstream of CBFA1 (RUNX2) that promotes the osteoblast phenotype except in the case of secondary chondrocytes (Buxton et al., 2003) Sox9 is further required for the establishment of chondrogenic mesenchymal condensations (Wright et al., 1995). Its expression coincides with the expression of the collagen α1(II) gene (Col2a1) (Ng et al., 1997). At this stage, Sox9 also controls antiapoptotic molecules such as Noggin and Chordin that inhibit signals responsible for formation of interdigital spaces. It also functions to antagonise BMPs as well (Chimal-Monroy et al., 2003). Later, Sox9 is needed for overt differentiation for chondrocytes, for the proliferation of these cells, for the establishment of parallel columns of proliferating chondrocytes in the growth plates of endochondral skeletal elements, and for proper joint formation. Finally, Sox9 also inhibits the transition of chondrocyte into hypertrophic chondrocytes and, hence, controls subsequent endochondral ossification (Zhao et al., 1997).

Mutations in the human Sox9 gene are known to cause campomelic dysplasia; a rare congenital dwarfism syndrome characterised by hypoplastic development of endochondrally formed skeletal tissues (appendicular long bones, scapulae, and pelvic girdles, vertebral column and some craniofacial structures) and various nonskeletal anomalies (anatomical sex reversal; kidney, heart and CNS defects). A decrease in Sox9 activity would inhibit production of collagen type II, and eventually other cartilage matrix proteins resulting in lethal skeletal abnormalities characterised by hypoplasia of most endochondral bones and XY sex reversal (Foster et al., 1994, Wagner et al., 1994).

Two other members of the Sox family, Sox5 and Sox6, also play an essential role in chondrocytic differentiation. Sox5 and Sox6 are coexpressed with Sox9 in all chondroprogenitors and all differentiated chondrocytes (Lefebvre et al. 1998). Sox 5 and Sox 6 have been shown to cooperate with Sox9 to activate the Col2a1 enhancer and the Col2a1 gene during in vitro studies.

Current cartilage repair strategies rely on the expansion of autologous chondrocytes followed by their reimplantation into cartilage defects. They may be injected into
joints or utilised for the engineering of cartilage 'plugs' *ex vivo* (Brittberg et al., 1994; Buckwalter and Mankin, 1998).

In order to obtain a sufficient number of cells, the above procedures require expansion of chondrocytes. However, it is well documented that primary mammalian cells in culture also have a finite replicative lifespan whereby they enter a state of senescence where they cease to proliferate but remain metabolically active (Hayflick, 1965). The mitotic potential of primary cells in culture is also dependent on the age of the donor, with cells from older individuals exhibiting a lower proliferative lifespan (Hayflick, 1965).

Hayflick’s original system of cellular senescence *in vitro* used human fibroblasts. Fibroblasts derived from human embryonic tissue undergo 50+/−10 population doublings in culture before losing their ability to divide. Cellular age is measured as the number of population doublings that the culture has achieved.

Evidence suggests that the finite lifespan of cultured diploid fibroblasts is connected with actual cellular ageing.

- There is a correlation between the lifespan of the donor species and the number of doublings that a population of its fibroblasts will undergo (Hayflick, 1977)
- There may be an inverse correlation between the age of the donor animal and the number of doublings that populations of its fibroblasts will achieve in culture (Hayflick, 1965, 1977 and Goldstein, 1978)
- Several changes found in the later passage cells *in vitro* also occur in old cells *in vivo* (Hayflick, 1977).

Recent work in our laboratory has identified a population of chondroprogenitor cells in the superficial zone of articular cartilage with an ability to form colonies *in vitro* from a low seeding density, with an extended cell cycle time and, a high affinity for fibronectin in cell adhesion assays (Dowthwaite et al., 2004).
The objective of this chapter was to determine if it was possible to isolate, clone and then extensively cultivate the chondroprogenitor cells. The cultured cells would be compared to chondroprogenitor enriched, middle/deep and full depth derived cultured chondrocytes. The preservation of both chondrogenic commitment and differentiation potential of different cell populations during their expansion in vitro would be monitored by the extent of Sox9 expression, and their ability to redifferentiate and form cartilage when transferred into a 3D culture system.
3.2. MATERIALS AND METHODS

3.2.1. Chondrocyte isolation

Surface (SZ), and deep (DZ) zone chondrocytes were isolated from 7-day, 18-month and over 30-month (OTM) bovine metacarpalpalangeal (MCP) joints by fine dissection as described in section 2.2.1 (fig 3.1). Full depth explants were also excised from the MCP joint and subjected to enzymatic digestion as described in section 2.2.1. To eliminate any contamination by the SZ cells, SZ layers were removed by fine dissection and the remaining MZ/DZ were excised and enzymatically digested as described in section 2.2.1.

3.2.2. Differential adhesion

Twenty-four well plates were incubated with various extracellular matrix molecules (diluted in either phosphate buffered saline (PBS) containing 1mM CaCl$_2$ and MgCl$_2$ (PBS+) or 0.4M acetic acid for collagen adhesion at concentrations shown in table 3.1 overnight at 4°C and washed the following morning in PBS+. Eighteen-month and OTM chondrocytes were subjected to differential adhesion to fibronectin and control plates only. SZ and DZ chondrocytes were seeded onto plates at an initial concentration of 700 cells/ml and incubated in a humidified 37°C, 5% CO$_2$ air for 20 minutes. After 20 minutes, media (and non-adherent cells) were removed and fresh DMEM/F12 + 10% FCS added to the dishes. Culture dishes were maintained in a humidified 37°C, 5% CO$_2$ air incubator for up to 4 days. Controls comprised of dishes treated with PBS only.

<table>
<thead>
<tr>
<th>LIGAND</th>
<th>CONCENTRATION</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>27nM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>27nM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>27nM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Type IV Collagen</td>
<td>27 nM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Laminin</td>
<td>27nM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tenascin</td>
<td>27nM</td>
<td>Gibco BRL</td>
</tr>
</tbody>
</table>

*Table 3.1. Concentration of ligands used in the differential adhesion assay.*
Figure 3.1. Schematic illustration of the bovine skeleton (A) (Adapted from University of Kentucky Agripedia web pages, 2002). 7d (B,D) and 18 month (C,E) bovine metacarpal-phalangeal joints (MCP) unopened of 7 day old bovine calf (B) and 18 month old bovine (C). Opened MCP joint of 7 day old bovine calf (D) and 18 month old bovine (E).
3.2.3. Cell Counts

Initial adhesion was assayed within 4 hours of plating by counting every adhered cell on the dish using an inverted microscope equipped with phase contrast optics. Colonies of 32+ (which include transit amplifiers) or more chondrocytes were counted using the same microscope at 4 days and colony forming efficiency (CFE) was calculated as follows:

\[
\text{CFE} = \frac{\text{no. colonies at X days}}{\text{Initial no. cells}}
\]

3.2.4. Characterisation of colonies

In order to characterise the colonies, SZ chondrocytes isolated from 7-day MCP joints were seeded onto 35mm plates (coated with 10μg/ml bovine plasma fibronectin overnight) at an initial concentration of 4000 cells/ml and incubated for 20 minutes in 5% CO₂ air at 37°C. After 20 minutes, media (and non-adherent cells) were removed and fresh DMEM/F12 10% FCS added to the dishes. Culture dishes were maintained in a humidified 37°C, 5% CO₂ air incubator for up to 6 days.

3.2.5. Isolation of colonies - clones

Following 6 days in culture and with the aid of an inverted microscope, colonies of 32+ cells were identified and marked. Media was removed from the dishes and a cloning cylinder was then applied around the selected colony by pressing the thicker edge (previously greased with sterile petroleum vaseline) lightly against the Petri-dish. Two drops of trypsin (0.05% trypsin, 0.53mM EDTA, in Hanks B.S.S) were added to the cylinder and incubated at 37°C for 5 minutes. The cells were collected and transferred to a 35mm Petri-dish and cultured in DMEM+.

3.2.6. Characterisation of ‘an enriched population of chondroprogenitors’

For comparative purposes to studies carried out by Boyer et al., (2003), following the differential adhesion to fibronectin, the colonies of 32+ cells were not isolated following 6 days in culture, but all adherent cells were allowed to expand until
confluence. We named this population ‘an enriched population of chondroprogenitors’ or ‘chondroprogenitor enriched’.

3.2.7. Characterisation of full depth (FD) chondrocytes in monolayer culture

Full depth chondrocytes were expanded in monolayer culture at an initial density of 100,000 cells per 25cm² culture flask (T25s) in order to compare morphology and growth kinetics with time in culture.

3.2.8. Characterisation of middle (MZ)/deep zone (DZ) chondrocytes in monolayer culture

MZ/DZ chondrocytes were cultured in monolayer culture at an initial density of 100,000 cells per T25 to eliminate any influence the SZ chondrocytes may have on morphology and growth kinetics of the full depth chondrocyte population with time in culture.

3.2.9. Expansion in monolayer culture

When colonies and the enriched population approached confluence (P0) in the 35mm dishes, cells were trypsinised and transferred to T25s (P1). FD and MZ/DZ derived chondrocytes on reaching confluence, were trypsinised and also transferred to T25s. Subsequent growth in T25s culture flasks was carried out by continual passage at a ratio of approx 1:10. At various passages, aliquots of cells were removed for pellet culture and RNA extraction. Cell numbers harvested and seeded at each stage were kept throughout the process to allow the number of population doublings (PDs) at each stage to be calculated. The PDs were calculated with the following equation:

\[
PD = \log_{10} \left( \frac{N}{N_0} \times 3.33 \right),
\]

where \( N \) is the number of cells at the end of the passage and \( N_0 \) is the number of cells at the beginning of the passage (Cristofalo et al., 1998; Piera-Velazquez et al., 2002). Following 60 days in culture, the average total PD (as PDs are likely to vary) for clones, chondroprogenitor enriched, middle/deep and full depth cultures were calculated.
3.2.10. Cultivation of expanded chondrocytes in pellets

At various passages, aliquots of cells were removed from all cultures; clones, 'chondroprogenitor enriched', FD and MZ/DZ and processed for pellet culture. Cells were aliquoted at 250,000 cells per 500μl of medium centrifuged at 2000rpm for 5 minutes in 1.5ml polypropylene conical tubes that were placed in a CO₂ incubator for up to 14 days. Media were changed twice weekly. Media comprised of either serum supplemented media (SSM) or chemically defined serum-free media (SFM). SFM was supplemented with 10ng/ml TGF-β1 (SFMT), SFM supplemented with TGF-β1 and $10^{-7}$M dexamethasone (SFMTD), SFM supplemented with 10ng/ml bone morphogenetic protein-2 (BMP-2) (SFMB). SSM was control medium (DMEM/F12) supplemented with 4.5mg/ml D-glucose, 0.1mM ascorbic 2-phosphate and 50μg/ml Gentamycin. SFM consisted of DMEM/F12 supplemented with ITS (Gibco, i.e., 10μg/ml insulin, 5.5mg/ml transferrin and 5ng/ml selenium), 4.5mg/ml D-glucose, 0.5mg/ml bovine serum albumin (BSA), 4.7mg/ml linoleic acid and 0.1mM ascorbic acid 2-phosphate and 50μg/ml Gentamycin. The ITS supplements, TGF-β, BMP-2 and dexamethasone concentrations were selected based on previous reports that they promoted chondrogenic differentiation of expanded human chondrocytes (Yaeger et al., 1997) and mesenchymal progenitor cells (Yoo et al., 1998).

Pellets were cultured in three different medium compositions: SSM, SFM and SFMT. Only clonally derived pellets were cultured with SFMTD and SFMB medium compositions. After 14 days in culture, with media changes twice weekly, pellets were processed for histological, immunohistochemical and mRNA analysis.

3.2.11. Cell senescence

Using a method by Dimri, et al., (1995), β-galactosidase (β-Gal) staining was used to detect cell senescence. The group demonstrated that several human cells express a beta-galactosidase, histochemically detectable at pH 6, upon senescence in culture. This marker was expressed by senescent, but not presenescent, fibroblasts and keratinocytes but was absent from quiescent fibroblasts and terminally differentiated keratinocytes. It was also absent from immortal cells but was induced by genetic manipulations that reversed immortality. Cells were washed in PBS, fixed for 3-5 minutes in 2% formaldehyde, washed and incubated at 37°C with fresh senescence
associated β-Gal (SA-β-Gal) stain solution: One mg of 5-bromo-4-chloro[3]inolol β-Gal/ml (stock = 20mg of dimethylformamide per ml)/40mM citric acid/ sodium phosphate, buffered at pH 6.0/ 5mM potassium ferrocyanide/ 5mM potassium ferricyanide/ 150mM NaCl/ 2mM MgCl₂. Staining was evident in 2-4 hr and maximal in 12-16 hr. To detect lysosomal β-Gal, the citric acid /sodium phosphate was buffered at pH 4.0 (Dimri et al., 1995).

3.2.12. Quantification of labelled senescent cells

Labelled cells were examined using a Leitz light microscope and recorded using 'Image Grabber' software. The total numbers of cells were identified with haematoxylin counterstain and the number of positive cells were identified as those stained with a blue precipitate. The results were expressed as the percentage of positively labelled cells. A total of 4 random fields of view from each dish (n=1) were examined. Results were analysed using one-way ANOVA (Analyse-it for Microsoft Excel 2003 – software supplier). Double blind analysis. All pairwise comparisons between groups were conducted using the Fischer’s LSD post-hoc test. Significance and confidence was assigned at P values 0.05.

3.2.13. Histological and immunolocalisation of pellets

Pellets were cultured for 14 days, rinsed in PBS and fixed in chilled 95% ethanol for 10 minutes, followed by a second wash in PBS. Pellets were then frozen at -80°C in OCT (Raymond Lamb, Sussex) and 10µm sections cut on a cryostat at -20°C (see section 2.2.4). For histological evaluation, sections were stained with Safranin O (see appendices) and counterstained with haematoxylin. Intracellular labelling required pre-treatment of the pellets with Monensin (See section 2.2.4). Immunohistochemistry for collagen types I and II was performed as described in section 2.2.4 and table 2.2. Fluoresence was examined using a Leitz Laborlux 12 fluorescent microscope. Images were captured using a RS Photometrics camera and Coolsnap image software and formatted with Adobe Photoshop (Adobe, version 5.5).
3.2.14. RNA extraction and cDNA synthesis

RNA was extracted from expanded clones, chondroprogenitor enriched, middle/deep zone and full depth cultures at each passage. Before the RNA was extracted, the cells were briefly washed with PBS to remove any serum, RNA was then extracted by adding 600\(\mu\)l of RLT buffer containing BME and extracted using the ‘RNeasy mini’ kits (Qiagen) as per manufacturer’s guidelines. The procedure also included an ‘on column’ DNase digest step.

RNA was stored in solution at -80°C until required. The quality of extraction was verified by 1% (w/v) agarose / TBE gel and by measuring light absorbance at 260nm and 280nm. The concentration of RNA in the extraction was calculated using the equation:

\[
[RNA] = A_{260} \times D \times 40\mu\text{g/ml}
\]

Where D = the final dilution factor, and \(A_{260}\) = the absorbance of the solution at 260nm. A total of 2\(\mu\)g of total RNA necessary to study all the mRNA transcribed within the cells.

Controls comprised extracting RNA from full depth explants isolated from 7-day-old bovine MCP calf joints.

3.2.15 Generation of first strand cDNA (RT)

cDNA was transcribed from 1\(\mu\)g of RNA from expanded clones, chondroprogenitor enriched, middle/deep zone and full depth culture samples within a 30\(\mu\)l reaction of 10U/\(\mu\)l Moloney’s murine leukaemia virus reverse transcriptase (MMLV RT), 0.5mM deoxynucleotide triphosphate (dNTPs; dATP, dCTP, dGTP and dTTP) 1U/\(\mu\)l RNase inhibitor, and 10\(\mu\)g/ml random hexamers (all from Promega Ltd.) at 25°C for 10 minutes, followed by 48°C for 60 minutes and a 95°C 10 minute step to denature the reverse transcriptase enzyme. The RT products were used immediately in PCR or stored at -20°C.
3.2.16. Polymerase Chain Reaction (PCR) Amplification

PCR primers were designed using ‘Oligo 4.0’ Software (National Biosciences) and synthesised by Invitrogen (Paisley). All primers were dissolved in 10mM Tris HCl, 1mM EDTA, pH 8.0 (TE buffer) at stock concentrations of 50μM. Primers that have been designed are shown in Table 3.2.

The primers were used in the following PCR reaction, using the above RT reaction products. For a 50μl reaction: 1μl cDNA, 5μl 10x PCR buffer, 1μl 25mM dNTPs, 150ng/ml Primer 1, 150ng/ml Primer 2, 5U/ml Taq polymerase, 25mM MgCl2 and remainder distilled DNase and RNase free water. The PCR reaction was cycled at 95°C for 30 seconds, optimal annealing temperature (see table 3.2 ), for 60 seconds and 72°C for between 30 and 120 seconds depending upon product length, for 35 cycles, followed by an elongation step of 72°C for 6 minutes. The product was left at 4°C for 10 minutes. Primers corresponding to the cDNA sequence of bovine glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were used as control for the presence of mRNA in each sample. Thirty μl of PCR product was run on a 2% (w/v) agarose / TBE gel at 70V for 1 hour. The gel was stained with ethidium bromide at 1μg/ml for 20 minutes and differentiated in tap water. The bands were visualised on an ultraviolet light box and the gel documented using a Geldoc 2000 and the quantity one™ programme.

For subsequent sequence analysis, PCR products were purified by using the ‘Purification kit’ (Quiagen). The purified products were sequenced using and ABI Prism sequencer, and sequence homology compared against published cDNA sequences.

<table>
<thead>
<tr>
<th>Target template</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Optimal Annealing temperature</th>
<th>Product size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH Acc. NM AJ000039</td>
<td>tcc acc cac ggc aag t</td>
<td>ggt cat aag tcc ctc cac gat</td>
<td>58°C</td>
<td>300</td>
</tr>
<tr>
<td>Sox9 Acc. NM AF278703</td>
<td>aac ggc gag ctc aag</td>
<td>acg aac ggc cgc ttc tc</td>
<td>64°C</td>
<td>315</td>
</tr>
</tbody>
</table>

Table 3.2. Primers used to generate PCR products.
Monolayer culture does not promote the chondrocyte phenotype. In order to quantify the changes in the expression of the transcription factor Sox9 over time within the clonal expansion, chondroprogenitor enriched and MZ/DZ cultures and compare this with the changes in expression within full depth chondrocyte monolayer culture, we decided to use the qPCR Taqman technique (Perkin-Elmer Applied Biosystems).

3.2.16. Real-time PCR amplification and quantitative analysis

We used the ABI Prism TaqMan quantitative polymerase chain reaction (PCR) system (Applied Biosystems, CA, USA) to study the relative expression levels of the transcription factor Sox9 in monolayer expanded cultures. Primers and dual-labelled fluorescent probes were designed using “Primer Express” software (Applied Biosystems, CA, USA) to detect bovine Sox9 (table 3.2). The relative expression levels of Sox9 was then compared to expanded sample RNA samples using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, CA, USA), normalising to 18S ribosomal RNA (rRNA; Applied Biosystems, CA, USA). The ABI ΔΔCt method of relative quantification was applied as the PCR reaction efficiencies were equal (Sox9 and rRNA) (see appendices). Control reactions replacing cDNA with water were used to certify that amplification was not affected by contamination.

The probe reporter dye used for detection of Sox9 was 6-carboxyfluorescein (FAM), quenched by 6-carboxy-tetramethyl-rhodamine (TAMRA). The rRNA reagents were purchased in kit form (Applied Biosystems, CA, USA). The probe reporter dye was VIC (proprietary to Applied Biosystems, CA, USA) and was quenched by TAMRA.

For relative quantification by the ΔΔCT method, reactions were run in triplicate, for passage both for the gene of interest and for the standard. In 96-well plates PCR reactions were prepared (from master mixes to minimise error), as per the manufacturer’s instructions (Eurogentec, Seraing, Belgium) using the primer and probe concentrations indicated in table 3.2. Ct values(cycle threshold; see PE Applied Biosystems, 1997 were normalised to the internal standard and to the control cartilage, and the mean and standard deviation values for each sample determined as
per the recommended calculations of Applied Biosystems (Biosystems, 1997). Our calculations gave a quantity value for each sample that was relative to a chosen sample; normally passage 0 or 1. No template control reactions were used on each plate.

Quantitative PCR reactions were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute within an ABI Prism 7700 Sequence Detection System (Applied Biosystems, CA, USA).

**Statistical analysis.** The mean and standard deviation values were determined for duplicate experiments Ct values as per Applied Biosystems' instructions (Biosystems, 1997).

All qPCR experiments were repeated 3 times, and the data were combined with propagation of error to give a representation of Sox9 expression changes within all samples. For propagation of error, we calculated the standard deviation (SD) of the quotient: \( CV = CV_1^2 + CV_2^2 \) where \( CV = \text{SD/mean value} \).

Relative mRNA quantities for Sox9 expression for each culture were evaluated for significant differences using a Student’s t-test of Ct values normalised to ribosomal RNA. Changes in mRNA quantities between passages were analysed using one-way analysis of variance (ANOVA) of normalised Ct values for pairwise contrasts with LSD error protection at a 95% confidence interval.

<table>
<thead>
<tr>
<th></th>
<th><strong>Forward primer</strong></th>
<th><strong>Reverse primer</strong></th>
<th><strong>Probe</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sox9</strong></td>
<td>cac gcc gag ctc aac aag</td>
<td>acg aac gcc cgc ttc tc</td>
<td>ttcacgcttcagagcttgcaca</td>
</tr>
<tr>
<td><strong>Accession number:</strong></td>
<td><strong>AF278703</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Concentration</strong></td>
<td>50μM</td>
<td>50μM</td>
<td>10μM</td>
</tr>
</tbody>
</table>

*Table 3.3: Quantitative PCR primer and probe sequences and reaction concentrations.*

*Probes are FAM-TAMRA labelled.*
3.3. RESULTS

The morphology of the articular cartilage explants extracted from 7-day, 18-month and over 30-month (OTM) bovine MCP was visualised by sectioning and staining with Safranin O and counterstaining with haematoxylin (fig 3.2). At 7 days, the articular cartilage appeared highly cellular and homogeneous. At 18 months and in OTMs there was a shift from the immature isotropic structure, to a highly anisotropic structure in the more mature tissue. The SZ cells within the 18 month explant were flattened (to 6 cell layers). The MZ cells were separated by matrix and the deeper zone contained columns of chondrocytes. The proteoglycan content ascertained by Safranin O staining was homogeneous in both 7-day and 18-month explants, however, within OTM explants there was a loss of staining at the surface and at the deeper regions of the excised explants.

7-day bovine chondrocytes

Initial adhesion ranged between 3 and 23% (fig 3.3). SZ cells derived from 7 day MCP joint were significantly more adherent to fibronectin (FN) than tenascin (p<0.0001). Significantly more SZ cells adhered to collagen type I than to collagen types II and IV, tenascin and laminin (p<0.0001). DZ cells adhered to all ligands at fairly similar percentages, the exception was tenascin with a significantly lower percentages of DZ cells adhering (p<0.0001).

The colony forming efficiency (CFE) of SZ chondrocytes subjected to differential adhesion to FN for 20 minutes and cultured for 6 days was significantly increased (p<0.001) relative to all other cohorts in experiment (fig 3.4). There was no significant difference within the remaining cohorts.

18-month old bovine chondrocytes

Initial adhesion ranged between 2 and 9.4% (fig 3.5a). SZ cells derived from 18-month MCP were significantly (p<0.05) more adherent to PBS than fibronectin. Significantly more SZ cells adhered to FN then DZ derived cells (p<0.05).
The CFE of SZ chondrocytes derived from 18-month MCP subjected to differential adhesion (DA) to FN for 20 minutes and cultured for 6 days was significantly increased (P<0.05) relative to all other cohorts in the experiment (fig 3.5b).

OTM
Initial adhesion ranged between 1-9.28% (fig 3.6a). All cohorts were significantly more adherent (p<0.01) than SZ cells to FN. The CFE of SZ chondrocytes subjected to DA to FN for 20 minutes and cultured for 6 days was significantly increased (p<0.01) relative to all other cohorts in the experiment (fig 3.6b).

The CFE following DA to FN from 7-day SZ was 0.185, from 18-month was 0.03 and OTM 0.16. Interestingly the CFE following DA to PBS was 0.02, 0.01 and 0.01 from 7 day, 18 month and OTM respectively. The CFE of DZ chondrocytes following DA to FN was 0.01, 0.0065 and 0.0085 from 7 day, 18 month and OTM respectively.

A typical 32+ cell colony is represented in figure 3.7.

Morphological observations showed that during expansion, cells derived from clones (fig 3.8), chondroprogenitor enriched (fig 3.9); middle/deep zones (fig 3.10) and full depth (fig 3.11) increased in size with time and also became multinucleate. Clonally derived cultures increased in size (or increased degree of spreading) following 19PD (fig 3.8 C-H), chondroprogenitor enriched derived cultures were all enlarged following 37PD (fig 3.9 D & E). Middle/deep derived cultures enlarged following 8PD (fig 3.10 A-D), and full depth derived cells enlarged between 8 and 15 population doublings (fig 3.11 B-D). It was difficult to compare morphologies as different PDs were analysed.

The growth kinetics of the 8 clones (fig 3.12) was investigated from primary culture through 17 passages, corresponding to an average 47 population doublings. P1 cultures reached confluence in about 2 weeks and an average 18 doublings, a doubling time of less than 24 hours. On replating, the clones slowed their proliferation rate, with a growth rate of about 3-4 days per population doubling. With further cultivation the growth rate slowed to around 1 PD per week. Between 10-20 PDs the growth is exponential.
It was assumed that on average, following differential adhesion, around 8% of SZ cells would adhere to fibronectin. We used this figure to determine the population doublings of the chondroprogenitor enriched population. The growth kinetics of the 5 chondroprogenitor enriched cultures (fig 3.13) was investigated from primary culture through 10 passages; 35 population doublings. P1 cultures reached confluence in about 2 weeks and an average 10 doublings. On replating the proliferation rate slowed, with cells reaching confluence in 20 days, an average of 7 days per PD. Further cultivation increased the proliferation rate, an average of 3-4 days per PD.

The growth kinetics of the 3 mid/deep cultures (fig 3.14) was investigated from primary culture through 7 passages, corresponding to an average 24 population doublings. P1 cultures reached confluence in about 4 days and an average 3 doublings, a doubling time of less than 24 hours. However, on replating, the middle/deep cultures slowed their proliferation rate, with a growth rate of about 3-4 days per population doubling. This trend continued throughout cultivation.

The growth kinetics of the 3 full depth derived cultures (fig 3.15) was investigated from primary culture through 8 passages, corresponding to an average 25 population doublings. P1 cultures reached confluence in about 1 week and an average 3 doublings. This trend continued throughout cultivation.

The growth rate of the combined 8 clonal cultures (fig 3.12), 5 chondroprogenitor enriched cultures (fig 3.13), 3 middle/deep derived cultures (fig 3.14) and 5 full depth derived cultures (fig 3.15) were analysed and compared by using the slope of the curve from the graph (fig 3.16).

Following 60 days in culture, the average total PD for clones, chondroprogenitor enriched, middle/deep and full depth cultures was 27.75, 19.6, 22.56 and 23.28 respectively. Therefore, over 60 days a significantly greater (p <0.001) amount of PDs were carried out by clonally derived cultures.

The initial slope demonstrated that the growth rate of the clones was significantly faster than all other cultures between 0 and 6 days (p < 0.0001) (fig 3.16). The growth
rates of both middle/deep and full depth cultures were significantly faster than the chondroprogenitor enriched cultures (p<0.0001).

Between 7 and 123 days, the growth rate of the clones had slowed down, and all cultures were dividing significantly faster than the clonal cultures (p<0.0001). The growth rates of both middle/deep and full depth cultures between 7 to 123 days were significantly faster than the clonal and chondroprogenitor enriched cultures (p<0.0001). Between 124 and 280 days, the growth rate of the chondroprogenitor enriched population was significantly greater than the clonal cultures (p<0.005).

During the expansion of the various cell populations we wanted to characterise the relative quantity of Sox9 mRNA found within the cultures. No significant differences were found between serial clonal expansions between 18 to 45 PDs (fig 3.17).

Within chondroprogenitor enriched cultures, there was a significant decrease in the relative quantity of Sox9 mRNA found within serial cultures with time in culture (p<0.0001) (fig 3.18).

Following the serial expansion of middle/deep chondrocytes there was an observed significant decrease in the relative quantity of Sox9 mRNA with time in culture (p<0.0001) (fig 3.19).

Within full depth derived expanded cultures there was a significant increase in the relative quantity of Sox9 mRNA between the first (3PD) and second (7PD) passage (p<0.0001) (fig 3.20). The relative quantity of Sox9 mRNA significantly decreased with further cultivation (p<0.0001).

Early passage (10PD) clonally derived cells (C9) expressed positive lysosomal β-galactosidase (β-Gal) labelling (optimally active at pH 4)(fig 3.21A) but did not label positive for senescence associated β-Gal (SA β-Gal) (optimally active at pH 6) (fig 3.21B).

Thirty three % of cells within one clonally derived culture (C1) labelled positive for SA β-Gal (fig 3.21A & fig 3.22). However, within two other clonally derived cultures
(C2 & C3) a significantly (p<0.05) lower amount of cells labelled positive; 8% and 6% respectively for SA β-Gal as demonstrated by the formation of a local blue precipitate (fig 3.21D & fig 3.22).

Within the middle/deep (MD1) derived cultures, following 18PD (fig 3.21G & 3.22) there was a significantly higher amount of SA β-Gal labelling (p<0.05) compared to late passaged clonally derived cultures (C2: 40PD & C3 42PD) (fig 3.21C, D & 3.22).

Late passaged MD3 (25PD) cultures contained a significantly lower amount of positive labelled SA β-Gal cells (p<0.05) compared to earlier passaged MD1 (18PD).

Full depth cultures were not analysed for the presence of SA β-Gal positive cells due to low numbers.

At various passages aliquots of cells were removed from each culture, clones, chondroprogenitor enriched, middle/deep (MD) and FD derived for pellet culture. The clonally derived pellets only survived in chemically defined serum-free media supplemented with 10ng/ml TGF-β1 (SFMT). For the remainder of the study and for comparative purposes we only used SFMT for all pellet cultures.

The ECM present in pellets was visualised by Safranin O staining after 14 days of culture. Following 14 days in culture, detectable amounts of glycosaminoglycans (GAGs) were visualised in all early passaged clones (fig 3.23 A, B, C). However, the amount and intensity varied depending on the clone. Within pellets derived from late passaged clones, detectable amounts of GAG were visualised in C2 (33PD) only (fig 3.23E). No GAG was detected within C1 pellets following 27PD during expansion (fig 2.23D) or C3 following 36PD during expansion (fig 2.23F). Collagen type II labelling was detected in all pellets analysed that had derived from both early (fig 3.24 A-D) and late passaged cultures (fig 3.24 E-H). Only punctate labelling for collagen type I was detected in all pellets analysed (fig 3.24 I-L).

Only pellets derived from late passaged chondroprogenitor enriched cultures were analysed morphologically. There were no detectable amounts of GAG present (fig 3.25). Collagen type II was detected within pellets derived from early passaged
chondroprogenitor enriched (fig 3.26A) and late passaged (fig 3.26B, C & D). Collagen type I labelling was continuous throughout late passaged derived pellets (fig 3.26 E, F).

GAG deposition was found continuously within pellets derived from early passaged middle/deep cultures (fig 3.27A, B). However, in pellets derived from later passaged sculture no GAG deposition was detectable (fig 3.27D). Both collagens type I (fig 3.28 A, B, C) and type II (fig 3.27 E, F,G) were detected continuously throughout the matrix in pellets derived from cultures up to 18PD. Punctate collagen type II labelling was detected in late passages.

Only pellets derived from early passaged full depth cultures showed focal areas of GAG deposition. No detectable areas of GAG deposition were found within pellets derived from late passaged cells (fig 3.29 D, E, F). Type II collagen was detected in pellets following 15PD. Type I collagen was detected continuously throughout the matrix of pellets derived from both early and late passages (fig 3.30 E-H).
Figure 3.2. Montage of Safranin 0 stained articular cartilage cross sections taken from 7-day (A), 18-month (B) and over 30-month (C) old bovine metacarpalphalangeal joints. Scale bar = 100μm
Figure 3.3. Initial number of cells isolated from 7-day bovine metacarpal phalangeal (MCP) joint articular cartilage adhering to various ligands within 20 minutes. Combined mean (+/- standard error of the mean) of 3 experiments, n=4 dishes per ligand per experiment. Statistically significant differences p < 0.0001 are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SFN v S CI</td>
<td>-8.196</td>
<td>-13.568 to -2.825</td>
<td>sig</td>
</tr>
<tr>
<td>SFN v S TN</td>
<td>6.112</td>
<td>0.579 to 11.846</td>
<td>sig</td>
</tr>
<tr>
<td>D FN v S CI</td>
<td>-6.530</td>
<td>-11.901 to -1.158</td>
<td>sig</td>
</tr>
<tr>
<td>D FN v S TN</td>
<td>7.779</td>
<td>2.245 to 13.312</td>
<td>sig</td>
</tr>
<tr>
<td>D FN v D TN</td>
<td>5.577</td>
<td>0.206 to 10.949</td>
<td>sig</td>
</tr>
<tr>
<td>D FN v S CIV</td>
<td>7.006</td>
<td>1.634 to 12.377</td>
<td>sig</td>
</tr>
<tr>
<td>D FN v S LN</td>
<td>6.220</td>
<td>0.849 to 11.592</td>
<td>sig</td>
</tr>
<tr>
<td>S PBS v S CI</td>
<td>-4.658</td>
<td>-15.401 to -6.143</td>
<td>sig</td>
</tr>
<tr>
<td>D PBS v S CI</td>
<td>-6.196</td>
<td>-11.568 to -0.825</td>
<td>sig</td>
</tr>
<tr>
<td>D PBS v S TN</td>
<td>8.112</td>
<td>2.579 to 13.646</td>
<td>sig</td>
</tr>
<tr>
<td>D PBS v D TN</td>
<td>5.911</td>
<td>0.539 to 11.282</td>
<td>sig</td>
</tr>
<tr>
<td>D PBS v S CIV</td>
<td>7.339</td>
<td>1.968 to 12.711</td>
<td>sig</td>
</tr>
<tr>
<td>D PBS v S LN</td>
<td>6.554</td>
<td>1.182 to 11.925</td>
<td>sig</td>
</tr>
<tr>
<td>S CI v D CI</td>
<td>5.768</td>
<td>0.396 to 11.139</td>
<td>sig</td>
</tr>
<tr>
<td>S CI v S CII</td>
<td>10.911</td>
<td>4.820 to 17.001</td>
<td>sig</td>
</tr>
<tr>
<td>S CI v D TN</td>
<td>12.107</td>
<td>7.134 to 17.080</td>
<td>sig</td>
</tr>
<tr>
<td>S CI v S CIV</td>
<td>13.536</td>
<td>8.563 to 18.509</td>
<td>sig</td>
</tr>
<tr>
<td>S CI v D CIV</td>
<td>8.625</td>
<td>3.652 to 13.598</td>
<td>sig</td>
</tr>
<tr>
<td>S CI v S LN</td>
<td>12.750</td>
<td>7.777 to 17.723</td>
<td>sig</td>
</tr>
<tr>
<td>S CI v D LN</td>
<td>8.357</td>
<td>3.384 to 13.330</td>
<td>sig</td>
</tr>
<tr>
<td>D CI v S TN</td>
<td>8.541</td>
<td>3.007 to 14.074</td>
<td>sig</td>
</tr>
<tr>
<td>D CI v D TN</td>
<td>6.339</td>
<td>0.968 to 11.711</td>
<td>sig</td>
</tr>
<tr>
<td>D CI v S CIV</td>
<td>7.768</td>
<td>2.396 to 13.139</td>
<td>sig</td>
</tr>
<tr>
<td>D CI v S LN</td>
<td>6.982</td>
<td>1.611 to 12.354</td>
<td>sig</td>
</tr>
<tr>
<td>D CI v S TN</td>
<td>9.755</td>
<td>3.931 to 15.579</td>
<td>sig</td>
</tr>
<tr>
<td>D CI v D TN</td>
<td>7.554</td>
<td>1.883 to 13.224</td>
<td>sig</td>
</tr>
<tr>
<td>D CI v S CIV</td>
<td>8.982</td>
<td>3.312 to 14.652</td>
<td>sig</td>
</tr>
<tr>
<td>D CI v S LN</td>
<td>8.196</td>
<td>2.526 to 13.867</td>
<td>sig</td>
</tr>
<tr>
<td>S TN v D CIV</td>
<td>-5.684</td>
<td>-10.631 to -0.736</td>
<td>sig</td>
</tr>
<tr>
<td>S TN v D LN</td>
<td>-5.952</td>
<td>-11.099 to -0.804</td>
<td>sig</td>
</tr>
<tr>
<td>S CIV v D LN</td>
<td>-5.179</td>
<td>-10.152 to -0.206</td>
<td>sig</td>
</tr>
</tbody>
</table>

(S: surface zone cells, D: deep zone cells, FN: Fibronectin, PBS, Phosphate buffered saline, CI: type I collagen, CII; type II collagen, CIV type IV collagen, TN: Tenascin, LN; Laminin)
**Figure 3.5a** Initial number of cells isolated from 18-month bovine metacarpal phalangeal (MCP) articular cartilage adhering to fibronectin (FN) or Phosphate buffered saline (PBS) within 20 minutes. Combined mean (+/− standard error of the mean (SEM)) of 3 experiments, n=4 dishes per ligand per experiment. Statistically significant differences p < 0.0001 are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SFN v SPBS</td>
<td>-1.875</td>
<td>-3.663 to -0.087</td>
<td>(significant)</td>
</tr>
<tr>
<td>SFN v DFN</td>
<td>2.107</td>
<td>0.320 to 3.895</td>
<td>(significant)</td>
</tr>
<tr>
<td>SFN v DPBS</td>
<td>-1.125</td>
<td>-2.913 to 0.663</td>
<td>(significant)</td>
</tr>
<tr>
<td>SPBS v DFN</td>
<td>3.982</td>
<td>2.195 to 5.770</td>
<td>(significant)</td>
</tr>
<tr>
<td>SPBS v DPBS</td>
<td>0.750</td>
<td>-1.038 to 2.538</td>
<td>(significant)</td>
</tr>
<tr>
<td>DFN v DPBS</td>
<td>-3.232</td>
<td>-5.020 to -1.445</td>
<td>(significant)</td>
</tr>
</tbody>
</table>

**Figure 3.5b.** CFE of surface and deep zone chondrocytes (derived from 18-month bovine MCP articular cartilage 6 days following initial adhesion to FN or PBS. Combined mean (+/- SEM) of 3 experiments, n=4 dishes per ligand per experiment. Statistically significant differences p < 0.05 are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SFN v SPBS</td>
<td>0.020</td>
<td>0.002 to 0.038</td>
<td>(significant)</td>
</tr>
<tr>
<td>SFN v DFN</td>
<td>0.024</td>
<td>0.006 to 0.042</td>
<td>(significant)</td>
</tr>
<tr>
<td>SFN v DPBS</td>
<td>0.021</td>
<td>0.003 to 0.039</td>
<td>(significant)</td>
</tr>
<tr>
<td>SPBS v DFN</td>
<td>0.004</td>
<td>-0.014 to 0.022</td>
<td>(significant)</td>
</tr>
<tr>
<td>SPBS v DPBS</td>
<td>0.001</td>
<td>-0.017 to 0.019</td>
<td>(significant)</td>
</tr>
<tr>
<td>DFN v DPBS</td>
<td>-0.003</td>
<td>-0.021 to 0.015</td>
<td>(significant)</td>
</tr>
</tbody>
</table>

(S; surface zone cells, D; deep zone cells, FN; Fibronectin, PBS, Phosphate buffered saline)
Figure 3.6a Initial number of cells isolated from over 30-month bovine metacarpal phalangeal (MCP) articular cartilage adhering to fibronectin (FN) or Phosphate buffered saline (PBS) within 20 minutes. Combined mean (+/- standard error of the mean (SEM)) of 3 experiments, n=4 dishes per ligand per experiment. Statistically significant differences p < 0.005 are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFN v SPBS</td>
<td>-3.024</td>
<td>-5.231 to -0.817 (significant)</td>
</tr>
<tr>
<td>SFN v DFN</td>
<td>-3.957</td>
<td>-6.272 to -1.642 (significant)</td>
</tr>
<tr>
<td>SFN v DPBS</td>
<td>-4.286</td>
<td>-6.753 to -1.818 (significant)</td>
</tr>
<tr>
<td>SPBS v DFN</td>
<td>-0.933</td>
<td>-3.248 to 1.381</td>
</tr>
<tr>
<td>SPBS v DPBS</td>
<td>-1.262</td>
<td>-3.729 to 1.206</td>
</tr>
<tr>
<td>DFN v DPBS</td>
<td>-0.329</td>
<td>-2.893 to 2.236</td>
</tr>
</tbody>
</table>

Figure 3.6b. CFE of surface and deep zone chondrocytes (derived from over 30-month bovine MCP articular cartilage 6 days following initial adhesion to FN or PBS. Combined mean (+/- SEM) of 3 experiments, n=4 dishes per ligand per experiment. Statistically significant differences p < 0.001 are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFN v SPBS</td>
<td>0.149</td>
<td>0.087 to 0.210 (significant)</td>
</tr>
<tr>
<td>SFN v DFN</td>
<td>0.152</td>
<td>0.090 to 0.214 (significant)</td>
</tr>
<tr>
<td>SFN v DPBS</td>
<td>0.152</td>
<td>0.086 to 0.218 (significant)</td>
</tr>
<tr>
<td>SPBS v DFN</td>
<td>0.003</td>
<td>-0.061 to 0.068</td>
</tr>
<tr>
<td>SPBS v DPBS</td>
<td>0.003</td>
<td>-0.065 to 0.072</td>
</tr>
<tr>
<td>DFN v DPBS</td>
<td>0.000</td>
<td>-0.068 to 0.069</td>
</tr>
</tbody>
</table>

(S; surface zone cells, D; deep zone cells, FN; Fibronectin, PBS, Phosphate buffered saline)
Figure 3.7. Typical morphology of a 32M14 cell line Scale bar = 100 μm
Figure 3.7. Typical morphology of a 32+ cell clone. Scale bar = 100µm.
Figure 3.8. Morphological differentiation of isolated clones following 264 days in culture. Images of clones were captured following 5 population doublings (PD) (A), 19 PD (B), 21 PD (C) 24PD (D), 26PD (E), 28PD (F), 36 PD (G) and 45 PD (H). Note the increase in size of the cells (degree of spreading) after 19 PD. Scale bar = 100µm. Images are representative of typical morphologies.
Figure 3.9. Morphological differentiation of isolated chondroprogenitor enriched following approximately 166 days in culture. Images of clones were captured following 10 population doublings (PD) (A), 16 PD (B), 35 PD (C) 37PD (D) and 39PD (E). Note the increase in size (or degree of spreading) after 37 PDs. Scale bar = 100μm. Images are representative of typical morphologies.
Figure 3.10. Morphological differentiation of isolated middle/deep chondrocytes following approximately 65 days in culture. Images of clones were captured following 8 population doublings (PD) (A), 12 PD (B), 22 PD (C) and 25 PD (D). Note cells increase in size (spread) following 8PDs. Scale bar = 100μm. Images are representative of typical morphologies.
**Figure 3.12.** Graph representing the cell population growth curve of 8 clones. Cells were harvested at the indicated times and population doublings determined.

**Figure 3.13.** Graph representing the population growth curve of 5 chondroprogenitor enriched cultures. Cells were harvested at the indicated times and population doublings determined.
Figure 3.14. Graph representing the population growth curve of 3 middle/deep derived articular chondrocyte cultures. Cells were harvested at the indicated times and population doublings determined.

Figure 3.15. Graph representing the population growth curve of 5 full depth derived articular chondrocyte cultures. Cells were harvested at the indicated times and population doublings determined.
Figure 3.16. Graph representing the growth rates of clones, the chondroprogenitor enriched population, middle/deep population and the full depth derived cultures. The growth rates were determined by using the slope of the curve of each culture and combining them. Statistically significant differences in growth rates between 0 & 6 days and 7 & 123 days (p<0.0001) and 124-280 days (p<0.05) are marked in the tables below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>95% CI</td>
</tr>
<tr>
<td>Clone v Enriched</td>
<td>0.693</td>
<td>0.668 to 0.718</td>
</tr>
<tr>
<td>Clone v Mid/Deep</td>
<td>0.588</td>
<td>0.558 to 0.618</td>
</tr>
<tr>
<td>Clone v Full depth</td>
<td>0.573</td>
<td>0.547 to 0.598</td>
</tr>
<tr>
<td>Enriched v Mid/Deep</td>
<td>-0.105</td>
<td>-0.138 to -0.072</td>
</tr>
<tr>
<td>Enriched v Full depth</td>
<td>-0.120</td>
<td>-0.149 to -0.092</td>
</tr>
<tr>
<td>Mid/Deep v Full depth</td>
<td>-0.015</td>
<td>-0.048 to 0.018</td>
</tr>
</tbody>
</table>

0-6 days

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>95% CI</td>
</tr>
<tr>
<td>clone v Enriched</td>
<td>-0.077</td>
<td>-0.132 to -0.022</td>
</tr>
<tr>
<td>clone v mid/Deep</td>
<td>-0.197</td>
<td>-0.264 to -0.131</td>
</tr>
<tr>
<td>clone v Full depth</td>
<td>-0.213</td>
<td>-0.268 to -0.157</td>
</tr>
<tr>
<td>Enriched v mid/Deep</td>
<td>-0.120</td>
<td>-0.193 to -0.048</td>
</tr>
<tr>
<td>Enriched v Full depth</td>
<td>-0.136</td>
<td>-0.198 to -0.073</td>
</tr>
</tbody>
</table>

7-123-days

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>95% CI</td>
</tr>
<tr>
<td>Clone v Enriched</td>
<td>-0.115</td>
<td>-0.184 to -0.046</td>
</tr>
</tbody>
</table>

124-280 days
Figure 3.17. Graph representing the relative quantity of Sox9 mRNA normalised to control (18 population doublings (PD)) within clonal derived cultures with time up to 45PD. No significant differences were observed (p<0.05).

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>18PD v 20PD</td>
<td>0.085</td>
<td>-2.902 to 3.072</td>
</tr>
<tr>
<td>18PD v 34PD</td>
<td>-2.139</td>
<td>-5.379 to 1.101</td>
</tr>
<tr>
<td>18PD v 45PD</td>
<td>-1.766</td>
<td>-4.754 to 1.221</td>
</tr>
<tr>
<td>20PD v 34PD</td>
<td>-2.224</td>
<td>-5.544 to 1.096</td>
</tr>
<tr>
<td>20PD v 45PD</td>
<td>-1.851</td>
<td>-4.925 to 1.223</td>
</tr>
<tr>
<td>34PD v 45PD</td>
<td>0.373</td>
<td>-2.948 to 3.693</td>
</tr>
</tbody>
</table>

Figure 3.18. Graph representing the relative quantity of Sox9 mRNA normalised to control samples (10PD) within chondroprogenitor enriched cultures up to 40PD. Statistically significant differences in (p<0.0001) are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10PD v 12PD</td>
<td>1.664</td>
<td>-0.988 to 4.315</td>
</tr>
<tr>
<td>10PD v 35PD</td>
<td>-3.230</td>
<td>-6.571 to 0.111</td>
</tr>
</tbody>
</table>
| 10PD v 40PD    | -4.583     | -7.234 to -1.932
| 12PD v 35PD    | -4.894     | -8.173 to -1.615
| 12PD v 40PD    | -6.247     | -8.819 to -3.674
| 35PD v 40PD    | -1.353     | -4.632 to 1.926

(significant)
**Figure 3.19.** Graph representing the relative quantity of Sox9 mRNA normalised to control samples, 4 population doublings (PD) within middle/deep derived cultures up to 25PD. Statistically significant differences in (p<0.0001) are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
<th>95% CI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4PD v 8PD</td>
<td>2.2267</td>
<td>1.0067</td>
<td>to 3.4466</td>
<td>(significant)</td>
</tr>
<tr>
<td>4PD v 25PD</td>
<td>-2.3333</td>
<td>-3.5533</td>
<td>to -1.1134</td>
<td>(significant)</td>
</tr>
<tr>
<td>8PD v 25PD</td>
<td>-4.5600</td>
<td>-5.7800</td>
<td>to -3.3400</td>
<td>(significant)</td>
</tr>
</tbody>
</table>

**Figure 3.20.** Graph representing the relative quantity of Sox9 mRNA normalised to control samples 3PD within full depth cultures up to 15PD. Statistically significant differences in (p<0.0001) are marked in the table below.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
<th>95% CI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3PD v 7PD</td>
<td>-0.023</td>
<td>-0.499</td>
<td>to 0.454</td>
<td></td>
</tr>
<tr>
<td>3PD v 15PD</td>
<td>-1.103</td>
<td>-1.579</td>
<td>to -0.626</td>
<td>(significant)</td>
</tr>
<tr>
<td>7PD v 15PD</td>
<td>-1.080</td>
<td>-1.557</td>
<td>to -0.603</td>
<td>(significant)</td>
</tr>
</tbody>
</table>
Figure 3.21. Photomicrographs of cultures stained for β-galactosidase (β-Gal) to detect lysosomal β-Gal at pH 4 (control) (A) and cell senescence associated β-Gal (SA-β-Gal) at pH 6 (B-H). Early passaged clonal (C) cultures (following 10 population doublings (PD)) were stained (B) as well as late passage, 35PD (C) and 45PD (D). Late passaged (E) and 44PD (F). Mid and late passaged middle-steady (MS) cultures were stained, 18PD (G) and 25PD (H) respectively. Senescent cells expressed a β-Gal that was detected in single cells by X-Gal, which forms a local blue precipitate upon cleavage. Scale bar 150μm.
Figure 3.21. Photomicrographs of cultures stained for β-galactosidase (β-Gal) to detect lysosomal β-Gal at pH4 (control) (A) and cell senescence associated β-Gal (SA-β-Gal) at pH 6 (B–H). Early passaged clonal (C) cultures (following 10 population doublings (PD)) were stained (B) as well as late passage; 39PD (C) and 40PD (D). Late passaged chondroprogenitor enriched (CE) cultures were stained; 35PD (E) and 44PD (F). Mid and late passaged middle/deep (MD) cultures were stained; 18PD (G) and 25PD (H) respectively.

Senescent cells expressed a β-Gal that was detected in single cells by X-Gal, which forms a local blue precipitate upon cleavage. Scale bar 100μm.
Figure 3.23. Photomicrographs of pellets stained with Safranin O for glycosaminoglycans and counterstained with haematoxylin. Clones were expanded in control medium for 18 population doubling (PD) (A, B, C), 27PD (D), 33PD(E), 36PD (F) and pelleted at 250,000 cells/ml and cultured in serum free media supplemented with 10ng/ml TGFβ1 (SFMT). Scale bar 100μm.

Pellets cultured in SFMT following the expansion up to 33PD in control medium contained extracellular matrix with detectable amounts of glycosaminoglycans (GAGs) (A-E). No detectable amounts of GAG were present in pellets following 36PD (F).
Figure 3.24. Photomicrographs of pellets immunolabelled for types I and II collagen. Clones were expanded in control medium for 18PD (A,B,C, I, J), 21 PD (D & K), 27PD (E), 33PD (F & L), 38 PD (G), 45PD (H) and pelleted at 250,000 cells/ml and cultured in serum free media supplemented with 10ng/ml TGFβ1 (SFMT). Scale bar 100μm. All pellets labelled for collagen type II continuously throughout their extracellular matrix (A-H). There appeared to be cellular labelling for collagen type I (I-L).
Figure 3.25. Photomicrographs of pellets stained with Safranin O for glycosaminoglycans and counterstained with haematoxylin. Chondroprogenitor enriched were expanded in control medium for up to 39 population doublings (PD) (A), 42 PD (B), 44PD (C) and pelleted at 250,000 cells/ml and cultured in serum free media supplemented with 10ng/ml TGFβ1 (SFMT). Scale bar 100μm. Pellets cultured in SFMT following the expansion in control medium between 39 and 44PD did not contain detectable amounts of glycosaminoglycans (GAGs).
**Figure 3.26.** Photomicrographs of pellets immunolabelled for types I & II collagen. Chondroprogenitor enriched cultures were expanded for 10 population doubling (PD) (A), 31PD (E), 33PD (B), 38PD (C), 44PD (D) in control medium and pelleted at 250,000 cells/ml and cultured in serum free media supplemented with 10ng/ml TGFβ1 (SFMT). Scale bar 100μm.
Figure 3.27. Photomicrographs of pellets stained with Safranin O for glycosaminoglycans and counterstained with haematoxylin. Middle/deep derived chondrocytes were expanded for 4 population doubling (PD) (A), 8PD (B), 15PD (C), 22 PD in control medium and pelleted at 250,000 cells/ml and cultured in serum free media supplemented with 10ng/ml TGFβ1 (SFMT). Scale bar 100µm.

Pellets cultured in SFMT following the expansion in control medium contained extracellular matrix with detectable amounts of glycosaminoglycans (GAGs) (A-C). No detectable amounts of GAG were present in pellets following 22PD (D).
Figure 3.28. Photomicrographs of pellets immunolabelled for types I and II collagen. Middle/deep derived chondrocytes were expanded for 3 population doubling (PD) (A,E), 8PD (B,F), 12 PD (G), 18PD (C), 22PD (D) in control medium and pelleted at 250,000 cells/ml and cultured in serum free media supplemented with 10ng/ml TGFβ1 (SFMT). Scale bar 100μm.

All pellets labelled continuously throughout their extracellular matrix for collagens type I and type II.
Figure 3.29. Photomicrographs of pellets stained with Safranin O for glycosaminoglycans and counterstained with haematoxylin. Full depth derived chondrocytes were expanded for 3 population doublings (PD) (A), 7PD (B), 10PD (C), 12PD (D), 15PD (E), 25PD (F) in control medium and pelleted at 250,000 cells/ml and cultured in serum free media supplemented with 10ng/ml TGFβ1 (SFMT). Scale bar 100μm.

Pellets cultured in SFMT following the expansion in control medium contained extracellular matrix with detectable amounts of glycosaminoglycans (GAGs) (A-C). No detectable amounts of GAG were produced following 12 PD (D-F).
Figure 3.30. Photomicrographs of pellets immunolabelled for types I & II collagen. Full depth derived chondrocytes were expanded for 3 population doublings (PD) (A, E), 10PD (B, F), 12PD (C, G), 15PD (D, H) in control medium and pelleted at 250,000 cells/ml and cultured in serum free media supplemented with 10ng/ml TGFβ1 (SFMT). Scale bar 100μm.

All pellets labelled for collagen type II continuously throughout their extracellular matrix.
3.4. DISCUSSION

In recent years, autologous chondrocyte implantation has been increasingly used to repair articular cartilage defects. The number of chondrocytes that can be harvested from a patient for this procedure is limited by biopsy size, and the age of the patient. Previous studies demonstrate that primary chondrocytes as well as having a limited growth potential in culture lose their cartilage-specific phenotype. This is characterised by a loss of spherical morphology and concomitant decrease in collagen II and aggrecan synthesis and a fibroblastic like morphology.

The morphology of the articular cartilage explants extracted from 7-day-old, 18-month and over 30-month (OTM) bovine MCP demonstrated a shift from the immature isotropic structure to a highly anisotropic structure in the more mature tissue. Previous work in our laboratory had demonstrated the presence of a subpopulation of chondrocytes within 7-day bovine articular cartilage, which exhibited differential adhesion to fibronectin and the ability to form large numbers of colonies from an initially low seeding density. Within this study, we set out to establish whether the chondroprogenitor cells could be isolated by differential adhesion to ligands other than fibronectin. We also wished to ascertain whether mature articular cartilage also contained a subpopulation of chondroprogenitor cells.

Initial adhesion to various ligands ranged between 3 and 23% of the original cell number. Significantly more SZ cells adhered to fibronectin than tenasin, and significantly more SZ cells adhered to collagen I than to collagen II, IV, tenasin or laminin. This result is not surprising as collagen type I is present at the surface of articular cartilage (Wardale and Duance, 1993). DZ cells adhered to all ligands at fairly similar percentages. The exception was tenasin, with a significantly lower percentage of DZ cells adhering. These results are contradictory to results carried out by Jones and Watt (1993) on skin cells who showed high initial adhesion was comparable with high CFE. They were able to isolate stem cells from culture human epidermis on the basis of their rapid adhesion to various ECM proteins.

We have potentially identified a presumptive chondrocyte progenitor population by their rapid adhesion (20 minutes) to fibronectin. No other ligand gave high numbers
of colonies from low seeding densities regardless of initial adhesion number. Jones and Watt (1993) showed that their epidermal cells with the highest CFE adhered to both fibronectin and type IV collagen. However, this is not surprising as type IV collagen is a major component of basement membranes to which keratinocytes adhere and is not 'defined' as a cartilage matrix component per se. Immunohistochemical analysis within our labs has detected this collagen in normal human and osteoarthritic articular cartilage (Vinall, 1997). Our contradictory results to Jones and Watt (1993) are due almost certainty to differences in species, age and the tissue investigated.

The results of the differential adhesion assays of 18 month and over 30 month (OTM) old bovine SZ chondrocytes were exciting. The results demonstrated the presence of presumptive chondrocytes progenitor population by their rapid adhesion to fibronectin within mature tissue. This gives hope to the possibility of finding chondroprogenitor cells within mature human tissue. Indeed, cells having the ability to form colonies have been isolated from human articular cartilage from a 40-year-old donor (Boyer, personal communication).

Interestingly the CFE for 7-day SZ was 0.185, for 18 month was 0.03 and for OTM was 0.16. There is no explanation as to why there is such a fall in CFE for SZ cells derived from 18-month-old bovine MCP.

Although no data regarding the differential adhesion of chondrocytes exists in the literature, there are many reports investigating the adhesion of surface zone and deep zone subpopulations to various ECM ligands. Studies by Lawlor (1995) showed that freshly isolated subpopulations of cells from porcine (6-9 months old) articular cartilage yielded very poor results as adhesion was as high to BSA as it was to collagen types I and II, fibronectin, vitronectin and foetal calf serum. Lawlor's (1995) study revealed that by increasing the length of the assay to 4 hours or longer, resulted in slightly higher adhesion to fibronectin. Ten days in culture appeared to increase the 'stickiness' of the cells to all substrates (except BSA or hyaluronic acid). Sommarin et al (1989) demonstrated that bovine articular chondrocytes adhered to fibronectin-coated plastic with a higher affinity than to albumin-coated plates. The attachment was inhibited by competition with an RGD containing peptide suggesting that the α5β1 integrin receptor is required for such adhesion (Sommarin et al., 1989). Reid et
al., (2000) demonstrated that there was no significant difference in the percentage of freshly isolated chondrocytes that adhered to immobilised collagen I or II. When subpopulations of chondrocytes were assayed for their attachment to either collagen, there were no differences evident between chondrocytes from superficial and deep layers. Binding studies using native preparations of iodinated collagen under saturated conditions, revealed that chondrocytes from full thickness cartilage and suspended chondrocytes from superficial and deep layers also bound significantly more type II collagen than type I (Reid et al., 2000). To date, few studies have demonstrated the affinity of chondrocytes to tenascin. However, studies by Murphy et al., (2000) demonstrated that various tenascin variants (Tn EFn-) were able to promote mesenchymal cell adhesion. Other variants (Tn-260) had anti-adhesive effects (Murphy et al., 2000).

The ability of SZ cells to adhere to fibronectin is due to their high expression of α5β1 integrin subunits as demonstrated by fluorescence-activated cell sorter (FACS) analysis carried out within our laboratory (Dowthwaite et al., 2004). This study also showed that although middle zone derived cells exhibited a higher affinity for fibronectin than SZ, they did, however, lack the ability to form colonies. Magnetic immuno-selection of Notch-1 positive cells from the SZ followed by differential adhesion to fibronectin increased CFE compared with unselected cells. Both α5β1 and Notch-1 were expressed by about 75% of SZ chondrocytes (Dowthwaite et al., 2004) but only 1-2% of the selected cells formed colonies. Neither, Notch-1 or α5β1, therefore, are specific markers for the progenitor cells.

A typical 32+ colony is represented, however, further characterisation would have benefited this study, e.g. are the cells positive for surface zone markers or indeed chondrogenic markers? Future work would involve characterising the individual cells within the colony as Barrandon and Green (1987) have within skin. They demonstrated that individual keratinocytes vary in their capacity for self-renewal. The clones were classified into 3 types, holoclones, paraclones and meroclones (Barrandon and Green, 1987). Distinct patterns of protein synthesis as demonstrated by immunolabelling have also been established within colonies for the characterisation of skin stem cells within in vitro culture dishes (Mackenzie, 2003).
The growth rates of the combined 8 clonal cultures, 5 chondroprogenitor enriched cultures, 5 middle/deep derived cultures and 3 full depth derived cultures were analysed and compared. Initially, the growth rate of the clones was significantly faster than all other cultures between 0 and 6 days. The growth rates of both middle/deep and full depth cultures were significantly faster than the clonal and chondroprogenitor enriched cultures. The growth rates of both middle/deep and full depth cultures between 7 to 123 days were significantly faster than the clonal and chondroprogenitor enriched cultures.

These results were comparable to studies on skin stem cells. However, following the physical stress of sorting, both the stem and transit amplifying (TA) cells required a higher than normal concentrations of cells to grow in culture (1-2 x10^6 cells per 35mm dish). This was not required for our study of chondroprogenitor cells. The TA cells grow rapidly reaching confluence in approximately 7 days and which we believe reside in the middle zone of articular cartilage. The progenitor cells, on the other hand, grew more slowly at first, taking at least 12 days to reach confluence, suggesting that stem cells may be slower to enter a proliferative phase or indeed have a longer cell cycle than transit amplifying cells. This is in agreement with cell cycle profiles, which show that twice as many TA cells are proliferative compared to stem cells (Dunnwald et al., 2001).

From the graphs it can be determined that cells in early passage tend to divide more rapidly than those in late passage cultures. Concurrent with this slowing of growth rate, was a change in appearance for all cultures, from the initial fibroblastic shape to a larger, more flattened, square, multinucleate morphology. The reasons, for increased multinucleate cells are unknown but are a common feature of cells in culture. It was not correct to directly compare the cultures. Each culture began with different initial inoculum as we were unable to culture an individual cell derived from isolated full depth chondrocytes. However, we did compare population doublings. Following 60 days in culture the average total PD for clones, chondroprogenitor enriched, middle/deep and full depth cultures was 27.75, 19.6, 22.56 and 23.28 respectively. Therefore, over 60 days a significantly greater amount of PDs were carried out by clonally derived cultures.
Sox9 expression was maintained throughout the expansion of clonal chondroprogenitor cells for 40 PDs in contrast to full depth cells, which showed significantly decreased Sox9 expression by 15 PDs.

Hardingham et al., (2002) studied the gene expression in full depth derived chondrocytes passaged in monolayer culture. The group also demonstrated that with time in culture, the chondrocyte-specific genes such as collagen II and aggrecan decrease whilst collagen I expression increases (Hardingham et al., 2002) complementing the results of Lefebvre et al., (1997) and Jakob et al., (2000). qPCR showed that the upregulation of collagen I is rapid following the isolation of chondrocytes and their transfer into monolayer culture, and it precedes the downregulation of collagen II. Downregulation of the transcription factors Sox9 and L-Sox5 also precedes changes in collagen II expression. These changes were largely complete by passage 5, with little difference between passages 5 to 12. We could not directly compare our full depth results with this groups study as they documented changes in gene expression by passage number rather than PD. Future experiments could also assess collagen type I and II gene expression within the clonal cultures.

Within our study, we did not compare Sox9 expression between the cultures, only within the same culture over time. It is possible, therefore, that the relative quantity of Sox9 mRNA found, is actually a lot higher within full depth cultures compared to clonally derived chondroprogenitors to begin with. Further qPCR experiments, comparing all early cultures with each other would resolve this issue.

Full depth chondrocytes’ ability to form cartilage in pellet culture declined with declining Sox 9 expression, cartilage progenitor progeny maintained the ability to form cartilage up to 40PDs. Collagen type I was found continuously throughout the matrix of chondroprogenitor enriched, middle/deep and full depth cultured pellets. Fibrocartilage consists of dedifferentiated chondrocytes and mainly collagen type I and is insufficient as a weight bearing tissue (Eyre and Wu, 1983; Minas and Nehrer, 1997). Therefore, it was encouraging that only small amounts of type I collagen was found within clonally derived pellets.

Pellets derived from clonal cultures were initially cultured in five different medium compositions: serum supplemented media (SSM), chemically defined serum free
media (SFM), serum free media supplemented with 10ng/ml TGF-β1 (SFMT), SFM supplemented with T and 10⁻⁷M dexamethasone (SFMFD) or SFM supplemented with 10ng/ml Bone morphogenetic protein-2 (BMP-2) (SFMB). The pellets derived from clonal culture only survived in SFMT media, the reasons for this are unknown. This result does, however, correlate with studies by Yoo et al. (1998), which revealed that the addition of TGF-β1 was necessary to induce the differentiation of the bone-marrow-derived progenitor cells to chondrocytes. This result is not surprising as TGF-β1 is found in abundant amounts within embryonic cartilage, and may play a role in the chondrogenic transformation of primitive mesenchymal condensations (Cancedda et al., 1995). Cell aggregates, incubated without TGF-β1 (with or without dexamethasone) did not survive. The group also demonstrated that the chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells was retained through multiple passages. Within the same study it was shown that SFM plus dexamethasone was not sufficient for the aggregates to survive, but SFMFD was. Dexamethasone has been shown to enhance the expression of Sox9, type II collagen, aggregan and link genes and, thus, promote cartilage-like tissues both morphologically and biochemically in cultures of isolated chondrocytes. Quarto et al. (1992) also reported that dexamethasone supported chondrocyte viability, leaving our results difficult to interpret other than we are using a specific cell. BMPs specifically regulate the early commitment of mesenchymal cells toward the chondrogenic lineage (Celeste et al., 1990; Francis-West et al., 1999). BMP-2 was demonstrated to be a key chondrogenic factor for a pleuripotent mesenchymal cell line (Ahrens et al., 1993), mouse embryonic stem cells (Kramer et al., 2000) and embryonic cells at a pre-chondrogenic stage (Quarto et al., 1997). BMP-2, however, did not have a chondrogenic influence on our chondroprogenitor population. The presence of serum did not aid the survival of clonal derived pellets either.

Boyer et al. (2003) using cells comparable to our chondroprogenitor enriched population demonstrated high expansion potential in comparison to full depth derived cultured chondrocytes. The cells retained the ability to synthesise a cartilage-like hyaline like matrix rich in collagen type II after 25 PD as did our chondroprogenitor enriched pellets.
Of interest were the differences in pellet morphology derived from three different clonal cultures. During early passages, less extensive modifications by the *in vitro* environment have taken place. However, a strong selection pressure is exerted by the technique of culture in determining the cell type that ultimately predominates. In this study, we cultured all cells in monolayer using the same media throughout, although FCS batches may have differed (this was not ascertained). Isolating a higher number of clonally derived chondroprogenitors in future, would ascertain the proportion of clones that produce cartilage containing high amounts of GAG and collagen type II.

Primary cultures (controls) of clonal cultures were negative for cytochemically detectable senescence associate β-galactosidase (SA β-Gal). The study, however, would have benefited from testing for SA β-Gal in earlier passages. The results for SA β-Gal again highlight the discrepancies of analysing a low number of cultures. Normal chondrocytes possess a limited mitotic potential of 30-35PD and inevitably enter a state of replicative senescence in which cellular proliferation ceases (Adolphe et al., 1983; Allsopp et al., 1992; Evans and Georgescu, 1983; Kolettas et al., 1995; Martin and Buckwalter, 2002). Clonal cultures although positive for SA β-Gal continued to proliferate. Clonal progenitor cells have, to date, reached a total of 50 PDs, 15 more PDs than had been predicted for chondrocytes. It has been hypothesised that early senescence within *in vitro* cultures, is a culture artefact caused by culture related factors such as oxidative stress. Most somatic cells become senescent because of progressive shortening of their telomeres following each round of DNA synthesis. Telomeres cap the ends of each chromosome and keep their genetic threads from fraying and disappearing with each ‘tug’ that happens when the cell divides itself again. Since most mammalian telomeres cannot repair themselves, they are usually slowly worn away over time; shorter telomeres are often found in older cells (Allsopp et al., 1992).

The higher proportion of SA β-Gal positive cells within chondroprogenitor enriched and middle/deep cultures compared to clonal cultures could be due to their resident cells being ‘older’. That is, the majority of cells have already travelled further down the differentiation pathway *in vivo* than the progenitor cells.
CHAPTER 4
The effects of growth factors on the expansion and differentiation of articular cartilage progenitor cells
4.1 INTRODUCTION

The challenge for both clinicians and basic scientists is to repair large full thickness defects with a ‘neo’ cartilage matrix similar and if not identical to the native. Likely candidates for the use in engineered cartilage include bone marrow-derived mesenchymal progenitor cells (Johnstone et al., 1998; Mackay et al., 1998; Yoo et al., 1998) and autologous chondrocytes (Brittberg et al., 1994; Jakob et al., 2001; Martin et al., 1999; Yaeger et al., 1997).

However, to generate cartilaginous constructs using autologous chondrocytes, the limited number of cells available following a biopsy needs to be efficiently utilised. Techniques that minimise the initial number of cells required and can, thereby, minimise donor site morbidity are desirable.

Articular chondrocytes are typically characterised by their rounded cell shape and by the synthesis of collagen type II and aggrecan. When chondrocytes are released from their extracellular matrix and cultured at low density in monolayer, they undergo phenotypic modulation (sometimes referred as ‘dedifferentiation’). Expansion in monolayer culture promotes a flattened fibroblastic morphology, and the expression of macromolecules typical of pre-chondrogenic mesenchymal such as collagen type I and versican (Benya and Shaffer, 1982; Binette et al., 1998).

This modulation of the chondrocyte phenotype can be reversed or prevented by culturing cells in an environment that supports a spherical morphology and inhibits cell flattening. These conditions include pellet culture (high cell density/micromass)(Ahrens et al., 1977; Stewart et al., 2000), in agarose (Benya and Shaffer, 1982), in alginate (Bonaventure et al., 1994), on three-dimensional (3D) scaffolds (Martin et al., 1999) or in the presence of actin disrupting agents (Brown and Benya, 1988; Zanetti and Solursh, 1984).

Studies have demonstrated that chondrocytes that were ‘modulated’ by serial passaging for prolonged periods exhibited a reduced ability to redifferentiate (Benya and Shaffer, 1982; Bonaventure et al., 1994). It was suggested that this reduced ability was either due to a significant decline in the rate of phenotype reversion or a loss of
the ability to fully re-enter the differentiation program. However, some studies showed that the redifferentiation of human chondrocytes in vitro to only fully develop in long-term cultures (Binette et al., 1998; Liu et al., 1998), that long term cultured human articular chondrocytes retained their differentiated characteristics (Kolettas et al., 1995) and to be dependent on the lot of serum used (Yaeger et al., 1997). Following this latter result, the group studied the redifferentiation under controlled conditions in a chemically defined serum-free medium (Yaeger et al., 1997). The ability of human expanded chondrocytes to redifferentiate was shown be enhanced by specific combinations of growth factors and hormones during pellet culture.

Articular chondrocytes are known to undergo only a limited number of cell divisions and that their proliferative potential decreases with age (Adolphe et al., 1983; Evans and Georgescu, 1983). Clinical procedures require the development of techniques therefore, that quickly expands but also maintain the chondrogenic potential of the isolated chondrocytes.

When specific growth factors were added to monolayer cultures, rabbit, bovine and human articular cartilage chondrocytes were induced to proliferate (Bradham and Horton, 1998; de Haart et al., 1999; Guerne et al., 1995; Martin et al., 1999; Vivien et al., 1990).

Studies using both chick (Quarto et al., 1997) bovine calf (Martin et al., 1999) and adult human (Jakob et al., 2001) chondrocytes demonstrated that expansion using specific growth factors not only modulated their proliferation and differentiation but also their ability to redifferentiate when transferred to high density cultures.

Within this study we wanted to ascertain if our putative chondroprogenitor cells behaved in a similar manner to differentiated chondrocytes when exposed to growth factors during expansion. The results from the previous chapter demonstrated that following extended expansion in culture (+260 day) the chondroprogenitor cells were still capable of redifferentiating when cultured as pellets and produced hyaline like-cartilage synthesising both GAGs and collagen type II.
Growth factors known for their mitogenic affects were supplemented to the culture medium for this study; fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), platelet derived growth factor (PDGFbb), transforming growth factor-β1 (TGF-β1) or the combination of FGF-2 and TGF-β1 (FGF-2/TGFβ1) and interleukin-β (IL-β) was also tested.

A complex interrelationship exists between the ECM and cytokine signalling in articular chondrocytes (Hill and Logan, 1992). FGF-2 is a member of the FGF family, which has at least 23 related mitogenic proteins that show 35-60% amino acid conservation. Cartilage contains a large amount of FGF-2 and that it is, therefore, highly responsive to this factor. In vitro studies have shown that FGF-2 acts primarily on the growth of cartilage, rather than on its maturation. The number of FGF-2 receptors decreases as the cells become hypertrophic (Iwamoto et al., 1991), which supports the concept of a higher sensitivity of immature proliferating cells to the factor. Stimulating rabbit or chicken chondrocytes with FGF-2 in soft agar culture initiated high levels of colony formation (Kato et al., 1987).

The main reported effects of FGF-2 on chondrocytes in vitro are i) increased proliferation and ii) reduced differentiation, inhibiting chondrocyte maturation preventing type X collagen synthesis and terminal differentiation (Bohme et al., 1995; Wroblewski and Edwall-Arvidsson, 1995), including decreased expression of cartilage-specific genes (Horton et al., 1989; Shida et al., 1996). FGF-2 (5ng/ml) used during monolayer expansion yielded a population of cells with superior differentiation ability, (Martin et al., 1999) the presence of FGF-2 modulated the subsequent responsiveness of the cells to BMP-2 producing a higher wet weight fraction of GAG, higher amounts of GAG/cell as well as more homogeneously distributed GAG. Chondrocyte expansion without FGF-2 in the same study yielded a population of fibroblasts with a contractile phenotype and very limited chondrogenic potential. Martin et al., (2001) speculated that the presence of FGF-2 during monolayer expansion prevented chondrocyte trans-differentiation into a terminal contractile fibroblast-like phenotype, referred to as a myochondrocyte or a ‘myofibrochondrocyte’ (Kambic et al., 2000; Wang et al., 2000) and instead supported chondrocyte modulation into a committed chondroprogenitor cell.
Recently, FGF-2 was shown to preserve Sox9 expression and to preserve the ability of bone marrow-derived mesenchymal progenitor cells expanded in monolayer to subsequently differentiate into bone-like and cartilaginous tissues, \textit{in vivo} and \textit{in vitro} (Martin et al., 1997; Martin et al., 1998).

It has been proposed that FGF-2 either supports a selective expansion of specific sub-populations of primary chondrocytes, or it stimulates all primary cells to reach a similar developmental stage.

TGF\(\beta\) is a stable, multifunctional polypeptide growth factor. While specific receptors for this protein have been found on almost all mammalian cell types, the effect of the molecule varies depending on the cell type and growth conditions.

TGF-\(\beta\) has been implicated as an important mediator in the induction and maintenance of matrix macromolecules in articular cartilage (Trippel, 1995). TGF-\(\beta\) receptors are produced by articular chondrocytes (Villiger and Lotz, 1992; Vivien et al., 1990). TGF\(\beta\) is stored in large amounts within the cartilage and bone matrix in a latent form (Lyons et al., 1990; Wakefield et al., 1988). ECM serves as a regulatory component of TGF\(\beta_1\) expression. TGF\(\beta_1\) is abundant in embryonic cartilage, playing an important role in the chondrogenic transformation of primitive mesenchymal condensations to cartilage primordia (for review see Cancedda et al., 1995).

TGF\(\beta_1\) mRNA expression was upregulated by exogenous TGF\(\beta_1\) and was downregulated by extracellular type I and type II collagens, a negative feedback mechanism used within cartilage to preserve tissue homeostasis (Qi and Scully, 2000). When cartilage destruction takes place, IGF-I and TGF\(\beta\) as a result of proteinase activation by matrix proteolysis, may act with each other to stimulate cell growth and synthesis of collagen and proteoglycan to restore homeostatic balance.

TGF-\(\beta\) is generally shown to stimulate or inhibit cell proliferation (Brenner et al., 1993; Vivien et al., 1990) as well as proteoglycan synthesis in cultured chondrocytes (Morales and Roberts, 1988; van der Kraan et al., 1992). Chondrocyte proliferation by
TGF-β has been reported to decrease with the number of in vitro subcultures (Guerne et al., 1994) and may then become inhibitory (Blanco et al., 1995).

Fukumoto et al., (2003) used TGFβ1 only for the first 2 days of culture because it has been observed that a longer duration, specifically 14 days of TGFβ1 exposure does not result in improved chondrogenesis versus two days of treatment (Fukumoto et al., 2003). In serum free medium, TGFβ caused a dose dependent inhibition of DNA synthesis by chondrocytes. In contrast, TGF-β potentiated both GAG and DNA synthesis stimulated by FCS, PDGF, EGF and FGF (Hiraki et al., 1988).

Jakob et al., (2001) showed that chondrocytes expanded in the presence of FGF-2/TGFβ displayed not only the highest proliferation rate and the most enhanced modulation in monolayer, but also the highest capacity to redifferentiate and generate a cartilaginous tissue in response to TGFβ and dexamethasone, supplemented during the 3D culture in pellets.

During limb development, epithelial cells in the apical ectodermal ridge keep the underlying mesenchymal cells in a proliferative state, preventing their differentiation by secreting signalling molecules such as EGF (Yoon et al., 2000). PDGF modulates limb chondrogenesis functioning to promote chondrogenesis at early stages of limb development and inhibitory at later stages (Ataliotis, 2000). Although not well documented, EGF has been shown to have a mitogenic effect on chondrocytes (Gospodarowicz and Mescher, 1977). An inverse relationship between GAG and DNA synthesis was identified when proliferation of rabbit articular chondrocytes was increased with EGF and PDGF (Prins et al., 1982a; Prins et al., 1982b). Unlike EGF, PDGF during expansion of human chondrocytes induced a significant decrease in doubling times (Jakob et al., 2001) compared to control media. Cells from young human donors responded better to PDGF than to TGF-β1, while the inverse pattern was seen in cells from adult donors (Guerne et al., 1995). Age related decrease in responsiveness of rat chondrocytes to EGF is associated with diminished number and affinity for the ligand of all surface binding sites (Nonaka et al., 1999).

Interleukin-1β is a potent immuno-modulator which mediates a wide range of immune and inflammatory responses. Although originally defined as a molecule synthesised
by monocytes and macrophages, it is now known that a number of non-immune cells synthesize IL-1, including chondrocytes. In cartilage, IL-1 is able to control the synthesis and activation of a number of proteases involved in cartilage destruction e.g. stromelysin and collagenase (Pelletier et al., 1993; Saklatvala and Bird, 1986). A negative regulator of chondrogenesis, IL-1, inhibits chondrocyte differentiation by directly suppressing transcription of the cartilage specific type II collagen gene, and also inhibits GAG production (Aydelotte et al., 1992; Chandrasekhar et al., 1994; Frisbie and Nixon, 1997; Gibson et al., 1984; Goldring et al., 1994). Inhibition of chondrocyte proliferation is an important effect IL-1 (Chin and Lin, 1988; Guerne et al., 1994) which may prevent compensatory repair responses that are associated with inflammatory cartilage remodelling. The growth regulatory effects of IL-1 on mesenchymal cells are dependent on the differentiation status of the cells. It is possible, therefore, that our chondroprogenitor cells may respond differently to IL-1 than do modulated chondrocytes. IL-1 is a potent stimulator of fibroblast proliferation (mediated through the induction of PDGF) (Kumkumian et al., 1989; Schmidt et al., 1982) and when chondrocytes dedifferentiate, their growth factor responsiveness of the cells also changes and the inhibitory effects of IL-1 decreases (Guerne et al., 1994).

In this study, we used high cell density pellet culture as a well defined differentiation-permissive environment to investigate whether the chondroprogenitors following their expansion and exposure to growth factors, maintained their chondrogenic potential. Factors used to redifferentiate cells in pellets were selected as they had previously been shown to be chondrogenic stimuli for dedifferentiated chondrocytes and bone marrow-derived mesenchymal cells (Jakob et al., 2001; Johnstone et al., 1998; Yaeger et al., 1997; Yoo et al., 1998).

In order to characterise the effect of the growth factors on chondroprogenitor expansion, we analysed both hours to double and the expression of the transcription factor Sox9. To analyse redifferentiation assessed the synthesis of GAG and collagen types I and II.
4.2 MATERIALS AND METHODS

4.2.1. Cell Isolation

Surface zone (SZ) chondrocytes were isolated from 7-day MCP joints by fine dissection as described in 2.2.1. SZ chondrocytes were seeded onto 35mm Petri dishes at an initial concentration of 4000 cells/ml and subjected to differential adhesion to fibronectin as described in 3.2.4. The adherent cells were then cultured for 6 days in DMEM+. Fifty-six colonies of 32+ cells were identified and trypsinised as described in 3.3.5. Following trypsinisation, the cells were collected and seeded into 10mm wells (24 well plates) and allowed to expand.

4.2.4. Clonal proliferation

Throughout this expansion phase, cells were cultured in control media (DMEM+ 1% or 10% FCS), or control media supplemented with different growth factors involved in cartilage metabolism and cultured in a humidified 37°C, 5% CO₂ air incubator.

The factors tested included: 5ng/ml of fibroblast growth factor-2 (FGF-2), (n=4) 10ng/ml of epidermal growth factor (EGF) (n=4), 10ng/ml of platelet derived growth factor (PDGFbb) (n=4), 1ng/ml of transforming growth factor-β1 (TGF-β1) (n=4), or the combination of 5ng/ml FGF-2 and 1ng/ml TGF-β1 (FGF-2/TGFβ1) (n=4) (R&D system) and finally 10ng/ml of interleukin-β1 (IL-1β) (Tebu-bio, Cambridge). The concentrations of these factors were selected based on studies by various groups (Jakob et al., 2001; Martin et al., 2001).

Activation of growth factors

Lypophilised TGF-β1 and PDGF was reconstituted in 4mM HCL containing 0.1% bovine serum albumin (BSA), neutralised and added to culture media to obtain the final concentration. Aliquots of b-FGF, EGF were diluted with PBS containing 0.1% BSA and IL-β was reconstituted in water before added to culture media to obtain the final concentration.

After approximately 12 days when cells were sub-confluent, first passage cells (P1) were detached using trypsin/1mM EDTA counted and replated at 5 x 10³ cells/cm²
into 10mm well (24 well plates). Following a further 4 days in culture, when cells again approached confluence, second passage (P2) cells were trypsinised counted and cultured as pellets.

The doubling time of a cell population during the exponential growth phase was calculated as the ratio of $T$ to $\log_2 \left( \frac{N}{N_0} \right)$, where $N_0$ and $N$ were the cells counted at the beginning and the end, respectively, of time in exponential growth phase ($T$) (Martin et al., 1999).

### 4.2.5. Cultivation of P2 chondrocytes as pellets

P2 chondrocytes from cultures expanded in control medium +10% FCS and control medium + 10% FCS supplemented with growth factors were resuspended in chemically defined serum free media (SFM) as described in 3.2.10. Aliquots of 250,000 cells in 500μl of medium were centrifuged at 2000rpm for 5 minutes in 1.5ml polypropylene conical tubes which were placed in a humidified 37°C, 5% CO$_2$ air incubator for 14 days with media changed twice weekly.

### 4.2.6. Histology and immunohistochemistry of cell pellets

Pellets were cultured for 14 days, rinsed in PBS and fixed in chilled 95% ethanol for 10 minutes, followed by a second wash in PBS. Pellets were then frozen in a freezer at -80°C in OCT and 10μm sections cut on a cryostat (see section 2.2.4). For histological evaluation, sections were stained with Safranin O and haematoxylin (see appendices). Intracellular labelling required pre-treatment of the pellets with Monensin (See section 2.2.4). Immunohistochemistry of collagens I and II was performed as described in section 2.2.4 and using antibodies described in table 2.2.

### 4.2.7. Total RNA extraction and cDNA synthesis

After exponential growth phase (P1 and P2), mRNA from subconfluent wells was extracted as described in section 3.2.14.
4.2.8. Real-time PCR amplification and quantitative analysis.

PCR reactions were performed and monitored using an ABI Prism 7700 Sequence Detection System as described in section 3.2.16. Again expression of the gene for the transcription factor SOX9 was analysed. We quantified the expression of this gene within cultures expanded in 1% FCS control medium, 1% FCS control medium supplemented with different growth factors, 10% control medium and finally 10% control medium supplemented with growth factors within the first passage and then again at the end of the second passage.

4.2.9 Statistical Analysis.

The mean and standard deviation values were determined for duplicate experiments’ Ct values as per Applied Biosystems’ instructions (Biosystems, 1997).

All qPCR experiments were repeated 3 times, and the data were combined with propagation of error to give a representation of Sox9 expression changes within all samples. For propagation of error, we calculated the standard deviation (SD) of the quotient: \( CV = CV_1^2 + CV_2^2 \) where \( CV = SD/\text{mean value} \).

Relative mRNA quantities for Sox9 expression for each culture were evaluated for significant differences using Student’s t-test of Ct values normalised to ribosomal RNA. Changes in mRNA quantities between passages were analysed using one-way analysis of variance (ANOVA) of normalised Ct values for pairwise contrasts with LSD error protection at a 95% confidence interval.
4.3 RESULTS

Morphological observations showed that the majority of cells expanded in control medium containing 1% FCS (4.1A) during the first passage retained a round morphology, whereas a small population of cells expanded in the presence of various growth factors displayed a typical fibroblast-like phenotype of dedifferentiated chondrocytes (fig 4.1 B, D, E, F & G). Cultures expanded in EGF appeared to enlarge and spread across the culture plastic (fig 4.1C). Within the 10% FCS cultures, the representatives images demonstrate that the majority of cells expanded in control medium retained a rounded morphology (fig 4.2A). Again a proportion of the cells expanded in different growth factors developed a fibroblastic phenotype (fig 4.2 B, D, E, F & G).

Control cultures (fig 4.3B and 4.4B) display the typical fibroblast-like phenotype of modulated chondrocytes during the second passage. All cultures expanded with growth factors during P2 were fibroblastic in appearance. Cultures treated with 1% plus TGFβ1 and FGF-2 (fig 4.3E) did not appear healthy and only a few cells remained attached to the plastic. Cultures treated with 1% and 10% FCS and TGFβ1 (4.3F and 4.4F respectively) appeared to proliferate prodigiously.

Not all growth factors tested during chondrocyte expansion induced a significant decrease in doubling times during exponential growth compared to control cultures. During P1, within 1% cultures, PDGF and the combined TGF/FGF significantly reduced doubling time by 12.4% and 14.9% respectively (p<0.01) (fig 4.5), providing highest cell proliferation rates.

Within 10% FCS cultures (fig 4.5) the combination of TGF/FGF again significantly reduced the doubling times by 14.7%. FGF and TGF alone were significantly slower (6.32% and 22.24% respectively) than control (p< 0.001).

During P2 in all experiments carried out using 1% FCS media, supplemented with both TGFβ1 and FGF-2 combined died (fig 4.6). Within passage 2, only cultures expanded in control medium containing IL-β had significantly reduced doubling times by 12% (p<0.0001), FGF significantly doubled the hours to divide compared to
control. Within 10% cultures, only cells expanded in TGFβ1 significantly reduced their doubling times (by 36.3%) (p<0.0001) (fig 4.8).

We also analysed the effects of 1% and 10% serum concentration on doubling time within the growth factor subgroups. During the first passage significant decreases in doubling times were seen by 10% FCS cultures compared to 1% cultures for EGF, FGF and IL-β subgroups by 19.7%, 20%, and 23% respectively. Following the second passage significant differences were seen within EGF and TGF subgroups, with the 10% FCS containing media reducing the doubling times by 9.4% and 42.0% respectively.

Following the expansion phase, we assessed the relative expression of Sox9 mRNA found within the cultures. No significant differences were observed between clones expanded in control media or control media supplemented with growth factors in either 1% or 10% cultures (fig 4.7). At the end of passage 2 there was a significant increase in the relative expression of Sox9 mRNA within 1% FCS cultures expanded in the presence of PDGF and FGF-2 (p<0.05) (fig 4.8). There were no significant differences within the 10% cultures.

We then analysed the effects of 1% and 10% serum concentration on the expression of Sox9 mRNA within the growth factor subgroups. Following the first passage, 10% FCS containing cultures supplemented with FGF showed a significant increase in the relative quantity of Sox9 mRNA compared to the 1% culture (p<0.05) (fig 4.7). However, within 10% FCS cultures supplemented with either TGF/FGF or TGF alone there was a significant decrease in the relative quantity of Sox9 mRNA compared to the 1% cultures (p<0.05) (fig 4.7).

Following the second passage, 10% FCS containing control cultures showed a significant increase in the relative expression of Sox9 mRNA compared to the 1% culture (p<0.05) (fig 4.8). However, within 10% FCS cultures supplemented with either PDGF or TGF alone, there was a significant decrease in the relative quantity of Sox9 mRNA compared to the 1% cultures (p<0.05) (fig 4.8s).
Following expansion in control medium containing 10% FCS and 2 weeks of culture in pellet form in SFMT, control pellets contained no detectable levels of GAG staining within the ECM (fig 4.9A). However, pellets formed from cells expanded in media containing control media supplemented with various growth factors displayed scattered areas of ECM containing GAGs (fig 4.9) and labelled positive for both collagen II (fig 4.10) and collagen I (fig 4.11).
**Figure 4.1.** Comparative morphology of clones cultured for approximately 12 days in control medium CTR + 1% foetal calf serum (FCS) (A) or CTR 1%FCS in the presence of platelet derived growth factor (PDGF) (B), epidermal growth factor (EGF) (C), fibroblast growth factor-2 (FGF-2) (D), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2) (E), TGF-β1 alone (F) and interleukin-1β (IL-1β) (G). Scale bar = 100μm. Cells expanded in control medium (A) retained a rounded morphology. A population of cells expanded with different growth factors displayed a typical fibroblast-like phenotype of dedifferentiated chondrocytes (B, D, E, F, G). Cells expanded in media containing EGF (C) enlarged.
Figure 4.2. Comparative morphology of clones cultured for approximately 12 days in control medium CTR + 10% foetal calf serum (FCS) (A) or CTR 10% FCS in the presence of platelet derived growth factor (PDGF) (B), epidermal growth factor (EGF)(C), fibroblast growth factor-2 (FGF-2) (D), (D), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2) (E), TGF-β1 alone (F) and interleukin-1β (IL-1β)(G). Scale bar = 100μm. The majority of cells in the control cultures and cultures supplemented with growth factors retained a rounded morphology. A small proportion of cells in each culture developed a fibroblastic like morphology.
Figure 4.3. Comparative morphology of passage 2 (P2) clones cultured for approximately 6 days in control medium CTR + 1% foetal calf serum (FCS) (A) or CTR 1%FCS in the presence of platelet derived growth factor (PDGF) (B), epidermal growth factor (EGF)(C), fibroblast growth factor-2 (FGF-2) (D), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2) (E), TGF-β1 alone (F) and interleukin-1β (IL-1β)(G). Scale bar = 100μm. All cultures displayed the typical fibroblast-like phenotype of dedifferentiated chondrocytes. Treatment with TGF/FGF (E) appeared to cause the cells to shrink and condense whereas cultures supplemented with TGF-β1 (F) only appeared at a higher density.
Figure 4.4. Comparative morphology of passage 2 (P2) clones cultured for approximately 6 days in control medium CTR + 10% foetal calf serum (FCS) (A) or CTR 10% FCS in the presence of platelet derived growth factor (PDGF) (B), epidermal growth factor (EGF) (C), fibroblast growth factor-2 (FGF-2) (D), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2) (E), TGF-β1 alone (F) and interleukin-1β (IL-1β) (G). Scale bar = 100μm. All cultures displayed the typical fibroblast like phenotype of dedifferentiated chondrocytes. Cultures treated with TGFβ1 appeared at a higher density when compared to the other cultures.
Figure 4.5. Graph representing the time in hours for cells expanded in control medium CTR + 1% foetal calf serum (FCS) (CTR 1% FCS) or CTR 10% FCS and in the presence of platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2), TGF-β1 alone and interleukin-β1(IL-β) during passage 1. Statistically significant differences between cultures (p < 0.01) are marked in the table below.

### P1 1%

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF v EGF</td>
<td>0.008</td>
<td>0.001 to 0.015</td>
</tr>
<tr>
<td>PDGF v FGF</td>
<td>0.011</td>
<td>0.004 to 0.018</td>
</tr>
<tr>
<td>PDGF v CTR</td>
<td>0.007</td>
<td>0.001 to 0.014</td>
</tr>
<tr>
<td>PDGF v IL-β</td>
<td>0.008</td>
<td>0.001 to 0.015</td>
</tr>
<tr>
<td>EGF v TGF/FGF</td>
<td>-0.008</td>
<td>-0.014 to -0.001</td>
</tr>
<tr>
<td>EGF v CTR</td>
<td>-0.001</td>
<td>-0.007 to 0.006</td>
</tr>
<tr>
<td>FGF v TGF/FGF</td>
<td>-0.011</td>
<td>-0.018 to -0.004</td>
</tr>
<tr>
<td>FGF v CTR</td>
<td>-0.004</td>
<td>-0.011 to 0.004</td>
</tr>
<tr>
<td>TGF/FGF v CTR</td>
<td>0.007</td>
<td>0.001 to 0.014</td>
</tr>
<tr>
<td>TGF/FGF v IL-β</td>
<td>0.008</td>
<td>0.001 to 0.014</td>
</tr>
<tr>
<td>CTR v TGF</td>
<td>-0.002</td>
<td>-0.010 to 0.005</td>
</tr>
<tr>
<td>CTR v IL-β</td>
<td>0.001</td>
<td>-0.006 to 0.007</td>
</tr>
</tbody>
</table>

### P1 10%

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF v FGF</td>
<td>0.005</td>
<td>0.000 to 0.010</td>
</tr>
<tr>
<td>PDGF v CTR</td>
<td>0.003</td>
<td>-0.001 to 0.008</td>
</tr>
<tr>
<td>PDGF v TGF</td>
<td>0.010</td>
<td>0.004 to 0.016</td>
</tr>
<tr>
<td>EGF v TGF/FGF</td>
<td>-0.005</td>
<td>-0.010 to -0.000</td>
</tr>
<tr>
<td>EGF v CTR</td>
<td>0.003</td>
<td>-0.002 to 0.007</td>
</tr>
<tr>
<td>EGF v TGF</td>
<td>0.009</td>
<td>0.003 to 0.015</td>
</tr>
<tr>
<td>FGF v TGF/FGF</td>
<td>-0.010</td>
<td>-0.015 to -0.004</td>
</tr>
<tr>
<td>FGF v CTR</td>
<td>-0.002</td>
<td>-0.006 to 0.003</td>
</tr>
<tr>
<td>FGF v IL-β</td>
<td>-0.006</td>
<td>-0.011 to -0.001</td>
</tr>
<tr>
<td>TGF/FGF v CTR</td>
<td>0.008</td>
<td>0.003 to 0.013</td>
</tr>
<tr>
<td>TGF/FGF v TGF</td>
<td>0.014</td>
<td>0.008 to 0.021</td>
</tr>
<tr>
<td>CTR v TGF</td>
<td>0.007</td>
<td>0.001 to 0.012</td>
</tr>
<tr>
<td>CTR v IL-β</td>
<td>-0.004</td>
<td>-0.009 to 0.001</td>
</tr>
<tr>
<td>TGFv IL-β</td>
<td>-0.011</td>
<td>-0.017 to -0.005</td>
</tr>
</tbody>
</table>

### P1

<table>
<thead>
<tr>
<th>Paired samples t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 % CTR v 10% CTR</td>
</tr>
<tr>
<td>1% PDGF v 10% PDGF</td>
</tr>
<tr>
<td>1% EGF v10% EGF</td>
</tr>
<tr>
<td>1% FGF v 10% FGF</td>
</tr>
<tr>
<td>1% TGF v 10% TGF</td>
</tr>
<tr>
<td>1% IL-β v 10% IL-β</td>
</tr>
</tbody>
</table>

(significant)
Figure 4.6. Graph representing the time in hours for cells expanded in control medium CTR + 1% foetal calf serum (FCS) (CTR 1% FCS) or CTR 10% FCS and in the presence of platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2), TGF-β1 alone and interleukin-β1 (IL-β) during passage 2. Statistically significant differences between cultures (p < 0.01) are marked in the table below.

### P2 1%

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF v FGF</td>
<td>0.006</td>
<td>0.004 to 0.009</td>
</tr>
<tr>
<td>PDGF v CTR</td>
<td>-0.004</td>
<td>-0.006 to -0.001</td>
</tr>
<tr>
<td>PDGF v IL-β</td>
<td>-0.007</td>
<td>-0.010 to -0.004</td>
</tr>
<tr>
<td>EGF v FGF</td>
<td>0.009</td>
<td>0.006 to 0.011</td>
</tr>
<tr>
<td>EGF v CTR</td>
<td>-0.001</td>
<td>-0.004 to 0.002</td>
</tr>
<tr>
<td>EGF v IL-β</td>
<td>-0.004</td>
<td>-0.007 to -0.001</td>
</tr>
<tr>
<td>FGF v CTR</td>
<td>-0.010</td>
<td>-0.012 to -0.007</td>
</tr>
<tr>
<td>FGF v TGF</td>
<td>-0.008</td>
<td>-0.011 to -0.006</td>
</tr>
<tr>
<td>FGF v IL-β</td>
<td>-0.013</td>
<td>-0.016 to -0.010</td>
</tr>
<tr>
<td>CTR v TGF</td>
<td>0.001</td>
<td>-0.001 to 0.004</td>
</tr>
<tr>
<td>CTR v IL-β</td>
<td>-0.003</td>
<td>-0.006 to -0.000</td>
</tr>
<tr>
<td>TGF v IL-β</td>
<td>-0.004</td>
<td>-0.007 to -0.001</td>
</tr>
</tbody>
</table>

### P2 10%

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF v EGF</td>
<td>-0.006</td>
<td>-0.010 to -0.002</td>
</tr>
<tr>
<td>PDGF v TGF/FGF</td>
<td>-0.007</td>
<td>-0.011 to -0.002</td>
</tr>
<tr>
<td>PDGF v CTR</td>
<td>-0.006</td>
<td>-0.010 to -0.002</td>
</tr>
<tr>
<td>PDGF v TGF</td>
<td>-0.018</td>
<td>-0.022 to -0.013</td>
</tr>
<tr>
<td>PDGF v IL-β</td>
<td>-0.008</td>
<td>-0.012 to -0.003</td>
</tr>
<tr>
<td>EGF v FGF</td>
<td>0.010</td>
<td>0.006 to 0.014</td>
</tr>
<tr>
<td>EGF v CTR</td>
<td>0.000</td>
<td>-0.004 to 0.005</td>
</tr>
<tr>
<td>EGF v TGF</td>
<td>-0.012</td>
<td>-0.016 to -0.007</td>
</tr>
<tr>
<td>FGF v TGF/FGF</td>
<td>-0.010</td>
<td>-0.015 to -0.006</td>
</tr>
<tr>
<td>FGF v CTR</td>
<td>-0.010</td>
<td>-0.014 to -0.005</td>
</tr>
<tr>
<td>FGF v TGF</td>
<td>-0.021</td>
<td>-0.026 to -0.017</td>
</tr>
<tr>
<td>FGF v IL-β</td>
<td>-0.012</td>
<td>-0.016 to -0.007</td>
</tr>
<tr>
<td>TGF/FGF v CTR</td>
<td>0.001</td>
<td>-0.004 to 0.005</td>
</tr>
<tr>
<td>TGF/FGF v TGF</td>
<td>-0.011</td>
<td>-0.016 to -0.007</td>
</tr>
<tr>
<td>CTR v TGF</td>
<td>-0.012</td>
<td>-0.016 to -0.008</td>
</tr>
<tr>
<td>CTR v IL-β</td>
<td>-0.002</td>
<td>-0.007 to 0.002</td>
</tr>
<tr>
<td>TGF v IL-β</td>
<td>0.010</td>
<td>0.005 to 0.014</td>
</tr>
</tbody>
</table>

**Paired samples t test**

- 1% CTR v 10% CTR (significant)
- 1% PDGF v 10% PDGF
- 1% EGF v 10% EGF
- 1% FGF v 10% FGF
- 1% TGF v 10% TGF
- 1% IL-β v 10% IL-β (significant)
Figure 4.7. Graph representing the relative quantity of Sox9 mRNA normalised to control samples for cells expanded in control medium CTR + 1% foetal calf serum (FCS) (CTR 1% FCS) or CTR 10% FCS and in the presence of platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2), TGF-β1 alone and interleukin-β1(IL-β) during passage 1. Statistically significant differences between cultures (p < 0.05) are marked in the table below.

### P1 1%

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF v CTR</td>
<td>-3.420</td>
<td>-6.903 to 0.063</td>
</tr>
<tr>
<td>PDGF v TGF</td>
<td>-2.812</td>
<td>-6.295 to 0.671</td>
</tr>
<tr>
<td>PDGF v IL-β</td>
<td>-3.970</td>
<td>-7.453 to -0.487</td>
</tr>
<tr>
<td>EGF v CTR</td>
<td>-0.201</td>
<td>-3.536 to 3.133</td>
</tr>
<tr>
<td>FGF v CTR</td>
<td>-0.164</td>
<td>-3.647 to 3.319</td>
</tr>
<tr>
<td>TGF/FGF v CTR</td>
<td>-0.145</td>
<td>-4.166 to 3.877</td>
</tr>
<tr>
<td>CTR v TGF</td>
<td>0.608</td>
<td>-2.875 to 4.091</td>
</tr>
<tr>
<td>CTR v IL-β</td>
<td>-0.550</td>
<td>-4.033 to 2.933</td>
</tr>
</tbody>
</table>

### P1 10%

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF v CTR</td>
<td>-1.033</td>
<td>-3.804 to 1.739</td>
</tr>
<tr>
<td>EGF v CTR</td>
<td>0.147</td>
<td>-2.624 to 2.919</td>
</tr>
<tr>
<td>FGF v CTR</td>
<td>-2.171</td>
<td>-4.942 to 0.601</td>
</tr>
<tr>
<td>FGF v TGF</td>
<td>-3.800</td>
<td>-6.694 to -0.906</td>
</tr>
<tr>
<td>TGF/FGF v CTR</td>
<td>-0.297</td>
<td>-3.068 to 2.475</td>
</tr>
<tr>
<td>CTR v TGF</td>
<td>-1.629</td>
<td>-4.401 to 1.142</td>
</tr>
<tr>
<td>CTR v IL-β</td>
<td>-0.575</td>
<td>-3.347 to 2.196</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P1</th>
<th>Paired samples t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 % CTR v 10% CTR</td>
<td>(significant)</td>
</tr>
<tr>
<td>1% PDGF v 10% PDGF</td>
<td>(significant)</td>
</tr>
<tr>
<td>1% EGF v 10% EGF</td>
<td>(significant)</td>
</tr>
<tr>
<td>1% FGF v 10% FGF</td>
<td>(significant)</td>
</tr>
<tr>
<td>1% TGF/FGF v 10% TGF/FGF</td>
<td>(significant)</td>
</tr>
<tr>
<td>1% TGF v 10% TGF</td>
<td>(significant)</td>
</tr>
<tr>
<td>1% IL-β v 10% IL-β</td>
<td>(significant)</td>
</tr>
</tbody>
</table>
Figure 4.8. Graph representing the relative quantity of Sox9 mRNA normalised to control samples for cells expanded in CTR 1% FCS or CTR 10% FCS and in the presence of PDGF, EGF, FGF-2, TGF/FGF-2, TGF-β1 alone and IL-β1 during passage 2. Statistically significant differences between cultures (p < 0.05) are marked in the table below.

There was a significant increase in the relative quantities of SOX9 mRNA in cultures expanded in the presence of PDGF and FGF within 1% FCS cultures.

<table>
<thead>
<tr>
<th>P2 1%</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>95% CI</td>
</tr>
<tr>
<td>PDGF v CTR</td>
<td>-2.068</td>
<td>-3.619 to -0.517 (significant)</td>
</tr>
<tr>
<td>PDGF v IL-β</td>
<td>-2.010</td>
<td>-3.637 to -0.384 (significant)</td>
</tr>
<tr>
<td>EGF v CTR</td>
<td>-1.230</td>
<td>-2.857 to 0.397</td>
</tr>
<tr>
<td>FGF v CTR</td>
<td>-2.170</td>
<td>-3.797 to -0.543 (significant)</td>
</tr>
<tr>
<td>FGF v IL-β</td>
<td>-2.112</td>
<td>-3.811 to -0.413 (significant)</td>
</tr>
<tr>
<td>TGF/FGF v CTR</td>
<td>-0.710</td>
<td>-2.261 to 0.841</td>
</tr>
<tr>
<td>CTR v TGF</td>
<td>1.066</td>
<td>-0.561 to 2.693</td>
</tr>
<tr>
<td>CTR v IL-β</td>
<td>0.058</td>
<td>-1.569 to 1.685</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P2 10%</th>
<th>Difference</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>95% CI</td>
</tr>
<tr>
<td>PDGF v CTR</td>
<td>-0.711</td>
<td>-1.769 to 0.347</td>
</tr>
<tr>
<td>PDGF v IL-β</td>
<td>-1.350</td>
<td>-2.297 to -0.403 (significant)</td>
</tr>
<tr>
<td>EGF v CTR</td>
<td>-0.748</td>
<td>-1.907 to 0.412</td>
</tr>
<tr>
<td>EGF v IL-β</td>
<td>-1.387</td>
<td>-2.445 to -0.328 (significant)</td>
</tr>
<tr>
<td>FGF v CTR</td>
<td>-0.635</td>
<td>-1.734 to 0.465</td>
</tr>
<tr>
<td>FGF v IL-β</td>
<td>-1.274</td>
<td>-2.266 to -0.281 (significant)</td>
</tr>
<tr>
<td>TGF/FGF v CTR</td>
<td>0.092</td>
<td>-0.966 to 1.151</td>
</tr>
<tr>
<td>CTR v TGF</td>
<td>-0.199</td>
<td>-1.257 to 0.859</td>
</tr>
<tr>
<td>CTR v IL-β</td>
<td>-0.639</td>
<td>-1.697 to 0.419</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Paired samples t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% CTR v 10% CTR</td>
</tr>
<tr>
<td>1% PDGF v 10% PDGF</td>
</tr>
<tr>
<td>1% EGF v 10% EGF</td>
</tr>
<tr>
<td>1% FGF v 10% FGF</td>
</tr>
<tr>
<td>1% TGF v 10% TGF</td>
</tr>
<tr>
<td>1% IL-β v 10% IL-β</td>
</tr>
</tbody>
</table>
Figure 4.9. Photomicrographs of pellets stained with Safranin O for glycosaminoglycans and counterstained with haematoxylin. Chondrocytes expanded in control medium + 10% foetal calf serum (FCS) (CTR 10% FCS) (A) or CTR 10% FCS in the presence of platelet derived growth factor (PDGF) (B), epidermal growth factor (EGF) (C), fibroblast growth factor-2 (FGF-2) (D), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2) (E), TGF-β1 alone (F) and interleukin-β1 (IL-β1) (G) were then cultured for 2 weeks in serum free-medium supplemented with TGFβ1 (SFMT) in the form of pellets. Scale bar 100μm.

Pellets cultured in SFMT following the expansion in control medium did not contain extracellular matrix with detectable amounts of glycosaminoglycans (GAGs). Pellets cultured in SFM following expansion in PDGF (B), EGF (C), FGF (D), TGF/FGF-2 (E), TGF (F) and IL-β (G) displayed scattered areas of ECM containing GAG.
Figure 4.10. Photomicrographs of pellets immunolabelled for type II collagen. Chondrocytes expanded in control medium + 10% foetal calf serum (FCS) (CTR 10% FCS) (A) or CTR 10% FCS in the presence of platelet derived growth factor (PDGF) (B), epidermal growth factor (EGF) (C), fibroblast growth factor-2 (FGF-2) (D), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2) (E), TGF-β1 alone (F) and interleukin-β1 (IL-β1) (G) were then cultured for 2 weeks in serum free-medium supplemented with TGFβ1 (SFMT) in the form of pellets. Scale bar 100μm.

Chondrocytes expanded in CTR or CTR supplemented with growth factors deposited in extracellular matrix that labelled positive for collagen II. The pellet formed from chondrocytes expanded in TGF-β, dispersed during sectioning.
Figure 4.11. Photomicrographs of pellets immunolabelled for type I collagen. Chondrocytes expanded in control medium + 10% foetal calf serum (FCS) (CTR 10% FCS) (A) or CTR 10% FCS in the presence of platelet derived growth factor (PDGF) (B), epidermal growth factor (EGF) (C), fibroblast growth factor-2 (FGF-2) (D), transforming growth factor-β1 (TGF-β1) and FGF-2 (TGF/FGF-2) (E), TGF-β1 alone (F) and interleukin-β1 (IL-β1) (G) were then cultured for 2 weeks in serum free-medium supplemented with TGFβ1 (SFMT) in the form of pellets. Scale bar 100μm. Chondrocytes expanded in CTR or CTR supplemented with growth factors deposited an extracellular matrix that labelled positive for collagen I.
4.4 DISCUSSION

Procedures for autologous repair of cartilage defects may be difficult in elderly patients due to loss of stem cells (Quarto et al., 1995) or chondrocytes (Adolphe et al., 1983) and slow in vitro proliferation of these cells in culture (Vignon et al., 1976).

Therapeutic protocols for cartilage repair are currently based on autologous transplantation of bone marrow stem cells with chondrogenic potential (Caplan, 1991) or chondrocytes removed by biopsy from articular cartilage (Brittberg et al., 1994; Green, 1977; Wakitani et al., 1994).

Previous reports show that primary chondrocytes serially grown in vitro in monolayer gradually lose the cartilage-specific characteristics and that the phenotype can be recovered by culturing cells in appropriate differentiation-permissive conditions (Benya and Shaffer, 1982; Gibson et al., 1982). This indicates the reversibility of the differentiated phenotype at the genetic level.

Within this study, we have demonstrated that the chondroprogenitor cells behave in a similar manner to differentiated chondrocytes during the first passage. Chondroprogenitor cells expanded in 1% and 10% in the presence of both TGFβ-1 and FGF-2 displayed the highest proliferation rate. Previous studies reported that factors stimulating the highest chondrocyte proliferation also induced the strongest cell dedifferentiation (Jakob et al., 2001). This was not the case for TGF/FGF as there was no significant difference in relative Sox9 mRNA expression compared to controls. As discussed in the previous chapter, Sox9 mRNA levels could be relatively low compared to that expressed in differentiated chondrocytes, as chondroprogenitors may already have a ‘dedifferentiated phenotype’ as a committed progenitor. This aspect needs to be characterised, as Sox9 is expressed in primitive mesenchymal condensations during limb development. Future studies on the affects of modulation could use differentiation markers such as collagen type II/collagen type I and aggrecan/versican ratios and quantify by using the real-time PCR assay used in this study for Sox9 expression.
Cultures supplemented with FGF and TGF alone were significantly slower than control samples but combined demonstrated an additive effect. This had previously been shown on avian (Horton et al., 1989), rodent (Bradham and Horton, 1998) and human (Jakob et al., 2001) chondrocytes. De Haart et al., (1999) also showed conflicting results (similar to ours) to the above studies, as bFGF had been reported to stimulate the proliferation of chondrocytes, this could be explained by the different origins of chondrocytes and different culture circumstances (Adolphe et al., 1984; de Haart et al., 1999; Froger-Gaillard et al., 1989).

Interestingly within 1% cultures, PDGF significantly reduced the doubling times compared to controls and following the second passage, demonstrated a significant increase in the relative quantity of Sox9 mRNA. Previous studies complement our results as PDGF has been shown to have a stimulatory effect on rabbit articular chondrocytes when present in basal media supplemented with 1% FCS (Prins et al., 1982b). PDGF has also been shown to modulate limb chondrogenesis functioning to promote chondrogenesis at early stages of limb development and inhibitory at later stages (Ataliotis, 2000). This may be related to the immature status of both cell types i.e. early chondrogenesis and progenitor cell.

Our study also demonstrated that IL-1β significantly reduced the doubling times compared to control cultures following the second passage. Although, often described as a cytokine associated with a modulation of the phenotype and a negative regulator of chondrogenesis no significant decrease in Sox9 expression was observed in this study. Pellets derived from chondroprogenitors expanded in IL-1β contained areas of GAG deposition as well as labelling for type II collagen.

IL-1 induced chondrocyte responses are central in pathogenic events involved in both rheumatoid arthritis and osteoarthritis including the induction of matrix metalloproteinases, other proinflammatory cytokines and the inhibition of ECM synthesis. Our study may demonstrate an attempt by cartilage to repair itself; the chondroprogenitor cells reside at the surface and are responsible during development for appositional growth. The response of the chondroprogenitor cells to proliferate in response to IL-1β, may be a ‘built-in’ protection by cartilage in an attempt to repair. Cartilage by design needs its progenitors’ to reside at the surface for appositional
growth, but the surface is vulnerable to damage. It is the surface cells that are removed during the first stages of osteoarthritis following damage to the articular cartilage (McDevitt and Muir, 1976).

The growth regulatory effects of IL-1 on mesenchymal cells are dependent on their differentiation status, inhibiting the proliferation of chondrocytes but stimulating fibroblastic growth. When chondrocytes modulate their phenotype the growth factor responsiveness of the cells also changes and the inhibitory effects of IL-1 decreases (Guerne et al., 1994). The mechanisms responsible for the differential growth regulatory effects are not characterised. Such a characterisation using our chondroprogenitor cells would be beneficial for cartilage repair as most studies use full depth derived chondrocyte and, thus, a mixed population of cells.

The chondroprogenitors did have the capacity to redifferentiate and generate a cartilaginous like tissue with scattered areas of ECM containing GAG. Although collagen type II labelling was present, indicating chondrogenesis there did appear to be a substantial amount of type I collagen which is indicative of fibrocartilage. Matrix accumulation in most cases was modest.

Chondroprogenitors expanded in 1% FCS and in the presence of TGF/FGF died for reasons unknown. The content of serum and its several peptide and hormonal components, however, do have divergent effects on growth and PG synthesis during cell culture. It is known that individual factors exhibit individual as well as overlapping, synergistic or agonistic effects. The effect of serum was exemplified during the first passage. Although no significant differences were seen between 1% and 10% FCS control cultures, however, the doubling times of 10% FCS cultures containing EGF, FGF and IL-β were significantly lowered. Following the second passage, 10% FCS cultures containing EGF (again) and TGF were significantly reduced compared to 1% FCS cultures. Interestingly, the 10% FCS containing TGF culture nearly halved the doubling time compared to 1% FCS cultures. This part of the study highlights that the chondroprogenitors’ response to each growth factor should be optimised when presented alone or in combination.
Recent studies on the expansion of chondroprogenitor enriched population demonstrated that the initial serum concentration has a great impact on cell growth during batch cultures (Melero-Martin et al., 2003). An increase of initial serum concentration from 10% to 50% resulted in a 4-fold increase of viable cell density by the end of the exponential phase of the culture. Work by this group also showed that TGF-β1 (supplemented to 10% containing cultures) had an impact on cell proliferation, supplementation of 1-5ng/ml improved the cell density as much as an increase in the initial serum concentration from 10% to 40%. No changes were seen when cultures were supplemented with 1-10ng/ml FGF-2. This study is in agreement with our study using clonally isolated chondroprogenitor cells.

Following the effects of the various different culture media, compositions on the chondroprogenitors in pellet culture in chapter 3, we decided to continue using the defined serum free medium supplemented with 10ng/ml TGF-β1. Our studies demonstrated qualitatively the presence or absence of GAG and collagen but we did not use assays to quantify this. Studies have shown that the conditions of chondrocyte expansion in monolayer modulate at their transcriptional level their commitment to redifferentiation and their ability to respond to regulatory molecules in a 3D environment (Jakob et al., 2001). The effects of growth factors are also dependent on the history of cell expansion and/or the stage of cell differentiation. This aspect was demonstrated by Jakob et al. (2001) who showed that TGFβ supplementation reduced the collagen type II/ collagen type I ratio and aggrecan/versican mRNA ratios of EGF and PDGF expanded chondrocytes while it increased the same differentiation markers in cells expanded with FGF-2/TGFβ. Another study highlighted, that the addition of TGF-β during primary cultivation and first passage, followed by addition of L-ascorbic acid in the 2nd and 3rd passage, resulted in a 7-fold increase in cell number compared to the control group, in about 4 weeks (de Haart et al., 1999). The study addressed the importance of adding the specific growth factors at the right time. It will be interesting to re-test the various culture medium compositions used in chapter 3 on pellets derived from chondroprogenitors following their expansion in the various growth factors, namely TGFβ/FGF and IL-1β.

Most approaches to tissue repair use autologous cells from healthy sites i.e. peripheral epiphyses. The limitations for this procedure include the small number of cells
available at the donor site and the limited ability of the harvested cells to proliferate and undergo differentiation.

Future goals could include simple, minimally invasive procedure to harvest the progenitor cells by taking a small biopsy from the patient followed by application of regulatory factors at distinct time points during expansion and redifferentiation.

Further investigation of combination factors, to improve composition of engineered pellets will be necessary as will eliminating serum from culture media.
CHAPTER 5
The plasticity of articular cartilage progenitor cells *in vitro*
5.1 INTRODUCTION

Mesenchymal stem cells (MSCs) derived from bone marrow stroma have the capacity for extensive self-renewal and selective differentiation into a variety of mesenchymal tissue lineages (Caplan, 1991). When placed in appropriate culture environments, the MSCs give rise to bone (Hanada et al., 1997; Pittenger et al., 1999), cartilage (Pittenger et al., 1999; Yoo et al., 1998), muscle (Wakitani et al., 1995), fat (Majumdar et al., 1998) and a stromal or fibrous phenotype (Caplan, 1991). Recently, cells co-purifying with mesenchymal stem cells, termed, multipotent adult progenitor cells (MAPC) were shown to differentiate, at the single cell level, not only into mesenchymal cells, but also cells with visceral mesoderm, neuroectoderm and endoderm characteristics in vitro (Jiang et al., 2002; Woodbury et al., 2002; Woodbury et al., 2000).

Although more restricted potentially than embryonic stem (ES) cells, MSC’s have been extensively used for repairing defects and disease management. ES cells do have many ethical and political issues that accompany them. However, harvesting the MSCs does result in pain, morbidity and low cell number. Current research involves the investigation of an alternate source to MSC’s.

Recent studies have demonstrated that adipose tissue also represents a source of stem cells (Zuk et al., 2002; Zuk et al., 2001). A putative stem cell population within human lipoaspirates, called ‘processed lipoaspirate’ (PLA) exhibited stable growth and proliferation kinetics in culture. PLA, following exposure to lineage specific factors, differentiated in vitro toward the osteogenic, adipogenic, myogenic, chondrogenic and neurogenic lineages.

Articular chondrocytes when expanded in culture at low density in monolayer culture undergo phenotypic modulation. Expansion in monolayer promotes a fibroblastic-like morphology, and the expression of macromolecules typical of pre-chondrogenic mesenchyme such as collagen type I and versican (Benya and Shaffer, 1982; Binette et al., 1998). Phenotypically modulated chondrocytes can be reversed by culturing cells in an environment that supports a spherical morphology and inhibits cell flattening, a 3-D pellet system as described in chapter 4. Redifferentiation also occurs
under controlled conditions in a chemically defined serum-free media containing specific combinations of growth factors and hormones (Martin et al., 1999; Yaeger et al., 1997).

Recent reports also demonstrate that dedifferentiated adult human chondrocytes share some functional features of MSCs (Barbero et al., 2003; Dell’Accio et al., 2003; Tallheden et al., 2003). Quarto et al., (1997) hypothesised that a loss of chondrogenic phenotype could be accompanied by the acquisition of new differentiation pathways. Although adipogenic and osteogenic development did not occur spontaneously in vitro the microenvironment to which the implanted cells were exposed to in the host were sufficient to induce alternative lines of differentiation. Recent studies confirmed the capabilities of dedifferentiated chondrocytes to redifferentiate toward the chondrocytic phenotype. However, unlike MSCs there was no induction of the hypertrophic marker collagen type X (Quarto et al., 1997). Dedifferentiated human chondrocytes also differentiated towards the osteoblastic following exposure to osteogenic supplements and bone morphogenetic protein-2 (BMP-2) and adipocytic lineages during a 3 week culture in adipogenic medium (Barbero et al., 2003; Dell’Accio et al., 2003; Quarto et al., 1997; Tallheden et al., 2003).

Barbero et al.,(2003) demonstrated that supplementation of their expansion medium with the three growth factors, transforming growth factor-β1 (TGF-β1), fibroblast growth factor-2 (FGF-2) and platelet derived growth factor type BB(PDGF-BB) enhanced dedifferentiation of human articular chondrocytes. The enhancement of cell dedifferentiation increased their chondrogenic and osteogenic capabilities but decreased adipocyte formation.

Attempts have been made at determining the sequence of branching involved in the commitment of undifferentiated cells toward adipogenesis, chondrogenesis, or osteogenesis. Cellular components of the haematopoietic system have been successfully established.

It is apparent that the chondrogenic phenotype is unstable in vitro and that the fate of the chondrocyte (although seemingly versatile) can be determined by both the in vivo and in vitro history of the cell. Quarto et al., (1997) highlighted this history in their
Book early to be sure of your order.

Please note - our terms and conditions for cancellations are available through the Call Centre.

Important: You will not receive the on-line hire discount if you order linen through the Call Centre. To access the RINDI Tyndall system, dial 1800 123 386 or 123 456 789 and follow the on-screen prompts. You will be recorded for data protection. Receipts for the order will be sent by email or post within 2 days of receipt.

If you do not have access to a computer, hire your own or call our Call Centre.

You can also buy your robes on-line.

You can also buy your own robes on-line.

Pay by credit or debit card (all major cards accepted) and receive a 10% discount.

www.gowndhire.co.uk

WOMBLEDGE 2004

Save Money!
5.2. MATERIALS AND METHODS - IN VITRO DIFFERENTIATION

5.2.1. Chondrocyte Isolation

SZ, MZ and DZ chondrocytes were isolated from 7-day bovine MCP joints as described in section 2.2.1. Following digestion, the cells were plated at 5x10^4 cells/cm² in 35mm culture dishes. Each dish was kept in basic medium (DMEM containing 10% FCS supplemented with 50µg/ml ascorbic acid and 50mg/ml Gentamycin (DMEM+)) until sub-confluent.

5.2.2. Colony isolation

SZ chondrocytes were isolated as described in section 2.2.1. SZ cells were subjected to differential adhesion on FN (10µg/ml in PBS+) in 35mm Petri-dishes for 20 minutes. Non-adherent cells were aspirated and chondrocytes were cultured for up to 4 days. Colonies of 32+ cells were subsequently cloned as described in section 3.3.5 and cultured in 35mm dishes until sub-confluent in basic medium (DMEM+).

5.2.3. In vitro differentiation media

 Cultures were stimulated with the appropriate differentiating medium (fig 5.1) according to the following conditions:

Osteogenic differentiation was induced in 2-D cultures, using well-established medium supplements (Jaiswal et al., 1997; Maniatiopoulos et al., 1988) in basic medium supplemented with 10% FCS, 10mM β-glycerophosphate, 10nM dexamethasone, and 0.1mM L-ascorbic acid-2-phosphate (osteogenic medium, OM) (all from Sigma) and cultured for 3 weeks, with medium changed twice weekly.

Adipogenic differentiation was performed in 2-D cultures using cycles of treatment with different media (Pittenger et al., 1999). The cells were cultured in basic medium with 10% FCS until confluent. Cells were then treated with adipogenic induction medium (AIM); 10μg/ml insulin, 1µM dexamethasone, 100µM indomethacin, and 500µM 3-isobutyl-1-methyl xanthine (IBMX) plus DMEM (all from Sigma-Aldrich) for 72 hours and subsequently with adipogenic maintenance medium (AMM) - 10µg/ml insulin plus DMEM for 24 hours. Adipogenic supplements were added to
Figure 5.1. Schematic illustration showing the overall strategy for chondrocyte isolation (1,2), their expansion (3) and subsequent stimulation with differentiation media (4); osteogenic media (OM), adipogenic media (AM), neurogenic media (NM) or control media (OCM, ACM and NCM). The final closed boxes indicate the procedures used to detect differentiation (5).
basic medium containing 10% FCS. After the 96 hour treatment cycle was repeated 4 times, cells were cultured for an additional week in adipogenic medium.

Neurogenic differentiation was induced by culturing the cells for 24 h in pre-induction medium (DMEM, 20% FCS, 1mM β-mercaptoethanol-BME). After pre-induction, the cells were induced for up to 6 hours in neurogenic medium (NM), according to an established protocol (Woodbury et al., 2000). Briefly cells were washed with PBS and transferred to neuronal induction media composed of DMEM/1-10mM BME.

As controls, confluent cultures were kept in basic medium for the same period of time as the corresponding differentiation protocols, but without the stimulating factors in order to analyse spontaneous changes in phenotype over time.

5.2.4. Histochemical and Immunohistochemical analysis

Cells exposed to osteogenic induction media and appropriate controls were fixed in 4% paraformaldehyde and stained for the presence of alkaline phosphatase (AP) using ‘86-C alkaline phosphatase kit’ (Sigma). Parallel cultures were fixed in 10% formalin and treated with von Kossa’s stain (see appendix) to detect areas of mineralization.

Cultures grown in the cyclic adipogenic media were fixed in 60% isopropanol for 2 minutes and incubated in oil red O solution (0.1% in 60% isopropanol) for 20 minutes.

Neurogenic induced cultures were fixed in chilled 95% IMS and tested for neurogenic phenotype with immunohistochemical detection of monoclonal anti-Tau and monoclonal anti-β-III tubulin.

Immunolocalisation was carried out in the 35mm culture dishes. The cells were washed with PBS-T before blocking serum with goat serum. The blocking solution was diluted with PBS-T at 1:20 and incubated for 20 minutes. The blocking solution was poured off and the primary antibodies anti-Tau (Chemicon) and anti-β-III tubulin (Chemicon) (table 6.1) added overnight at 4°C. The primary antibodies were washed off with 3x5 minute washes in PBS-T and binding visualised using Alexa Fluor 488
goat anti-mouse used at 10μg/ml (Molecular Probes, Cambridge) diluted in PBS-T which was applied for 1 hour. A final 3x5 minute washing step in PBS-T ensued.

### 5.2.5. Controls

Positive control for neurogenic assay consisted of a section of rat brain. Negative controls for both antibodies were processed without the primary antibody. Further controls were performed using mouse immunoglobulins (MlgG) (DAKO) to ensure that no non-specific binding of the primary mouse antibody occurred.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Polyclonal(p)/ Monoclonal(m)</th>
<th>Recognises</th>
<th>Optimal working dilution</th>
<th>Pretreatment</th>
<th>Source/ Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-tubulin, beta III isoform (MAB 1637)</td>
<td>M</td>
<td>Peptide C-ESEQGPK</td>
<td>1:100</td>
<td>None</td>
<td>Chemicon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(specific for neurons)</td>
<td></td>
<td></td>
<td>Mitsui et al., 2002</td>
</tr>
<tr>
<td>Anti-TAU</td>
<td>M</td>
<td></td>
<td>1:200</td>
<td>None</td>
<td>Chemicon LoPresti et al., 1995</td>
</tr>
</tbody>
</table>

**TABLE 5.1. Antibodies used during immunohistochemistry**

### 5.2.6. Microscopic Characterisation

Fluorescence was examined using a Leitz Laborlux 12 fluorescent microscope. Images were captured using a RS Photometrics Coolsnap camera and Coolsnap image software and formatted with Adobe Photoshop (version 5.5). Histochemical staining were viewed with a Leica (Leitz DMRB) light microscope and Images taken using Image grabber software and formatted using Adobe photoshop (version 5.5).

### 5.2.7. Total RNA extraction and cDNA synthesis

Following 21 days of culture in osteogenic media, adipogenic media or control media, mRNA from culture dishes was extracted as described in section 3.2.14.
5.2.8. Real-time PCR amplification and quantitative analysis.

PCR reactions were performed and monitored using an ABI Prism 7700 Sequence Detection System as described in section 3.2.16. Genes analysed included osteocalcin and lipoprotein lipase (see table 5.2 and 5.3). We quantified the differences in expression of these genes between clonal, SZ, MZ or DZ derived cultures expanded in osteogenic, adipogenic media and control medium. The ABI relative standard curve method was used as PCR reaction efficiencies were not equal (osteocalcin and rRNA; lipoprotein lipase and rRNA).

For the relative standard curve method, a dilution series of control cDNA (lipoprotein lipase; fat mRNA, and osteocalcin; bone mRNA) was run (in triplicate) for both the gene of interest and the standard (rRNA) on the same 96-well plate as the samples (see appendix). Ct values and total RNA quantities were used to generate a relative standard curve. Treated and non-treated qPCR reactions using 0.1μl of cDNA (derived from approximately 2ng RNA) were indexed in triplicate to the relative standard curve. The calculations recommended by Applied Biosystems (Biosystems, 1997) were employed to calculate relative expression of the RNA species of interest, normalised to the internal standard and to the non-treated control sample. Controls replacing cDNA template with water were performed on each plate.

Quantitative PCR reactions were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute within an ABI Prism 7700 Sequence Detection System (Applied Biosystems, CA, USA).

5.2.9. Statistical analysis.

All values are presented as the mean +/- standard deviation. Differences between experimental groups were assessed as by a Student’s 2-tailed paired t-test for distributions with unequal variances. P values less than 0.05 were considered significant.
<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Osteocalcin</strong></td>
<td><strong>aaa ggc gca gcc ttc gt</strong></td>
<td><strong>agg tag cgc ctg agt ctc ttc a</strong></td>
<td><strong>aacctgctgccccctgctgtg</strong></td>
</tr>
<tr>
<td>Acc. X53699</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lipoprotein lipase</strong></td>
<td><strong>aac tgg atg ggc gat gaat</strong></td>
<td><strong>ggg ccc caa ggc tgt atc</strong></td>
<td><strong>aactatcctggggcaatgtgcat ctc</strong></td>
</tr>
</tbody>
</table>

**Table 5.2:** Quantitative PCR primer and probe sequences. All probes are FAM-TAMRA labelled

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Osteocalcin</strong></td>
<td><strong>50μM</strong></td>
<td><strong>50μM</strong></td>
<td><strong>10μM</strong></td>
</tr>
<tr>
<td><strong>Lipoprotein lipase</strong></td>
<td><strong>50μM</strong></td>
<td><strong>50μM</strong></td>
<td><strong>10μM</strong></td>
</tr>
</tbody>
</table>

**Table 5.3:** Quantitative PCR primer and probe reaction concentrations.
5.3. Results

5.3.1. Adipogenic Assay
At day 0, all cultures had different morphologies, clonal cultures contained a mixed population of rounded and fibroblastic like cells (fig 5.2A), and surface zone (SZ) derived cultures contained smaller rounded cells (fig 5.2B). Middle zone (MZ) and deep zone (DZ) derived cells were fibroblastic (fig 5.2C & D). The experiments began when a culture became sub-confluent in this instance it was DZ cultures (fig 5.2D).

Following a further 21 days in culture, the treated and non-treated cultures appeared different morphologically. The most obvious difference was that treated cultures (fig 5.2 E-H) appeared to contain a higher density of cells compared to controls (fig 5.2 I-L). Clonally derived cultures treated with adipogenic media (AM) were very closely packed compared to controls (fig 5.2 E & I). There were, however, enlarged cells, typical of adipocyte within both clonal (fig 5.2E) and SZ cultures (5.2F). SZ derived cultures produced ‘swirl like’ patterns in the culture dish which was not observed in the control cultures (fig 5.2F & J). It was very difficult to distinguish individual cells within MZ and DZ treated cultures as there was such a high density of cells compared to their controls (fig 5.2G&K and H&L respectively).

Histological examination using oil red O revealed the formation of small lipid droplets within the isolated clone cultures following four cultures of adipogenic inductive treatment (fig. 5.3A). No lipid droplet formation was evident in the corresponding control dish (fig. 5.3B).

Lipid containing cell clusters were infrequently found in SZ, MZ, and DZ derived cultures treated with adipogenic media (fig. 5.3 C, E, G).

In no case were clusters of lipid containing cells found in control (untreated) samples (fig. 5.3 B, D, F, H).
Real time reverse transcriptase polymerase chain reaction (RT-PCR) analysis of the expression of mRNA for lipoprotein lipase, in the cultures exposed to adipogenic treatment verified the above histological observation seen within clonal cultures.

There was a significant up regulation of the relative quantity of lipoprotein lipase mRNA ($p<0.005$) within the clonal culture treated with adipogenic media as compared with the control within experiment 1 (fig. 5.4A). This was not repeated in the 2nd experiment.

In both experiments (1 and 2) (graphs 5.4A and B) there was a significant downregulation in the lipoprotein lipase mRNA level ($p<0.01$ and $p<0.05$ respectively) with DZ derived chondrocytes treated with adipogenic media as compared to control.
Figure 5.2. Expansion of isolated clones, surface zone (SZ), middle zone (MZ) and deep zone (DZ) derived chondrocytes. Phase microscopic appearance of representative clones (A, E, I), SZ (B,F,J), MZ (C, G,K) and DZ (D,H,L) at time zero (A,B,C,D) and following 21 days in culture in adipogenic media (AM) (E,F,G,H) and control media (I,J,K,L). Scale bar = 100μm. At day 0, all cultures had different morphologies. Following 21 days of treatment, cultures treated with AM contained a higher density of cells compared to controls. Treated clonally and SZ derived cultures contained enlarged cells typical of adipocytes.
Figure 5.3. Adipogenic differentiation of isolated clones, surface zone (SZ), middle zone (MZ) and deep zone derived (DZ) chondrocytes. Oil red O staining of representative cultures of isolated clones (A,B), SZ (C,D), MZ (E,F) and DZ (G,H) expanded with adipogenic media (A,C,E,G) or with control media (B, D, F, H). Scale bar = 100μm.

Several adipocytic colonies were observed with oil red O staining following cyclic induction with adipogenic media within clonal cultures (A).

Lipid containing cells were infrequently found in SZ (C), MZ (E) and DZ (G) derived cultures treated with adipogenic media (see arrows).

Without the stimulatory factors no adipocytic lipid droplet were found in untreated controls.
Figure 5.4. Real-time reverse transcriptase-polymerase chain reaction analysis of the expression of mRNA for lipoprotein lipase in cultures exposed to both control and adipogenic treatment. Levels are expressed as the mean difference from those measured from control cultures. Statistically significant differences were determined using paired sample t-test.

(A) Experiment 1

There was a significant upregulation (30-fold) of the relative quantity of lipoprotein lipase mRNA within clonal cultures exposed to cyclic induction with adipogenic media as compared to control samples (p<0.0005).

A significant downregulation of lipoprotein lipase mRNA within adipogenic treated DZ derived chondrocytes (p<0.01).

(B) Experiment 2

The only observed significant difference was the downregulation of lipoprotein lipase mRNA within the adipogenic treated cultures derived from DZ chondrocytes compared to control (p<0.005).
5.3.2. Osteogenic media

Osteogenic media was added and the experiment commenced when a culture reached subconfluence, in this case MZ. Clonal cultures contained both rounded and fibroblastic like cells (fig 5.5A) while SZ contained small rounded chondrocytes (fig 5.5B). MZ and DZ cultures contained mostly fibroblastic cells (fig 5.5C&D).

Following a further 21 days of culture, the treated cultures (fig 5.5E-H) contained a higher density of cells than the non-treated controls (fig 5.5I-L) making it difficult to distinguish one cell from another. In all cases the DZ derived chondrocytes following 21 days of culture detached from plastic (fig.5.5H). Within the control cultures, both clonal (fig 5.5I), SZ (fig 5.5 J), MZ (fig 5.5H) contained fibroblastic like cells, DZ cells were compacted (fig 5.5L).

Following 21 days in culture no culture stained positive for alkaline phosphatase or von Kossa, indicative of the absence of a calcium rich matrix.

The only detectable upregulation of mRNA for osteocalcin was within the SZ culture following 3 weeks of OM, but this was not significant (fig 5.6). No osteocalcin mRNA was detected in clonal derived cultures or DZ derived chondrocytes. Osteocalcin was detected in MZ derived chondrocytes. Within the second experiment no osteocalcin mRNA was detected at all – PCR problems.
Figure 5.5. Expansion of isolated clones, surface zone (SZ), middle zone (MZ) and deep zone (DZ) derived chondrocytes. Phase microscopic appearance of representative clones (A, E, I), SZ (B,F,J), MZ (C, G,K) and DZ (D,H,L) at time zero (A,B,C,D) and following 21 days in culture in osteogenic media (OM) (E,F,G,H) and control media (I,J,K,L). Scale bar = 100μm. At day 0, all cultures had different morphologies. Following 21 days of treatment, cultures treated with OM contained a higher density of cells compared to controls. In all cases the DZ derived chondrocytes following 21 days of culture detached from the plastic (H).
Figure 5.6. Real-time reverse transcriptase-polymerase chain reaction analysis of the expression of mRNA for osteocalcin in cultures exposed to both control and adipogenic treatment. Levels are expressed following normalisation to control cultures.

There was only a detectable upregulation of osteocalcin mRNA within superficial zone derived cultures following 3 weeks of osteogenic media.
Relative quantities of osteocalcin mRNA

control

- treated

Surface  Middle  Deep  Clone

0  200  400  600  800  1000  1200  1400  1600  1800
5.3.3. Neuronal differentiation

To induce the neuronal phenotype, clones, SZ, MZ and DZ derived chondrocytes were cultured in serum-containing medium supplemented with 1mM β-mercaptoethanol (BME) for 24 hours. To affect neuronal differentiation, the cells were transferred to serum-free medium containing 1-10mM BME. The greatest change in morphology (within the shortest time) was observed using 10mM BME. Within 2 hours of treatment/exposure, changes in morphology in all cultures were apparent; clonal (fig. 5.7C), SZ derived (fig. 5.8C), MZ derived (fig. 5.9C) and DZ derived (fig. 5.10C). Responding cells appeared to retract their cytoplasm and take on a rounded morphology. Some cells exhibited process like extensions.

To characterise neuronal differentiation further we fixed BME treated cultures after 6 hours and examined immunohistochemically for the presence of tau, neuron-specific microtubule associated protein expressed by differentiating neurons (Kosik and Finch, 1987). Cells exhibiting a neuronal morphology (fig. 5.11 A, C, E & G) expressed tau protein in the cell body. There was positive labelling identified within the cell process of the isolated clone culture (fig. 5.11A). Flat cells were tau-negative.

We also labelled for the neuronal marker β-III tubulin an intermediate filament characteristic of mature neurons present in virtually all cells. Interestingly only the isolated clones (5.12A) labelled positive for β-III tubulin with SZ, MZ and DZ (5.12 C, D, G) derived cultures all β-III tubulin negative. All control cultures were negative (5.12 B, D, F, H).
Figure 5.7. Morphological differentiation of isolated clones. Images of clones were captured following 24 hours in pre-induction medium (A,C,E,G) and control media (B,D,F,H) – 0 hours and after 2 hours (C,D), 4 hours (E,F) and 6 hours (G,H) of exposure to neuronal induction media (NIM) and control media respectively.
Scale bar = 100μm.
Images are representative of typical morphologies before and during treatment but are not from the same field.
Clones rapidly transform from a fibroblastic morphology (A) to a more rounded morphology (C, E, and G) when exposed to NIM.
Figure 5.8. Morphological differentiation of isolated surface zone (SZ) derived chondrocytes. Images of clones were captured following 24 hour in pre-induction medium (A, C, E, G) and control media (B, D, F, H) – 0 hours and after 2 hours (C, D), 4 hours (E, F) and 6 hours (G, H) of exposure to neuronal induction media (NIM) and control media respectively. Scale bar = 100μm.

Images are representative of typical morphologies before and during treatment but are not from the same field. SZ derived chondrocytes rapidly transform from a fibroblastic morphology (A) to a more rounded morphology (C, E, and G) when exposed to NIM.
Figure 5.9. Morphological differentiation of isolated middle zone (MZ) derived chondrocytes. Images were captured following 24 hour in pre-induction medium (A,C,E,G) and control media (B,D,F,H) - 0 hours and after 2 hours (C,D), 4 hours (E,F) and 6 hours (G,H) of exposure to neuronal induction media (NIM) and control media respectively. Scale bar = 100μm. Images are representative of typical morphologies before and during treatment but are not from the same field. MZ derived chondrocytes rapidly transform from a fibroblastic morphology (A) to a more rounded morphology (C, E, and G) when exposed to NIM.
Figure 5.10. Morphological differentiation of isolated deep zone (DZ) derived chondrocytes. Images were captured following 24 hour in pre-induction medium (A, C, E, G) and control media (B, D, F, H) – 0 hours and after 2 hours (C,D), 4 hours (E,F) and 6 hours (G,H) of exposure to neuronal induction media (NIM) and control media respectively. Scale bar = 100μm.

Images are representative of typical morphologies before and during treatment but are not from the same field. DZ derived chondrocytes rapidly transform from a fibroblastic morphology (A) to a more rounded morphology (C, E, and G) when exposed to NIM.
Figure 5.11. Neurogenic differentiation of isolated clones, surface zone (SZ), middle zone (MZ) and deep zone derived (DZ) chondrocytes. Immunohistochemical labelling for the neuronal protein marker tau 6 hours after induction of representative cultures of isolated clones (A, B), SZ (C, D), MZ (E, F) and DZ (G, H) expanded with neurogenic media (A, C, E, G) or with control media (B, D, F, H).
Scale bar = 100μm.

Cells exhibiting a neuronal morphology labelled for tau within the cell body and extending into process – asterix (A) whereas flattened cells are tau-negative. Untreated samples are tau negative.
Figure 5.12. Neurogenic differentiation of isolated clones, surface zone (SZ), middle zone (MZ) and deep zone derived (DZ) chondrocytes.

Immunohistochemical labelling for the neuronal protein marker β-III tubulin, 6 hours after induction of representative cultures of isolated clones (A,B), SZ (C,D), MZ (E,F) and DZ (G,H) expanded with neurogenic media (A,C,E,G) or with control media (B, D, F, H). Scale bar = 100μm.

Isolated clones exposed to neurogenic media labelled for β-III tubulin expression. SZ, MZ and DZ derived chondrocytes were negative for β-III tubulin as were control cultures.
Figure 5.13. Immunohistochemical and histochemical controls. A) Phase contrast image of rat brain cortex. B) Positive labelling for tau in brain cortex. C) positive labelling for β-III tubulin in brain cortex. D) negative control, omitting primary antibody on rat brain cortex sections. E) Negative control, omitting primary antibody from cultured clones. F) Negative control, using MIG. G) Positive staining for fat using oil red O staining protocol, on adipose tissue obtained from the hoof of 7 day bovine calf. H) Positive staining for alkaline phosphatase within 1 week chick tibias. Scale bar = 100μm.
5.4 DISCUSSION

Phenotypically modulated adult human chondrocytes share some functional features of mesenchymal stem cells, to not only redifferentiate towards the chondrocyte phenotype but to also differentiate towards the adipogenic and osteogenic lineages. Differentiation towards the adipogenic, osteogenic and chondrogenic (following 18 population doublings) does not occur spontaneously in vitro but requires exposure to specific lineage specific factors.

Within our study, clonally derived cells accumulated lipid droplets following the 3 week cyclic exposure to adipogenic medium (AM). Lipid containing cell clusters were infrequently found in surface zone (SZ), middle zone (MZ) and deep zone (DZ) derived chondrocyte cultures. No lipid droplet formation was evident in the corresponding control cultures. The study was qualitative as positive cells were not numerically analysed. Tallheden et al. (2003) demonstrated with each cyclic induction, the number of lipid droplet containing cells increased, however, the frequency within each culture varied from donor to donor. Clonal cultures treated with AM contained clusters of enlarged cells which are characteristic to adipocytes. Enlarged cell were also apparent in the SZ cultures, which would also contain the chondroprogenitor population too.

The expression of lipoprotein lipase (LPL) a lipid exchange enzyme upregulated during adipogenesis (Ailhaud et al., 1992) was quantified within adipogenic samples using real time reverse transcriptase polymerase chain reaction (RT-PCR). There was a significant up-regulation of the relative expression of LPL mRNA within the clonal culture treated AM as compared to the control verifying the histological observation.

Zuk and colleagues (2002) demonstrated that induction of PLA cells with adipogenic media resulted in an expanded cell morphology and a time dependent increase in Oil red O staining but LPL levels beyond 7 days were shown to decrease. The group also demonstrated an altered sequence of adipose gene expression in PLA derived cells compared to MSC's, that could be due to a distinct developmental program characteristic of stem cells or the asynchronous development of cell subpopulations within the heterogeneous PLA.
Our study did not verify that the adipogenic induction conditions used were specific for fat lineage and did not result in the expression of genes consistent with for example bone. It would be of interest in future to study the several genes/proteins involved in lipid biosynthesis e.g. PPARγ2 a fat specific transcription factor that functions in preadipocyte commitment (Totonoz et al., 1994). Dell’ Accio et al (2003) revealed that both early and late passaged human articular chondrocytes accumulated oil red O-positive vacuoles, and an upregulation of the adipogenic markers PPARγ and aP2 following exposure to AM. Barbero et al. (2003) demonstrated that dedifferentiated articular cartilage also accumulated lipid droplets during 3 weeks of culture. However, no up-regulation of adipsin mRNA expression was observed. They concluded that no overt differentiation into adipocytes had been demonstrated, and that the formation of lipid droplets was a possible result of metabolic changes induced by the strong pharmacologic stimuli used in the form of IBMX and indomethacin. It is possible that this may have been true for our study. Indeed, during development, it is not uncommon to see lipid-like droplets within chondrocytes.

Unlike our clonally derived chondroprogenitors and previously tested expanded human chondrocytes (Barbero et al., 2003), fibroblast cell lines and human dermal fibroblast were not responsive to the same adipogenic stimuli (De Bari et al., 2001; Pittenger et al., 1999). It was proposed that the expanded adult human chondrocytes have an intrinsic capacity to generate adipocyte-like cells. Chondrocytes derived from mouse embryonic stem cells and subcultured also transdifferentiated towards the adipogenic lineage (Hegert et al., 2002).

Within our study following exposure to osteogenic supplements (OM), there was no differentiation toward the hypertrophic lineage or osteogenic lineage as assessed by histochemical staining. We did not label for type X collagen which, could be analysed in future experiments. Tallheden et al. (2003) demonstrated that all chondrocytes (control and OM treated) expressed alkaline phosphatase, however, following the completion of the experiment there was a 1.8 fold higher alkaline phosphatase/DNA ratio in OM treated samples compared to controls. The OM treated samples in the study above, had a higher DNA content than controls, indicative of a postconfluent growth. Although only qualitative, we did identify an increase in cell density when cultured under osteogenic conditions.
Recent work has questioned the efficacy of glucocorticoids, such as dexamethasone in mediating osteogenesis (Cooper et al., 1999). Zuk and colleagues (2001), demonstrated that osteogenic medium containing 1, 25-dihydroxyvitamin D₃ (vitamin D) rather than dexamethasone produced increased levels of alkaline phosphatase within processed lipoaspirate (PLA) cell population cultures. They also showed that treatment of MSC’s with dexamethasone produced increased alkaline phosphatase levels compared with vitamin D induction. This result is suggestive of a differential response to induction conditions between the two populations — glucocorticoid having an inhibitory effect. It is possible that the induction medium used in the present study is not sufficient to induce alkaline phosphatase activity.

Cells were examined by RT-PCR for the expression of osteocalcin. The absence of osteocalcin expression confirmed that the cells had not undergone a osteogenic change. Osteocalcin expression has been detected in bone cells or chondrocytes undergoing transformation to osteogenic cells (Boivin et al., 1990; Ishizeki et al., 1997; Nakase et al., 1998). Future experiments will analyse a range of genes that are regulated during this transformation including type I collagen and osteopontin since osteocalcin is a late marker of osteogenesis. It has been demonstrated that stimulation of bovine deep zone derived-chondrocytes with β-glycerophosphate (without dexamethasone) was sufficient to induce mineralization, and upregulation in gene expression of collagen type I, X, osteopontin and alkaline phosphatase (Sun and Kandel, 1999). The induction was achieved when the cells were plated on filter inserts and - a 3d environment. In contrast to MSCs, chondrocytes formed cartilage only, and not bone, in an *in vivo* osteochondrogenic assay (Tallheden et al., 2003).

Bone specific gene osteocalcin was observed in both PLA cells and MSC. Osteocalcin expression in osteo-induced PLA cells seemed to be biphasic — with expression at 7 days, no expression at 14 days and then expression at late phases (21-42 days). This biphasic response may be the response of PLA subpopulations at distinct developmental stages to osteogenic induction (Zuk et al., 2001). Osteo induced MSC revealed consistent expression of osteocalcin. Exposure of PLA cells to dexamethasone inhibited the expression of osteocalcin at all time points — replacement of dexamethasone with vitamin D for last 48 hours of induction was
sufficient to overcome the inhibitory effect. Inhibitory effect has been observed in rat MSC’s and human bone cultures (Jaiswal et al., 1997; Leboy et al., 1991).

Within 2 hours of exposure to neuronal induction media (NM) there were changes in morphology in all cultures. The precise mechanisms by which BME induces neuronal differentiation are unclear. Its antioxidant and properties, which enhance neuronal survival \textit{in vitro} (Ishii et al., 1993), may be partially responsible for the neuronal induction. Following 6 hours in culture, all cultures were Tau positive but only clonally derived cultures were β-III tubulin positive. Woodbury et al., 2002 hypothesised that the rapidity of expression (5 hours) was due to the prior expression of neuronal genes by the MSC’s may explain the rapid response, this could be true for our chondroprogenitor cells but seems less likely.

Tau is a microtubule stabilisation factor, that appeared to label axons exclusively but has since been demonstrated in somatodendritic, astrocytic compartments of the brain, oligodendrocytes as well as neurofibrillary tangles associated with Alzheimer’s disease (Grundke-Iqbal et al., 1986; LoPresti et al., 1995). Nuclear and cytoplasmic tau protein from human non-neuronal cells have been shown to share common structural and functional features with brain tau (Cross et al., 2000). It is possible that the tau labelled is not a marker of neuronal differentiation but it is due to a change in cell shape or the preparation of the cells to produce processes as flattened cells are tau negative. The enhanced fluorescence could be due to cell rounding concentrating the fluorescence. Overexpression of tau in non-neuronal cell were shown to induce long cellular processes (Knops et al., 1991).

Beta-III tubulin isotope is widely used as a neuronal marker in normal and neoplastic tissues shortly after the onset of differentiation (Lee et al., 1990; Moody et al., 1989). The results indicated that the clonally derived chondroprogenitor cultures had differentiated toward the neuronal phenotype. Reports have demonstrated, however, that embryonic expression of class III-β tubulin is not solely restricted to neuronal cell types (for review see Molea et al., 1999). Interestingly β-III tubulin has shown non-neuronal labelling around bone (Molea et al., 1999). Hence, the expression of class III-β tubulin is not, in itself, a reliable indicator of neuronal differentiation during embryonic development. Gene expression of various neuronal markers was not tested.
in our study due to difficulties in obtaining tissue. Future analysis could look at many more markers to assess the progress of differentiation as well as enzymes involved in neuronal transduction which would demonstrate a functional significance.

Many groups have proposed that within articular cartilage, there is either an undifferentiated cell population due to the ‘focal’ multilineage differentiation capabilities of expanded articular cartilage (Barbero et al., 2003; Dell'Accio et al., 2003) or that articular chondrocytes display phenotypic plasticity. This chapter illustrates that to some extent, the putative chondroprogenitor cells have the ability to differentiate towards different lineages.

It could be argued that contamination has taken place. However, during isolation only a slither of cells was taken from the surface and so there was no possibility of bone marrow contamination. Cartilage was never taken from the borders that would risk contamination from the synovial membrane.

Barbero et al. (2003), demonstrated that supplementation of their expansion medium with the three growth factors, transforming growth factor-β1 (TGF-β1), fibroblast growth factor-2 (FGF-2) and platelet derived growth factor type BB (PDGF-BB) enhanced dedifferentiation of human articular chondrocytes. The enhancement of cell dedifferentiation increased their chondrogenic and osteogenic capabilities but decreased adipocyte formation. Further expansion of the chondroprogenitor may improve their ability to differentiate towards the various lineages but perhaps not the cartilage one.

The downfall of the study was the limited number of clones analysed, and that the clones analysed were only subjected to one kind of induction media. We will also need to use cells that are known to be induced with the various differentiation induction media as both a comparator and control.

Of the 500 clones isolated and expanded from adipose tissue (PLA), 30 clones exhibited differentiation into at least one of three mesodermal lineages (osteogenic, adipogenic and chondrogenic). Seven clones exhibited differentiation into all 3 lineages (Zuk et al., 2002; Zuk et al., 2001). Other PLA derived clones exhibited a
exhibited a more dual lineage potential osteogenic/adipogenic, osteogenic/chondrogenic and adipogenic/osteocalcin. PLA cells were also induced to express markers consistent with neurogenic phenotype.

It is possible therefore, that during our study we isolated single clones that were capable of differentiating into all lineages. To clarify this issue, future studies will need to clone a larger number of chondroprogenitor to fully characterise their differentiation potential.
CHAPTER 6
The plasticity of articular cartilage progenitor cells *in vivo*
6.1. INTRODUCTION

The ultimate totipotent cell is the fertilised egg which can give rise to all cell types. The development of an animal is often described as the progressive loss of totipotency, then of pleuripotency and finally the differentiation into specific cell types. Pleuripotency has been defined experimentally by assessing whether cells can differentiate into multiple lineages, thus giving rise to more progressively restricted daughter cells that possess trans-germ potential.

Until recently, the embryonic stem cell was believed to be the “gold standard”, capable of differentiating into cells from all three embryonic germ layers (Evans and Kaufman, 1981; Shamblott et al., 1998). However, research on adult-derived stem cells has suggested a more restricted potential. It has been traditionally believed that stem cell progeny progressed in a linear, irreversible manner that eliminated their stem cell propensity which then restricted their fate to within a germ line.

Concepts currently accepted as defining a stem cell are the self renewing capability, the high capacity for cell division (lifespan) maintained throughout the lifetime of the organism, and the multipotential differentiation capability (Morrison et al., 1997). However, such criteria may not apply to cells of postnatal mesenchymal tissues, where there is no need for a real stem cell compartment (due to the low tissue turnover) as an apparent cell plasticity plays the same role (Beresford, 1989; Owen, 1988). The existence of the MSC in the postnatal organism is still debatable.

Recent observations indicating that stem cells isolated from one adult tissue can also give rise to mature cells of other cell lineages (irrespective of classical germ layer designations) contradict dogmas of commitment and differentiation of stem and progenitor cells (Avots et al., 2002). Adult stem cells were considered to be stringently tissue restricted in their development although the mechanisms need to be elucidated but potentially opens the door to a new era in cell based therapies for human diseases.

It is questionable as to whether plasticity reflects in vitro adaptation, transdifferentiation/cell-type switching or the persistence in adult tissues of stem cells.
with extensive endogenous or \textit{bona fide} developmental potentials (Joshi and Enver, 2002).

Studies which exemplify such a theory include: clonal strains of marrow adipocytes can be directed to form bone (Bennett et al., 1991) and chondrocytes can dedifferentiate toward the osteogenic lineage (Galotto et al., 1994). Neurogenic potential of MSCs have been confirmed as well as the induction of haematopoietic stem cells into hepatocytes (Lagasse et al., 2000) and the conversion of neurogenic precursors into muscle and blood (Bjornson et al., 1999; Galli et al., 2000).

The developmental potential of adult stem cells was first demonstrated by Ferrari et al. (1998) by the contribution of transplanted bone marrow derived cells to the muscle compartment of dystrophic mice (Ferrari et al., 1998). The developmental repertoire of neural stem cells was to follow, by their successful haematopoietic engraftment (Bjornson et al., 1999) and contribution to all three germ lines in chimaeric and chicken and mouse embryos (Clarke et al., 2000). Haematopoietic stem cells were shown to migrate and differentiate to neural cells (Brazelton et al., 2000) and muscle derived cells were shown to contribute to the haematopoietic system. The studies triggered a cascade of excitement in stem cell biology as an “alternative and less contentious therapeutic avenue to embryonic stem cells”. However, more stringent analyses of outcome measures such as cross gender transplantations and analyses of homotypic markers, with, for example, transplanted Y-chromosome carrying cells has highlighted the role of cell fusion in these types of experiments. While significant evidence still exists for transdifferentiation, it is thought to occur less frequently than once thought.

Mechanisms for such plasticity remain unknown however at the molecular level several genes appear to participate in more than one lineage pathway. The genes include leptin which participates in both adipogenesis and osteogenesis (Chen et al., 1997), CBFA-1 is not only expressed in marrow stromal cells but is retained as these and other cells differentiate into multiple cell types (e.g. osteogenic and chondrogenic) (Buxton et al., 2003; Satomura et al., 2000).
Experimental methods that have been previously used question plasticity, for example, experiments using populations rather than single cell, therefore, grafts could be heterogeneous with two 2 cell types contributing to the lineages. The long-term rescue of a functional system from a single cell has yet to be demonstrated which would include i.e. self renewal, proliferation, fully differentiation and contribution to active function. Possible explanation for plasticity is fusion with host tissue, bone marrow cells when cultured with embryonic stem cells fuse and the resulting hybrid can differentiate into any lineages. Therefore, adult stem cells thought to be transdifferentiating or plastic could, in fact, result from donor cells that had fused with host cells within various local tissue microenvironments. The hybrid cells, rather than the bone fide donor cells, exhibit the properties of cells normally resident and functional at that site (Terada et al., 2002; Ying et al., 2002). To demonstrate plasticity experiments must include rigorous reproducibility and must avoid the introduction of artefacts during ex vivo manipulation and culture.

The haematopoietic cells that were proposed to have been derived from transdifferentiation of muscle cells were in fact bona fide haematopoietic cells resident within the muscle tissue and not a product of muscle cell transdifferentiation hence demonstrating contamination and the importance of single cell isolation (Kawada and Ogawa, 2001).

Reyes and colleagues (2001) discovered the pluripotency of MSC’s derived from adult human bone marrow. Their report in favour of adult tissue stem cell plasticity describes the isolation, purification, expansion and phenotypic characterisation of cells co-purifying with MSCs (Reyes and Verfaillie, 2001). The generated mesodermal progenitor cells (MPC) were able to regenerate into a wide range of cell types that had the characteristics of osteoblasts, chondroblasts, adipocytes, skeletal myoblasts and endothelium. It is ‘possible that stem cells, unlike more committed precursors are capable of switching phenotypes at a later stage of development.

Another emerging concept is that stem cells are found in a multitude of organs/tissues including the muscle, heart, liver the skin, placenta and fat (Lucas et al., 1992; Toma et al., 2001; Zuk et al., 2001).
Joshi and Enver (2002) postulate that many examples of plasticity may not reflect reprogramming but the presence of residual stem cells within the adult that possess a wide range of developmental potentials. Furthermore, that a population of stem cells are generated during ontogeny which are resident in various tissues and organs where they are quiescent until regeneration or repairs are needed. In this regard, as all stem cells are assumed to originate from the same population, their characteristics and abilities could be similar, with changes made to adapt to their microenvironment (unique microhabitat). Therefore, the exposure of these tissue stem cells to appropriate conditions could potentially allow for regeneration of any organ system. Minasi et al., (2002) provided compelling evidence for the existence of postnatal stem cells residing within mesoderm-derived tissues (Minasi et al., 2002). Quail or mouse embryonic aorta (mesangioblastic cells) once transplanted into host chick embryos gave rise to multiple mesodermal tissues including blood, cartilage, bone, smooth, skeletal and cardiac muscle.

Cancer is thought to be a disease of stem or stem like cells (Reya et al., 2001). The tissue phenotypes of malignancies may, therefore, yield additional clues regarding stem cell plasticity as uterine carcinosarcomas contain both carcinomatous and sarcomatous elements.

Transplantation of long-term in vitro passaged neurosphere-derived cells into mice resulted in tumour formation. It is possible that cultured MPCs harbour cancer-predisposing mutations that might be revealed upon subsequent exposure of the engrafted animals to a second hit mutation (Morshead et al., 2002).

From a therapeutic viewpoint it must be argued whether adult stem cell plasticity has its roots in a transdifferentiation, a reprogramming mechanism, or reflects the existence/persistence of stem cells within the adult that retain extensive and unexpected differentiation potentials as part of their normal developmental programs. The important issue is quality and functionality of the effector cells produced, and understanding the mechanistic route of cell-fate determination for the long term success of stem cell derived grafts.
Wagers et al., (2002) demonstrated that single marked transplanted HSCs could restore haematopoietic system in recipients and their parabiotic partners but did not make any significant functional contribution to non-haematopoietic tissues either normally or under injury-induced repair and regeneration (Wagers et al., 2002). They concluded that transdifferentiation of HSCs is an extremely rare event, if it occurs at all.

Cell-type interconversion in other systems have been familiar to developmental biologists e.g. during limb regeneration in the newt, postmitotic cells at the site of injury form a bud-like structure called the blastema. The cells re-enter the cell cycle by spontaneous de-differentiation (Velloso et al., 2001). McGann et al., (2001) then showed that newt blastema extracts could induce murine myotubes to de-differentiate and undergo morphogenesis and develop into complete new limbs. Thus postmitotic cells have an innate ability to sense and respond to exogenous dedifferentiating factors from the environment suggestive that plasticity may not be an exclusive feature of stem cells (McGann et al., 2001).

Transcription factors have been implicated as key regulators in cell-fate switches and reprogramming. Such example is the induction of myogenesis by the forced expression of the myogenic regulator MyoD within fibroblast, melanoma, neuroblastema, liver and adipocyte cell lines (Weintraub et al., 1989). When hepatic oval cells were cultured in a high-glucose medium their re-differentiation into hormone-producing pancreatic islets demonstrated the role of the microenvironment and the cell’s extrinsic factors in plasticity (Yang et al., 2002).

The definition of a stem cell may need to be amended; their plasticity as well as their ability to cross germ layers.

Within the limb bud it is proposed that one common mechanism for pattern formation (in a developing system) is based on positional information, whereby cells acquire a positional identity with respect to boundaries and then interpret this positional value by changing their state. This may involve differentiation, proliferation or a change in shape. Functional integrity of the musculoskeletal system relies on the co-ordinated development of each of its constituent members, muscle, tendon and cartilage.
Current understanding of how vertebrate limbs develop comes mainly from studies on chick embryos. Chick embryos are classical models for experimental manipulation as they are readily accessible. Pattern formation within the limb has been considered from the viewpoint of positional information by a two-step process. Cells are either informed of their position with respect to some reference point(s) or cells interpret this information in order to participate in forming the structure appropriate to that position and contributing to the correct arrangement of tissue and cells (Wolpert, 1969).

Within this study we wanted to assess the differentiation potential of the clonally derived progenitor population. We infected a lineage label into the cells and injected them into the proximal limb of stage 19-21 chick embryos and tracked them for 1 week in ovo. Within the complex tissue arrangement of the limb bud it is possible that cells could differentiate according to their position within the bud or cells could differentiate and then move to the appropriate position.
6.2. MATERIALS AND METHODS

6.2.1. Culturing the cell line

Culturing and harvesting of GP 293 cells were based on those described by Burns et al. (1993). Briefly, monolayers of 293 GP cells expressing the gag and pol proteins were grown to near confluence in DMEM+. Cells were then transfected with 1µg plasmid DNA encoding VSV-G and lac-Z (donated by Rosa Beddington, ICRF Oxford) using the Quiagen Effectene kit following the manufactures’ instructions. Transfected cells were cultured for 3 days in DMEM+ and viral supernatant harvested every 24 hours, clarified (by centrifugation; 10000rpm for 10 minutes) to remove any cellular debris and stored at -80°C for future use.

6.2.2. Clonal isolation and infection

Superficial zone chondrocytes were isolated as described in section 2.2.1. SZ cells were subjected to differential adhesion to FN (10µg/ml in PBS+) in 35mm Petri-dishes for 20 minutes. Non-adherent cells were aspirated and chondrocytes were cultured for up to 4 days. Colonies of 32+ cells were subsequently cloned and cultured as described in section 3.2.4 and 3.2.5. Middle/deep derived cells were isolated as described in section 2.2.1 and cultured in 60mm Petri-dishes at 1 x 10^5/ml. Clones and middle/deep cells were infected with pseudotyped retrovirus conditioned media (5.6 x 10^6 CFU, Gary Dowthwaite personal communication) containing 10µg ml-1 polybrene (hexadimethrine bromide; Sigma) for 24 hours prior to injection. Media was removed and cells washed in DMEM (containing no additives), trypsinised, centrifuged and resuspended at 1x10^5 cells/10µl.

6.2.3. In ovo injection

Fertilised Rhode Island Red chicken eggs (Winter egg farm, Herts) were incubated at 37°C in a humidified incubator for 2.5 days. The embryo was then exposed by windowing. Before windowing, 1ml of thin albumin was removed using an adapted 18 gauge needle and syringe from the blunt end of the egg (Figure 6.1A arrow i). The removal of albumin allowed the developing chorio-allantoic membrane to drop. At later stages of development, the membrane rises to be adjacent to the shell membranes, and thus, the blood vessels are at risk of being damaged during the
Figure 6.1. Micrographs showing preparation of the egg prior to injections (A,B) and typical hindlimb anatomy for embryos (C,D). A view of the egg at 2.5 day of incubation, an adapted 18 gauge needle syringe (arrow i) is used to remove 1ml of albumin from the blunt end of the egg (A). (B) shows the creation of the second window approximately 1cm diameter using sterile forceps. (C) shows a typical example of the appearance of a developing hindlimb bud (arrow ii) of a stage 21 embryo fixed in Axsoro's fixative (adapted from Oldfield, 2003). (D) shows the appearance of the limb (dotted outline) and an example of the injection of labelled cells; the contents of the micropipette (arrow iii) is injected into the centre of the hindlimb.
windowing procedure. Adhesive tape was used to seal the hole created by the needle. A second window of approximately 1cm diameter was carefully cut into the top of the egg using forceps (Figure 6.1B); this allowed the embryo to be viewed. The window was again sealed with adhesive tape and the egg returned to the incubator until the following day. Aseptic technique was used during this process to prevent infections using 70% industrial methylated spirits (IMS) (Fisher Scientific).

Prior to injection of cells, the window was opened under a dissecting microscope and the embryo viewed and staged according to the criteria of Hamburger and Hamilton (1951). Embryos in ovo typically are situated so that their right side is facing upwards, the right leg is, thus, more accessible and usually the limb to inject (Figure 6.1D). Using a surgically sharp, flamed tungsten needle, a small incision was made in the vitelline membrane overlying the proposed injection site. Previously pulled injection-quality glass micropipettes (Clark Instruments) were backfilled with transfected cells and connected to a PV820 pneumatic picopump (World Precision Instruments) set at approximately 10 psi. Injections were made into the proximal limb bud of stages 19-21 (3.5 days, Hamburger and Hamilton) (Figure 6.1D arrow iii). Detailed records were made of each injection, before sealing each egg with adhesive tape and returning to the incubator. For each set of injections, several embryos received sham injections, which contained only DMEM.

6.2.4. Histochemical detection of β-galactosidase

All injected embryos were incubated until stages 36-37 i.e. halfway through gestation, according to Home Office Regulations. In order to detect β-gal activity, each embryo was quickly killed by cervical dislocation (in accordance with schedule 1 of Animal Act 1986) and staged. Embryos were subsequently fixed in 2% paraformaldehyde (BDH) for 60 min at room temperature on a rolling platform. Following an extensive period of washing in PBS (0.01 M phosphate-buffered saline, pH 7.2), embryos were transferred to a minimal volume of x-gal staining solution containing 20mM K3Fe(CN)6, 20mM K4Fe(CN)6.3H2O, 2mM MgCl2, and 1mg/ml x-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Sigma) within PBS. All embryos were incubated in the dark overnight at 37°C to allow development of the blue reaction product. Embryos were refixed in 2% paraformaldehyde and, after washing in PBS, were transferred to 70% ethanol.
Embryos were first analysed as whole mounts for the obvious appearance of the blue precipitate before careful dissection to reveal any deeper labelling. All markings were fully recorded and photographs were taken on an Olympus SZX12 dissecting microscope using an Olympus camedia digital camera (C-4040ZCOM) and formatted with Adobe Photoshop (version 5.5).

In cases where identification of a labelled tissue or structure were difficult, the tissue of interest was processed for routine histology. Briefly, dissected regions containing the labelled structures were dehydrated through a graded of ethanol and xylene and embedded in paraffin wax. Sections were cut at 10μm and mounted on APES coated slides. Sections were lightly counterstained with eosin before coverslipping and viewed with a Leica (Leitz DMRB) light microscope and Images taken using Image grabber software and formatted using Adobe photoshop (version 5.5).
6.3. RESULTS

Seventy two hours following transfection with the plasmid DNA encoding VSVG and lacZ, the 293GP packaging cells were stained for the β-galactosidase (β-gal) enzyme. The majority of cells turned blue (fig 6.2A).

Following their infection with the pseudotyped retrovirus conditioned media, both early (fig 6.2B) and late passaged chondroprogenitors (fig 6.2C) and middle/deep derived chondrocyte (fig 6.2D) cultures were stained with β-gal enzyme. These cells were surplus following injection of cell suspension into the hindlimb of the chick embryo. This demonstrated that the cells were still viable and that the cells continued to express the enzyme. Not all cells stained blue, indicating that not all cells have undergone division and incorporated the gene. Very few stained cells were found within late passaged chondrocytes (fig 6.2C).

Within this study we wanted to ascertain whether the chondroprogenitor cells are able to reincorporate into cartilage and whether they are irreversibly committed to the chondrogenic lineage.

Superficial ventral proximo-medial domains of the limb bud were injected with clonally expanded progenitor cells or middle/deep derived chondrocytes previously transfected with the lacZ gene. In some cases, progenitor cells were cultured for between 35 and 49 population doublings (PD) before transfected with the lacZ gene. This result would allow us to ascertain if long term culture affected not only the phenotype of the cells but their ability to re-incorporate into the developing limb.

In total, 72 embryos were injected; 29 embryos were injected with early passaged clonally derived progenitors, 17 with late passaged clonally derived progenitors, 17 with middle/deep derived chondrocytes and 6 were injected with media only (sham/control embryos). A total of 13.88% of all embryos died following the injection procedure.

When studying wholemounts, it was possible to visualise endogenous background labelling (fig 6.3). Typical endogenous labelling following staining for the β-gal
enzyme was seen within the midline seam of the body cavity (fig 6.3A), isolated label within epidermis overlying calvaria (fig 6.3B), small intestine (fig 6.3C) and distal regions of the digits (fig 6.3D). Histological examination of the digits demonstrated endogenous punctate labelling (fig 6.3E & F).

Twenty-four percent of the total early passaged clonally derived progenitor cases were identified to be positive for β-gal cell labelling within the hindlimb of stage 36-37 embryos following their injection at stages 19-21. Labelled cells were visualised, using a dissecting microscope in wholemount. Where areas of labelled cells were visualised the embryos were processed for routine histology.

A higher proportion of late passaged clonally derived progenitor cells were visualised to have incorporated into the hindlimb of stage 36-37 embryos, 35% of the total number of injected embryos. Only 11.67% of the total middle/deep derived chondrocytes injected into the limb bud were visualised in wholemount.

At no stage were β-gal positive cells present within processed hindlimbs that had received sham injections.

Results showed that both early and late passaged progenitor cells as well as middle/deep chondrocytes maintained in culture were able to engraft into various tissue structures within the limb.

Labelled progenitor cells were identified within distal end of shank (fig 6.4A & B), within the thigh (fig 6.5A & B) and dorsal regions of the shank (fig 6.6) within wholemount samples following a short period of culture. Following longer culture periods, labelled cells were identified within ventral foot region associated with tendons\or connective tissue (fig 6.8 A&B), the shank (fig 6.9 A, D, F&G) and tendons on the shank (fig 6.10A) within wholemount samples. Middle/deep labelled cells were identified within the dorsal aspect of the distal shank associated with tendon/ligamentous structures (fig 6.11A&B) and within the ventral aspect of the foot region (fig 6.12 A&B).
On histological analysis, incorporation of labelled early passaged progenitor cells and their progeny were found within muscle (fig 6.4 C&D, Fig 6.7 A), within connective tissue (fig 6.4 E-I, fig 6.5 E-I), epimysium (fig 6.4 G), dermal connective tissue (fig 6.5 G), tendon and tendon connective tissue (fig 6.7 B&C).

Late passaged chondroprogenitors were found within loose connective tissue (fig 6.8D), tendon sheath (fig 6.8E), blood vessel (fig 6.8 F&G), muscle connective tissue (fig 6.8H, fig 6.9 B&C), loose connective tissue (fig 6.9E, fig 6.10D&E), and the perichondrium (fig 6.10C).

Labelled middle/deep derived chondrocytes were found within muscle (fig 6.11C), perichondrium (fig 6.11D), muscle associated connective tissue (fig 6.11 E-G), connective tissue (fig 6.12C&D) and within the tendon sheath (fig 6.12E).

Cells were either isolated and widely distributed throughout the tissue or found in small discrete clusters.
Figure 6.2. Micrograph of GP293 cells (A) 72 hours following transfection with the lac z gene and stained with x-gal. The majority of cells turned blue.

Micrographs of early (B) and late (C) passaged chondroprogenitors and early middle/deep derived chondrocytes cultures (D) (transfected with the pseudovirus) stained for the β-gal enzyme. These cells were surplus following injection of cell suspension into the hindlimb of the chick embryo. They represent the viability and the ability of these cells to continue expressing the enzyme. Not all cells have stained blue indicating that not all cells have undergone division and incorporated the gene. Very few stained cells were found within late passaged chondrocytes (C).
Figure 6.3. Typical endogenous labelling following x-gal staining for
the β-gal enzyme. Within midline seam of body cavity (A), isolated
label within epidermis overlying calvaria (B), small intestine (C),
distal regions of the digits (D) within wholemount embryos.
Histological sections of endogenous punctate labelling within the
distal regions of the digits (E,F). Inset is a high magnification image
(E). Arrows indicate endogenous labelling. Scale bar =100μm.
Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21. Wholemount embryo at low magnification showing the region of diffuse label at the distal end of the shank region overlying perichondrial regions, S – shank, F – foot (A&B). At a higher magnification, labelling was identified within muscle (C&D), and within the connective tissue (E-I). The labelling is near the muscle, and therefore it could be epimysium (G). Inset are low magnification images. Arrows indicate areas of labelling. Scale bar = 100 μm.
FIGURE 6.5. Injection reference: JCB 22/07/03 # 6.
Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21. Wholemount embryo at low magnification showing the region of diffuse label within the thigh (A&B). At a higher magnification, labelling was identified within the connective tissue (D-G). Superficial position indicating possible dermal connective tissue (C). Inset are low magnification images. Arrows indicate areas of labelling. Scale bar = 100 μm.
FIGURE 6.6. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21.

Wholemount embryo at low magnification showing the region of diffuse label on the dorsal margin of the shank, injection reference: JCB 25/07/03 # 2 (A&B).

Wholemount embryo at low magnification showing the region of diffuse label within dorsal regions of shank and foot possibly related to tendon matrix, injection reference: JCB 04/07/03 # 8 (C&D).

Wholemount embryo at low magnification showing the region of diffuse label within dorsal regions of shank and foot possibly related to tendon matrix, injection reference: JCB 01/08/03 # 4 (E&F).

Wholemount embryo at low magnification showing the region of diffuse label within dorsal regions of shank and foot possibly related to tendon matrix, injection reference: JCB 01/08/03 # 13 (E&F).

S – shank, F – foot and T-thigh
FIGURE 6.7. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21.

Injection reference: JCB 04/07/03 # 7 (D&E) At high magnification, labelling was identified within muscle (A), tendon (B) or tendon connective tissue (C). Inset are low magnification images. Arrows indicate areas of labelling. Scale bar = 100 μm.
Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a long period of time approximately 35 population doublings, were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21. Wholemount embryo at low magnification showing the region of diffuse label within ventral foot region, associated with ventral tendons or connective tissue (A&B). Low power image of the cross-sectional area analysed. At a higher magnification, labelling was identified within the loose connective tissue (D), tendon sheath (E), blood vessel (F&G) and muscle connective tissue (H). Inset are low magnification images. Arrows indicate areas of labelling. Scale bar = 100 μm.

F-foot
FIGURE 6.9. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a long period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21. Inset are low magnification images. Arrows indicate areas of labelling. Scale bar = 100 μm.

Injection reference: JCB 25/07/03 # 2 (A-C) Wholemount embryo at low magnification showing the region of diffuse label on the shank (A) following 37 population doublings. At a higher magnification, labelling was identified within the muscle connective tissue (B&C).

Injection reference: JCB 20/06/03 # 9 (D&E) Wholemount embryo at low magnification showing the region of diffuse label within dorsal aspect of distal shank (D) following 35 population doublings. At a higher magnification, labelling was identified within connective tissue (E).

Injection reference: JCB 04/07/03 # 12 (F-G) Wholemount embryo at low magnification showing the region of diffuse label on the shank (F&G) following 34 population doublings.

S-shank, F-foot
FIGURE 6.10. Clonally derived chondroprogenitors, transfected with the lacZ gene and cultured for a long period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21. Inset are low magnification images. Arrows indicate areas of labelling. Scale bar = 100 μm.

Injection reference: JCB 04/07/03 # 18 (A-C) Wholemount embryo at low magnification showing the region of diffuse label within tendon on the shank (A) following 49 population doublings. At a higher magnification, labelling was identified within the perichondrium (C) and connective tissue (D&E).

Injection reference: JCB 20/06/03 # 12 (A-C) Wholemount embryo at low magnification showing the region of diffuse label within the dorsal aspect of the shank and foot (D&E) following 45 population doublings.

S-shank, F-foot
FIGURE 6.11. Middle/deep derived chondrocytes, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21. Inset are low magnification images. Arrows indicate areas of labelling. Scale bar = 100 μm.

Injection reference: JCB 25/07/03 # 13 Wholemount embryo at low magnification showing the region of diffuse label within the dorsal aspect of the distal shank associated with tendon/ligament (A&B). At a higher magnification, labelling was identified within muscle (C) perichondrium (D) and muscle connective tissue (E-G).

S-shank, F-foot
FIGURE 6.12. Middle/deep derived chondrocytes, transfected with the lacZ gene and cultured for a short period were incorporated into the tissues of a developing chick hindlimb following their injection into the proximal limb bud of stages 19-21. Inset are low magnification images. Arrows indicate areas of labelling. Scale bar = 100 μm.

Injection reference: JCB 25/07/03 # 8. Wholemount embryo at low magnification showing the region of diffuse label within the ventral aspect of the foot region (A&B). At a higher magnification, labelling was identified within connective tissue (C&D) and within the tendon sheath (E). S-shank, F-foot
6.4. DISCUSSIONS

Within this study, we assessed the differentiation potential of the clonally derived progenitor population compared to chondrocytes derived from middle/deep zones of articular cartilage. We infected a lineage label into the cells and injected them into the proximal limb of stage 19-21 chick embryos and tracked them after 1 week in ovo. Within the complex tissue arrangement of the limb bud, it is possible that cells could differentiate according to their position within the bud.

Overall these results suggest that neither the progenitor nor the middle/deep zone derived chondrocytes contributed toward the formation of cartilaginous tissue however, a proportion of cells from each population were able to re-incorporate into various tissue structures within the limb. Previous studies within our laboratory using chondroprogenitor enriched population of cells revealed β-galactosidase (β-gal) positive cells in numerous tissue types including cartilage, bone, tendon and muscle connective tissues (Dowthwaite et al., 2004).

We utilised the lacZ reporter gene encoding for β-gal to transfect chondroprogenitor cells in vitro before injecting these cells into the developing hindlimb bud. The injections were made into the proximal region of the limb bud. Comparisons were made between early passaged chondroprogenitors and late passaged chondroprogenitors and middle/deep derived chondrocytes. Embryos were then re-incubated following injection and processed histochemically for the β-gal enzyme at stages 36-37. The identification of labelled cells was carried out using a dissecting microscope in wholemount. Where areas of labelled cells were visualised the embryos were processed for routine histology.

Transfection of 293GP packaging cells with the plasmid DNA encoding VSVG and lacZ were still active within the virions produced as the majority of cells turned blue in appearance following staining for the β-gal enzyme.

Following the infection with the pseudotyped retrovirus conditioned media, chondroprogenitor cells were stained for β-gal enzyme. By reference of the intensity of blue of the cells it was clear that some cells were more prominently labelled than
others and some not at all, indicating that cells may not have divided and incorporated the gene into its genome. Following the injection procedure any remaining cells were cultured to test for both vitality and for the presence of the lacZ enzyme. When cultured over a short period of time only a small number of cell stained for the β-gal enzyme. Therefore, it is possible that not all chondroprogenitors that have been injected into the limb have divided (although the cell may have incorporated the lacZ gene) and would not stain for the β-gal enzyme. The evidence of staining does demonstrate that chondroprogenitors are suitable for transfection using the pseudovirus.

When looking at whole-mount, it was very important to distinguish between positive cellular labelling of infected incorporated cells and endogenous background labelling. Endogenous labelling is characteristically found in the calvaria, within the sternum, intestine, within tendons of the limbs and in digits. Endogenous labelling is normally paler, diffuse and is non-cellular and has been hypothesised to be due to ECM labelling by the x-gal substrate (personal communication with Dr Darryl Evans).

In total, 72 embryos were injected; 29 embryos were injected with early passaged clonally derived progenitors, 17 with late passaged clonally derived progenitors, 17 with middle/deep derived chondrocytes and 6 were injected with media only (sham/control embryos). Twenty four percent of the total early passaged clonally derived progenitor injected cases were identified to be positive for β-gal cell labelling within the hindlimb. A higher proportion of late passaged clonally derived progenitor were visualised to have incorporated into the hindlimb of stage 36-37 embryos, 35% of the total number of injected embryos. Only 11.67% of the total middle/deep derived chondrocytes injected into the limb bud were positive for labelling.

The low number of positive cases could be due to a variety of reasons. Embryos were only processed histologically if labelled cells were found in wholemount, it is possible, therefore, that had all embryos injected been sectioned that positive labelling may have increased.

It has been demonstrated that chondrocytes are able to proliferate in culture, however, following their transfer into a 3D pellet, their proliferation slows, which may be
indicative of the low number of positive cells visualised. Future studies would involve increasing the number of cases, including analysing the incorporation of late passaged middle/deep chondrocytes.

A number of cells may be unable to incorporate into the limb mesenchyme and would subsequently apoptose. Tickle and co-workers (1978) grafted tissue fragment or pellets of reaggregated cells from embryonic quail liver, neural retina and mesonephros into developing chick wing. Embryonic liver and neural tube are tissues which sort externally to limb bud mesenchyme in mixed aggregates – it would be expected from a differential adhesiveness mechanisms that heterotypic adhesions along the borders of graft and host would be favoured over cell-cell adhesion in the graft. The grafted cells, however, maximised like-like contacts did not invade into the wing, cells within tissues, therefore, are not actively moving around. Later studies demonstrated that unlike normal rat brain tissue, cells from a glioma and carcinogen treated brain survived and invaded the limb (Tickle et al., 1978, 1979) as might be expected from tumour derived tissue.

The chick wing bud has been used to gain an insight into cell movement and the mechanism of invasiveness of normal and malignant cells implanted (into the developing chick wing bud). In high density culture, cells from distal tips of developing limb buds differentiate into a continuous cartilage sheet, rich in type II collagen. When grafted back into limb buds, cells cultured for a short time differentiate into cartilage and a wide range of other connective tissues, whereas cells taken from older cultures give rise only to cartilage and perichondrium. Grafts placed distally give rise to more cell types than grafts placed proximally. The results strongly suggest the chondrogenesis in culture is the result of removing the signals that pattern differentiation within the limb bud (Cottrill et al., 1990).

The cells in the present study were resuspended at a concentration of $1 \times 10^5$ cells/10μl; therefore, in the case of chondroprogenitors this may have been insufficient for incorporation. Oldfield and Evans (2003) hypothesised that their low numbers of incorporation could be due to insufficient numbers of fibroblasts being injected. Within our study we did not vary the length of time cells were injected and, therefore, it was difficult to determine the actual amount of cells injected. The above study,
however, did not rule out any correlation between the amount of cellular labelling absent and the duration of the injection.

At the end of each injection session there did appear to be an aggregation of chondroprogenitor and middle/deep cells within the micropipette which is characteristic of chondrocytes when placed at high density in suspension. Again, the number of cells being injected may not have been equal per embryo. Future experiments could involve regular changes of cell suspension and regular agitation.

Labelled progenitor cells were identified within distal end of shank, within the thigh and dorsal regions of the shank within wholemount samples following a short period of culture. Following longer culture periods, labelled cells were identified within ventral foot region associated with tendons or connective tissue, within the shank (within tendon on the shank within wholemount samples. Middle/deep labelled cells were identified within the dorsal aspect of the distal shank associated with tendon/ligament and within the ventral aspect of the foot region. A high proportion of labelling was identified within similar areas, therefore the controls were studied to ensure that we were not mistaking background labelling for positive labelling. However, at no stage were β-gal positive cells present within processed hindlimbs that had received sham injections and all injected embryos appeared anatomically normal.

On histological analysis, incorporation of labelled early passaged progenitor cells and their progeny were found within muscle, connective tissue, epimysium, dermal connective tissue, tendon and tendon connective tissue. Late passaged chondroprogenitors were found within loose connective tissue, tendon sheath, blood vessel and muscle connective tissue, connective tissue, and the perichondrium. Labelled middle/deep derived chondrocytes were found within muscle, perichondrium, muscle associated connective tissue, connective tissue and within the tendon sheath. Interestingly, each of these different lineages listed are derived from progenitor cells within the limb mesenchyme, which originate from the somatic mesoderm of the lateral plate complimenting studies by Oldfield and Evans (2003) using tendon fibroblasts. We did not visualise β-gal activity within cartilaginous structures. The reason for this is unknown as previous studies in our lab injecting
chondroprogenitor enriched cells into the limb bud revealed positive cells within cartilage at the same time point (Dowthwaite et al., 2004).

Cells were either isolated and widely distributed throughout the tissue or found in small discrete clusters. Clusters of cells may indicate that the cells present are the progeny of the originally infected cell due to proliferation or that migration had taken place. In some cases it was difficult to distinguish which tissue lineage the cells had incorporated into, this may have been due to extensive processing.

There did not appear to be an observed pattern with regards to each tissue type with cellular labelling. However, in some cases the cells had adapted their phenotype to the surrounding tissue. Studies by Dowthwaite et al., (2004) demonstrated that if chondroprogenitor enriched cells were injected into the central proximal region of the limb bud, cells engrafted into the humerus. More lateral injections engrafted into tendons and perimysim. In order to test for functional engraftment, the group used an antibody specific for bovine type I collagen. The group revealed within tendons, parallel arrays of fibrillar collagen running along the tendon length. Bovine cells and collagen type I were also co-localised in articular fibrocartilage, perimysium and bone. Future studies would analyse the functionality of the engrafted early and late passaged chondroprogenitor cells as well as middle/deep derived chondrocytes within the hindlimb.

The study by Oldfield and Evans (2003) demonstrated the presence of β-gal positive fibroblasts were located in a variety of tissue lineages and in several tissue types within an individual specimen, connective tissue layers, perichondrium, chondrocytes, feather bud dermis, dermis and muscle.

We did not harvest any embryos earlier than stages 36-37, this would have allowed us to demonstrate β-gal positive cells within the site of injection and followed how they dispersed/migrated or integrated into the limb bud with time.

Although the injections within this study were angled proximally it is possible that due to movements of the embryo that the limb buds may have been injected more distally and, thus, may affect reproducibility. Ongoing studies include increasing the
CHAPTER 7

*In vitro* transplantation of articular cartilage progenitor cells
7.1. INTRODUCTION

The limited healing response of cartilage has been reported for more than three centuries (Hunter, 1743). A partial thickness defect, that is a defect which is restricted to the articular cartilage does not heal spontaneously. The major biological problems with the repair of articular cartilage are; the low cellularity of the tissue, low mitotic activity of the chondrocytes present, lack of blood supply, the stimulation of degrading enzymes, and the absence of a source of cells outside of the tissue that are capable of repair (Giurea et al., 2002).

A repair response is elicited when a full thickness defect is created with penetration of marrow spaces of subchondral bone. The defect becomes populated with undifferentiated, marrow-derived mesenchymal cells that differentiate into new subchondral bone and overlying cartilage. The fibrocartilage that results does not resemble the pre-existing cartilage biochemically or biomechanically and, ultimately, deteriorates leading to osteoarthritis.

The restoration of joint function and prevention of further deterioration of joint requires repair tissue to duplicate articular cartilage in structure, composition and mechanical properties.

The challenge for surgeons is the treatment of symptomatic full thickness articular cartilage injuries. Popular treatments promote bleeding from the subchondral bone inducing clot formation and subsequent healing by the autologous tissue or cultured cells to facilitate cartilage regeneration. These include abrasion arthroblasty first introduced by Johnson (1986), subchondral drilling and microfracturing (see chapter 1).

Nehrer et al., (1999) evaluated the composition of reparative tissue retrieved during revision surgery from full thickness chondral defects and concluded that only long-term clinical studies would prove the efficacy of the above procedures. The group highlighted the importance of evaluating and interpreting repair tissue biopsies in the development of new repair strategies, and benefits of a grading system that would include histomorphometric evaluation of biopsy samples from repair tissue with
consistent parameters of measurements for the comparison of results within and between studies (Nehrer et al., 1999). As the field of cartilage tissue engineering increases in publications, many tissue engineering techniques and various analysis methods are developed making comparisons difficult due to the lack of uniformity. Chondral and osteochondral lesions within some studies are classified in accordance with the system recommended by the international cartilage repair society (ICRS).

Current treatment options depend on a variety of factors including how the defect is graded using outerbridge classifications (Outerbridge, 1961), the size of the defect, the defect location on load bearing surfaces, the patient’s activity level, age, structural biomechanical problems and therefore its natural history must be understood (Onstott et al., 2000).

Chondrocytes in young animals possess the capability for articular cartilage regeneration. Two millimetre defects were drilled in the femoral condyles of 5 week old rabbits and the healing response compared to 4 month old rabbits (Huibregtsen et al., 2000). Following 12 weeks, new subchondral bone and cartilaginous tissue, and integration was visualised compared within the 5-week-old animals’ lesions but the 4-month-old rabbits did not demonstrate a regenerative capacity. The likely reason for this discrepancy is that the 5-week-old animals are still actively growing.

The ability of native chondrocytes to proliferate and elaborate substantial new matrix in vivo is limited. When cartilage is injured, chondrocytes will proliferate and increase production of matrix molecules locally but will fail to bridge a defect (Stockwell, 1979).

It may, therefore, be necessary to provide a new source of chondrocytes to the areas of cartilage injury, in conjunction with chondrocyte manipulations that function to increase matrix synthesis. Such a technique involves the implantation of a suspension of cultured autologous chondrocytes under a periosteal graft that is then sutured to the defect (Brittberg et al., 1994). Similar studies have been attempted in both animals and humans and have included the transplantation of mesenchymal stem cells (MSCs) and perichondrium implanted alone or within natural or synthetic biodegradable scaffolds (Grande et al., 1989; Hendrickson et al., 1994; Itay et al., 1987; Shortkroff et
al., 1996; Wakitani et al., 1994). Baragi et al., (1997) demonstrated that transduced allogeneic chondrocytes survived for at least 10 days when inserted into focal cartilaginous defects in rabbits. A rapid loss of transduced cells from the cartilage defect by 24 hours was indicative of the anti-adhesive properties of the wound margin (Baragi et al., 1997). No procedure has led to the full regeneration of structure and restoration of function of articular cartilage in animal experiments. The efficacies within human subject are difficult to assess as controversy exists about the long term clinical outcome. No procedure is a proven method for treating defects in the joint surface.

Recent efforts have focused on tissue engineering of articular cartilage in attempts to create cartilage in vitro suitable for tissue repair. Techniques have isolated chondrocytes and subsequently seeded them within synthetic or biological scaffolds or placed on the surface of substrate materials and grown in culture. A problem with this technique includes obtaining the cartilage biopsy, which requires additional damage to the joint surface. This problem could be potentially minimised by optimising cell expansion as discussed in previous chapters.

Studies within our laboratory have investigated the early reaction near an experimental lesion and changes in extracellular matrix. We have previously used an in vitro bovine cartilage explant culture system to examine the cellular changes at the wound edge caused by a trephine. The chondrocytes reacted in a similar manner to that of chondrocytes located near comparable defects in vivo (Stockwell, 1979). Following experimentally created defects or any surgical intervention a zone of cell death characteristically extends approximately 150μm from the wound edge, previously termed the ‘zone of necrosis’. Work in our laboratory has demonstrated that the cell death is both necrotic and apoptotic (Redman et al., 2004; Tew et al., 2001). Behind this zone of cell death there is a zone of cellular proliferation and altered matrix metabolism (Mankin, 1962). It is this cell death that is potentially hindering successful integration during repair procedures. Attempts to improve integration have included the treatment of articular cartilage with highly purified collagenase, which resulted in a significant increase in chondrocyte density at the wound edge where cell death had occurred (Bos et al., 2002; Hunziker and Kapfinger, 1998). It was proposed that the observed increase in chondrocytes was due to
repopulation rather than a breakdown of vital tissue due to cell migration and/or cell proliferation.

D'Lima (2001) showed data that the apoptosis induced by loading human full-thickness cartilage explants in vitro could be inhibited using caspase inhibitors. The apoptotic pathway is mediated by a cascade of aspartate-specific cysteine proteinases or caspases (3,8,9), that act by degrading intercellular proteins, that are essential for cell survival. Thus, this novel approach inhibits events within this pathway; protecting the chondrocytes. But inhibition of caspases doesn't reduce cell death in lesions (Tew et al., 2000).

Recent studies by Buckley et al. (1999) provide an alternative molecular explanation for the potent properties of RGD other than blocking signal transduction. They studied the effects of RGD peptides on T cells and found that it directly activates the protease caspase in the cell cytoplasm. It is possible that when the ECM is disrupted that the RGD peptide present in the fragmented macromolecules are internalised by the cell thus amplifying and propagating apoptotic signals, executing the apoptotic programme.

Further studies demonstrated that wounds made with a sharp scalpel showed restricted cell death, with both chondrocyte proliferation and new matrix visualised at the wound margin (Redman et al., 2004). These results show clinical relevance to the use of sharp precise instruments during the surgical management of cartilage defects to minimise cell death, promoting matrix production and, thus, integration.

Within this study we wanted to assess the affects of introducing high density pellets to the wound site. We have shown in previous chapters that chondroprogenitors and chondrocytes isolated from various zones were capable of producing cartilage-like matrix when cultured at high density. Defects of a defined dimension were created in the condyle of 7-day-old bovine metacarpal-phalangeal joint using a partial thickness defect tool (Hunziker and Rosenberg, 1996). We analysed the morphological effects of introducing pellets into the wound, based on the amount of glycosaminoglycan (GAG) staining and quantified cell death at the wound margin.
7.2. MATERIALS AND METHODS

7.2.1. Chondrocyte Isolation

Surface (SZ), middle (MZ), deep (DZ) zone and full-depth (FD) explants were isolated from 7-day bovine metacarpal-phalangeal (MCP) joints by fine dissection as described in section 2.2.1. MZ, DZ and FD chondrocytes were plated onto 60mm dishes at $5 \times 10^5$ cells/ml and cultured for 4 days in DMEM+.

7.2.2. Differential Adhesion

SZ chondrocytes were seeded onto fibronectin coated 60mm dishes (coated with 10µg/ml bovine plasma fibronectin overnight) at an initial concentration of $1 \times 10^6$ cells/ml and incubated for 20 minutes in 5% CO$_2$/37°C in a humidified incubator. After 20 minutes media (and non adherent cells) were removed and cells maintained in monolayer in DMEM+ for 4 days. The cell populations were now known as chondroprogenitor enriched (CE) (see section 3.2.6).

7.2.3. Cultivation of SZ, MZ and DZ chondrocytes in pellets

After 4 days, chondrocytes were treated with 2ml of 2.5% trypsin-EDTA (GIBCO) for 5 minutes in 5% CO$_2$/37°C. Following trypsinisation 3ml of DMEM was added to each dish, and the CE, MZ, DZ and FD suspensions were pooled into separate subpopulation stocks. The pooled solutions were centrifuged at 1500rpm for 5 minutes, and the cells resuspended in 10ml DMEM+. Following counting, SZ, MZ and DZ cells were resuspended in DMEM+ containing 1% HEPES buffer at 2x106 cells ml$^{-1}$ and 500µl aliquots placed in 1.5ml polypropylene conical tubes. Tubes were centrifuged at 2000 x g for 5 minutes to pellet the cells and the pellets maintained in DMEM+ for up to 3 days.

7.2.4. Wounding and Pellet transplantation

Full depth articular cartilage was excised from the MCP joint of 7-day-old calves using a #23 scalpel blade and explants wounded using an autoclaved 1.5mm drill bit (see fig 7.1A). Defects of a defined dimension were created in the condyle of MCP using a partial thickness defect tool (Hunziker and Rosenberg, 1996) (see figure 7.1C). The instruments used to create the superficial defects were made to our
Fig 7.1.
specifications (Rolf Hänggi Engineering Grenchen, Switzerland). Each defect was 0.8 mm wide with a depth of 0.2 mm, the length of each defect was controlled by the operator to be between 3 to 5 mm. The explant was then excised. Immediately following excision, 3-day cultured pellets derived from CE, MZ, DZ and FD were then carefully placed in the cartilage lesion and the explants (+ transplanted pellets) maintained in DMEM+ for 7 days in 5% CO₂/37°C. Controls consisted of wounded explants containing no pellet. After 7 days, explants were either analysed for cell death or fixed in 10% NBFS, wax embedded and processed for routine histology.

7.2.5. Cell death characterisation

Following 7 days of culture the wounded controls and CE, MZ, DZ and FD wound-pellet systems were treated with ethidium homodimer (EtHD) (2 mM) for 90 minutes in DMEM+ at 5% CO₂/37°C followed by washing in DMEM for 90 minutes. Subsequently, media was removed and explants were fixed in 10% NBFS overnight, wax embedded, sectioned at 10 μm and examined using a fluorescent microscope. Serial sections through the wound were cut, and labelled cells were counted on sections cut midway through the defect; 250 μm. Two regions were analysed, superficial to and deep to the surface.

7.2.6. Histological procedures

Following overnight fixation in 10% NBFS, pellets or wound-pellet systems were processed for routine histology as described in section 2.2.4. Sections were then dewaxed and stained with Safranin O and counterstained with Mayer's haematoxylin as described in 2.2.4. Samples incubated with EtHD for cell death analysis were dewaxed washed in PBS and coverslips were mounted with Vectashield (Vector, Peterborough) for observation.

7.2.7. Microscopic characterisation

Fluorescence (EtHD) was examined using a Leitz Laborlux 12 fluorescent microscope. Images were captured using a RS Photometrics Coolsnap camera and Coolsnap image software and formatted with Adobe Photoshop (version 5.5). Histochemical staining was viewed with a Leica (Leitz DMRB) light microscope and
images taken using Image grabber software and formatted using Adobe photoshop (version 5.5).

7.2.8. Statistical Analysis

For the quantitative analysis of cell death, the mean percentage of dead cells was calculated for each sample, $n = 18$ (3 experiments, 6 controls or 6 pellets per experiment) was then calculated.

For propagation of error, we calculated the standard deviation (SD) of the quotient: $CV = CV_1^2 + CV_2^2$ where $CV = \text{SD/mean value}$. Data are expressed as mean +/- standard error of the mean and the effects of the addition of pellets on cell death were assessed using one-way ANOVA (Analyse-it for Microsoft Excel 2003 software supplier). All pairwise comparisons between groups were conducted using the Fischer’s LSD post-hoc test. Significance was assigned at p-values less then 0.05.
7.3. RESULTS

Wounds created within full depth explants using a drill bit (fig 7.1 A) were not reproducible (fig 7.2). The wounds were of different depths (fig 7.2A & B) and in some cases shearing occurred (fig 7.2B). Cell death analysis of the wounds created showed extensive cell death, more so than when a trephine was used (data not shown).

Following wounding using the partial thickness defect tool, extensive cell death was observed around the wound margin and along the articular surface of the cartilage indicated by apoptotic bodies (fig 7.3 arrows) and ethidium homodimer (EtHD) labelling (fig 7.4). The addition of a chondrocyte pellet into the wound site decreased matrix loss.

Matrix loss as shown by lack of Safranin O staining was also detected around the wound margin of the control explants (fig 7.3) following 7 days in culture post wounding with the partial defect tool. Weak staining was present at the articular surface (fig 7.3B) and wound margin lip (fig 7.3C). Apoptotic bodies were present around the wound margin and deep into the wound (fig 7.3 C-F). Matrix loss was also demonstrated at the wound lip (fig 7.3F). Wounded control samples exhibited extensive labelling with EtHD around the wound margin to a depth of around 8-10 cell diameters in areas B & C (fig 7.4). Deeper into the explant the EtHD labelling was 3-4 cell diameters. At the articular surface of the wound labelling was seen to decrease distally from the wound and at the articular surface - (data not shown).

The addition of a chondroprogenitor enriched (CE) pellet appeared to decrease the loss of Safranin O staining deep to the wound (fig 7.5 C & D). However, at the wound lip there was a lack of stain, and this may be pellet matrix and not host matrix where the pellet was associated. Staining appeared to be immediately adjacent to the lesion edge (fig 7.5 C-E) compared to control (fig 7.3 D-F). In fig 7.5 C-E there appeared to be a cluster of CE chondrocytes adhered to the host matrix. This observation suggests that before processing, the whole pellet was associated with the host. The CE pellet itself contained small rounded cells and was only weakly stained with Safranin O (fig 7.5E).
A decrease in EtHD labelling was prominent in samples containing a CE derived pellet which contained little if any labelling with EtHD around the wound margin (fig 7.6) EtHD labelling correlated with the apoptotic bodies indicated by arrows in fig 7.5. Autofluorescence was apparent in the CE pellets (fig 7.6B).

The wounded explants transplanted with MZ pellets demonstrated weak staining at the articular surface (fig 7.7B) and loss of staining at the wound lip (fig 7.6C). GAG was present deep in the wound (fig 7.7D) and on lip (fig 7.7E). Note an attempt at integration between host and pellet (fig 7.7F). The adherence was stronger here than elsewhere relative to the forces applied when sectioning. Cell death analysis revealed labelling with EtHD was noted at area A to a depth of 4-5 cell diameter (fig 7.8A) and around 5-6 cell diameters at area B (fig 7.8B). EtHD labelling correlated with the apoptotic bodies indicated by arrows in fig 7.7. The MZ pellet itself contained both small rounded and larger vacuolated cells and was only weakly stained with Safranin O (fig 7.8E). EtHD labelling was present within the pellet (fig 7.8B), correlating with apoptotic bodies within fig 7.7F.

The wounded explants transplanted with DZ pellets demonstrated only weak staining at the surface of the explant near the wound (fig 7.9B). Staining appeared to be immediately adjacent to the lesion edge (fig 7.9C-E). Cell death analysis revealed EtHD labelling at area A (fig 7.10A) to a depth of around 9-10 cell diameters and around 11-12 cell diameters at area B (fig 7.10B). EtHD labelling correlated with the apoptotic bodies indicated by arrows in fig 7.9. The DZ pellet contained large vacuolated chondrocytes and stained strongly for GAG. Autofluorescence was apparent in the DZ pellets (fig 7.10B).

Following the transplantation of FD pellets into the defects of partially wounded full depth explants there was weak Safranin O staining at the articular surface (fig 7.11B) and within the wound (fig 7.11C). GAG was present at the lesion edge deep in the wound (fig 7.11C & D) and on the lip (fig 7.11E). Cell death analysis revealed extensive labelling with EtHD at area A (fig 7.12A) to a depth of 12-13 cell diameters and around 13-14 cell diameters at area B (fig 7.12B). EtHD labelling correlated with the apoptotic bodies indicated by arrows in fig 7.11. The FD pellet contained a
mixture of both small rounded and large vacuolated chondrocytes and stained strongly for GAG. EtHD labelling was present within the pellet (fig 7.12B).

To assess quantitatively the amount of cell death that occurred within control wounds and defects containing pellets we counted the number of cells labelling positive for EtHD in a given area as a proportion of the total amount of cells in the same area (using corresponding phase contrast images).

The percentage of dead cells was determined in regions A and B at 0-100μm and 100-200μm from the wound edge (fig 7.13 & 7.14). Within region A at both 0-100 and 100-200μm the amount of cell death caused by the wound was significantly (p<0.05) reduced after transplantation of a CE pellet (fig 7.13b & c) compared to controls. Within region B at 0-100μm cell death was significantly (p<0.05) reduced following the transplantation of a CE pellet (fig 3.14b). A significantly (p<0.05) lower amount of cell death was observed following transplantation of a CE pellet compared to FD pellets within region A 0-100μm (fig 7.13b) and region B at both 0-100μm and 100-200μm (fig 7.14b & c).
FIGURE 7.2. Full depth explants were wounded using a drill bit and stained with Safranin O (A & B). The wounds were not reproducible (A & B). Scale bars = 500μm.
FIGURE 7.3. Low power image of a representative section of a full depth wound created using a partial thickness defect tool stained with Safranin O (A). Weak stain was present at the articular surface (B) and wound margin lip (C). Apoptotic bodies and empty lacunae were present around the wound margin and deep in the wound (indicated by arrows) (C- F). A thin layer of matrix lacking stain was demonstrated at the lip of the wound (F). Scale bars A =500μm, B-F 100μm.
Figure 7.4. Top shows diagrammatic representation of a full depth partially wounded explant. Full depth 7-day bovine articular cartilage explants were wounded and cultured for 7 days and labelled with ethidium homodimer (EtHD) to assess cell death (A-E). Images are representative of typical morphologies but are not from the same field as Safranin O images. Typically, labelling with EtHD was noted at the articular surface to a depth of 4-5 cell diameters (A). Within areas B & C this increased to 8-10 cell diameters. Deeper into the explant (D) EtHD labelling was 3-4 cell diameter. At site E, there was a high proportion of labelling due to tearing. Corresponding phase image (F-J). Scale bar= 100μM.
FIGURE 7.5. Wounded representative full depth explant transplanted with chondroprogenitor enriched (CE) derived pellet and stained with Safranin O (A). There was intense Safranin O staining throughout the matrix, which extended deep into the wound (E), and along the articular surface distal to the wound (B). Loss of matrix was noted around the wound lip (C). Apoptotic bodies were present around the wound margin and deep into the wound (indicated by arrows) (C, D, E). Note; clusters of CE chondrocytes adhered to host matrix (C,D,E). The CE pellet itself contained small rounded cells and was weakly stained (F). Scale bars A =500μm, B-F= 100μm.
Figure 7.6. Top shows diagrammatic representation of a partially wounded full depth explant. Full depth 7-day bovine wounded articular cartilage explant transplanted with chondroprogenitor enriched (CE) derived pellet and cultured for 7 days and labelled with ethidium homodimer (EtHD) to assess cell death. Images are representative of typical morphologies but are not from the same field as Safranin O images. Note only weak labelling with EtHD around wound margin. Autofluorescence was apparent in CE pellet. Corresponding phase image (C & D). Scale bar = 100μm.
FIGURE 7.7. Wounded representative full depth explant transplanted with middle zone (MZ) derived pellet and stained with Safranin O (A). There was weak staining at the articular surface (B) and loss of staining at the wound lip (C). GAG was present deep into the wound (D) and on the lip (E). Apoptotic bodies and empty lacunae were present around the wound margin and deep in the wound (indicated by arrows) (C, D, E). Note an attempt at integration between host and pellet indicated by an asterix* (F). The MZ pellet contained a mix of small rounded cells and large vacuolated cells and was weakly stained (D). Scale bars A = 500µm and B-F = 100µm.
Figure 7.8. Top shows a diagrammatic representation of a full depth partially wounded explant. Full depth 7-day bovine wounded articular cartilage explant transplanted with middle zone (MZ) derived pellet, cultured for 7 days and labelled with ethidium homodimer (EtHD) to assess cell death. Images are representative of typical morphologies but are not from the same field as Safranin O images. Labelling with EtHD was noted at area A to a depth of 4-5 cell diameter (A) and around 5-6 cell diameters at area B (B). EtHD labelling was also present within the pellet indicated by an asterix* (B). Corresponding phase image (C & D). Scale bar = 100μm.
FIGURE 7.9. Wounded representative full depth explant transplanted with deep zone (DZ) derived pellet and stained with Safranin O (A). There was weak staining at the articular surface (B). GAG was present at the wound lip and deep in the wound (C, D) and on lip (E). Apoptotic bodies were present around the wound margin and deep in the wound (indicated by arrows) (B, C, D, E). The DZ pellet contained large vacuolated cells and stained strongly for GAGs (D). Scale bars A = 500μm and B-F = 100μm.
Figure 7.10. Top shows a diagrammatic representation of a full depth partially wounded explant. Full depth 7-day bovine wounded articular cartilage explant transplanted with deep zone (DZ) derived pellet and cultured for 7 days and labelled with ethidium homodimer (EtHD) to assess cell death. Images are representative of typical morphologies but are not from the same field as Safranin O images. Labelling with EtHD was noted at area A to a depth of 9-10 cell diameters (A) and around 11-12 cell diameters at area B (B). Autofluorescence was apparent in the DZ pellet. Corresponding phase image (C & D). Scale bar = 100μm.
FIGURE 7.11. A wounded representative full depth explant transplanted with full depth (FD) derived pellet and stained with Safranin O (A). There was strong staining at the articular surface (B) and within the wound (C). GAG was present in the wound margin (C,D) and on lip (E). Apoptotic bodies were present around the wound margin and deep in the wound (indicated by arrows) (B, C, D, E). The FD pellet contained a mixture of both small rounded cells and large vacuolated cells and stained strongly for GAGs (D). Scale bars A = 500μm and B-B =100μm.
Figure 7.12. Top shows a diagrammatic representation of a full depth partially wounded explant. Full depth 7-day bovine wounded articular cartilage explant transplanted with full depth (FD) derived pellet and cultured for 7 days and labelled with ethidium homodimer (EtHD) to assess cell death. Images are representative of typical morphologies but are not from the same field as Safranin O images. Labelling with EtHD was noted at area A to a depth of 12-13 cell diameters (A) and around 13-14 cell diameters at area B (B). EtHD labelling was present within the FD pellet as indicated by an asterix* (B). Corresponding phase images (C & D). Scale bar = 100μm.
Figure 7.13a. Top shows diagrammatic representation of a full depth wounded explant.

Figure 7.13b. Graph representing the percentage of dead cells found within region A at a depth of 0–100 μm caused by wounding (control) and the amount of cell death following wounding and transplantation of chondroprogenitor enriched (CE), middle zone (MZ), deep zone (DZ) and full depth (FD) derived pellets. Statistically significant differences between cultures \( (p < 0.05) \) are marked in the table below.

<table>
<thead>
<tr>
<th>REGION A 100 μM</th>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL v CE</td>
<td>0.448</td>
<td>0.248 to 0.647</td>
</tr>
<tr>
<td></td>
<td>CONTROL v MIDDLE</td>
<td>0.237</td>
<td>0.038 to 0.437</td>
</tr>
<tr>
<td></td>
<td>CONTROL v DEEP</td>
<td>0.258</td>
<td>0.058 to 0.457</td>
</tr>
<tr>
<td></td>
<td>CONTROL v FULL DEPTH</td>
<td>0.149</td>
<td>-0.051 to 0.348</td>
</tr>
<tr>
<td></td>
<td>CE v MIDDLE</td>
<td>-0.210</td>
<td>-0.410 to -0.011</td>
</tr>
<tr>
<td></td>
<td>CE v DEEP</td>
<td>-0.190</td>
<td>-0.389 to 0.010</td>
</tr>
<tr>
<td></td>
<td>CE v FULL DEPTH</td>
<td>-0.299</td>
<td>-0.499 to -0.099</td>
</tr>
<tr>
<td></td>
<td>MIDDLE v DEEP</td>
<td>0.020</td>
<td>-0.179 to 0.220</td>
</tr>
<tr>
<td></td>
<td>MIDDLE v FULL DEPTH</td>
<td>-0.089</td>
<td>-0.288 to 0.111</td>
</tr>
<tr>
<td></td>
<td>DEEP v FULL DEPTH</td>
<td>-0.109</td>
<td>-0.309 to 0.090</td>
</tr>
</tbody>
</table>

Figure 7.13c. Graph representing the percentage of dead cells found within region A at 100–200 μm caused by wounding (control) and the amount of cell death following wounding and transplantation of CE, MZ, DZ and FD derived pellets. Statistically significant differences between cultures \( (p < 0.05) \) are marked in the table below.

<table>
<thead>
<tr>
<th>REGION A 200 μM</th>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL v CE</td>
<td>0.501</td>
<td>0.195 to 0.806</td>
</tr>
<tr>
<td></td>
<td>CONTROL v MIDDLE</td>
<td>0.380</td>
<td>0.074 to 0.685</td>
</tr>
<tr>
<td></td>
<td>CONTROL v DEEP</td>
<td>0.367</td>
<td>0.062 to 0.672</td>
</tr>
<tr>
<td></td>
<td>CONTROL v FULL DEPTH</td>
<td>0.207</td>
<td>-0.098 to 0.513</td>
</tr>
<tr>
<td></td>
<td>CE v MIDDLE</td>
<td>-0.121</td>
<td>-0.426 to 0.184</td>
</tr>
<tr>
<td></td>
<td>CE v DEEP</td>
<td>-0.133</td>
<td>-0.439 to 0.172</td>
</tr>
<tr>
<td></td>
<td>CE v FULL DEPTH</td>
<td>-0.293</td>
<td>-0.598 to 0.012</td>
</tr>
<tr>
<td></td>
<td>MIDDLE v DEEP</td>
<td>-0.012</td>
<td>-0.318 to 0.293</td>
</tr>
<tr>
<td></td>
<td>MIDDLE v FULL DEPTH</td>
<td>-0.172</td>
<td>-0.477 to 0.133</td>
</tr>
<tr>
<td></td>
<td>DEEP v FULL DEPTH</td>
<td>-0.160</td>
<td>-0.465 to 0.145</td>
</tr>
</tbody>
</table>
**Figure 7.14a.** Top shows diagrammatic representation of a full depth wounded explant. **Figure 7.14b.** Graph representing the percentage of dead cells found within region B at 0-100μm caused by wounding (control) and the amount of cell death following wounding and transplantation of chondroprogenitor enriched (CE), middle zone (MZ), deep zone (DZ) and full depth (FD) derived pellets. Statistically significant differences between cultures (p < 0.05) are marked in the table below.

### REGION B 100μM

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL v CE</td>
<td>0.497</td>
<td>0.177 to 0.817</td>
</tr>
<tr>
<td>CONTROL v MIDDLE</td>
<td>0.352</td>
<td>0.032 to 0.672</td>
</tr>
<tr>
<td>CONTROL v DEEP</td>
<td>0.190</td>
<td>-0.130 to 0.509</td>
</tr>
<tr>
<td>CONTROL v FULL DEPTH</td>
<td>0.027</td>
<td>-0.293 to 0.346</td>
</tr>
<tr>
<td>CE v MIDDLE</td>
<td>-0.146</td>
<td>-0.465 to 0.174</td>
</tr>
<tr>
<td>CE v DEEP</td>
<td>-0.308</td>
<td>-0.627 to 0.012</td>
</tr>
<tr>
<td>CE v FULL DEPTH</td>
<td>-0.471</td>
<td>-0.790 to -0.151</td>
</tr>
<tr>
<td>MIDDLE v DEEP</td>
<td>-0.162</td>
<td>-0.482 to 0.158</td>
</tr>
<tr>
<td>MIDDLE v FULL DEPTH</td>
<td>-0.325</td>
<td>-0.645 to -0.005</td>
</tr>
<tr>
<td>DEEP v FULL DEPTH</td>
<td>-0.163</td>
<td>-0.483 to 0.157</td>
</tr>
</tbody>
</table>

**Figure 7.14c.** Graph representing the percentage of dead cells found within region B at 100-200μm caused by wounding (control) and the amount of cell death following wounding and transplantation of CE, MZ, DZ and FD derived pellets. Statistically significant differences between cultures (p < 0.05) are marked in the table below.

### REGION B 200μM

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference</th>
<th>LSD 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL v CE</td>
<td>0.454</td>
<td>0.125 to 0.783</td>
</tr>
<tr>
<td>CONTROL v MIDDLE</td>
<td>0.392</td>
<td>0.063 to 0.721</td>
</tr>
<tr>
<td>CONTROL v DEEP</td>
<td>0.236</td>
<td>-0.093 to 0.565</td>
</tr>
<tr>
<td>CONTROL v FULL DEPTH</td>
<td>0.056</td>
<td>-0.273 to 0.385</td>
</tr>
<tr>
<td>CE v MIDDLE</td>
<td>-0.062</td>
<td>-0.391 to 0.267</td>
</tr>
<tr>
<td>CE v DEEP</td>
<td>-0.218</td>
<td>-0.547 to 0.111</td>
</tr>
<tr>
<td>CE v FULL DEPTH</td>
<td>-0.398</td>
<td>-0.728 to -0.069</td>
</tr>
<tr>
<td>MIDDLE v DEEP</td>
<td>-0.156</td>
<td>-0.485 to 0.174</td>
</tr>
<tr>
<td>MIDDLE v FULL DEPTH</td>
<td>-0.336</td>
<td>-0.665 to -0.007</td>
</tr>
<tr>
<td>DEEP v FULL DEPTH</td>
<td>-0.181</td>
<td>-0.510 to 0.149</td>
</tr>
</tbody>
</table>
7.4. DISCUSSION

The challenge for both basic scientists and clinicians is the restoration/repair of damaged joint articular surfaces. The long term success of any attempt at repair of an articular cartilage defect is governed by the integration of transplanted cartilage or locally induced repair tissue and the native tissue. Repair attempts have included periosteal and perichondral grafting (Amiel et al., 1988; O’Driscoll, 1998), osteochondral grafts and bioengineered grafts (Wakitani et al., 1994). Integration is thought to be hindered by the chondrocytic death at the wound margin as well as the lack of matrix producing cells and the apparent inability of chondrocytes to migrate.

Following experimental in vitro wounding of full depth articular cartilage, extensive cell death was observed around the wound margin and at the articular surface, with concurrent matrix loss. Early work on experimental cartilage defects revealed histologically a wound response, which was characterised by a zone of cell death up to 200μm deep at the wound margin (Key A, 1931, Bennett et al., 1932). The band was termed the ‘zone of necrosis’ (Stockwell RA, 1979) and was recently characterised by Tew et al. (2001), using the TUNEL assay method to determine the ratio of cell death due to necrosis and apoptosis. The introduction of pelleted chondrocytes from chondroprogenitor enriched (CE), middle zone (MZ), deep zone (DZ) and full depth (FD) cell populations to the wound site reduced cell death suggesting that glycosaminoglycan (GAG) loss was reduced because of this. Therefore, cellular depletion (death) of the local regulator (the chondrocytes) of matrix synthesis and assembly would result in a loss of matrix. However, there appears in some cases to be a loss of matrix (not shown) away from the wound edge. This is possibly due to a diffusion signal from the apoptotic area. Levin et al., (2001), observed that cell death increased with duration of post-impact culturing (canine shoulder articular cartilage explant) and that the location of dead cells spread from the damaged region to the surrounding unloaded region with increasing time suggesting that a diffusible cell death signal released at a site of damage penetrates to peripheral areas. However following insult, much of the cell death could be necrotic followed then by an apoptotic episode. Increased apoptosis is also associated with osteoarthritis (Hashimoto et al., 1998) correlated with loss of chondrocyte viability and degradation of the ECM. The progression of OA is due to a loss of survival
signals and increased susceptibility of cells to undergo apoptosis in response to other stimuli e.g. cytokines and nitric oxide.

Chondrocyte apoptosis results in the formation of apoptotic bodies, which are removed in other tissues by phagocytic bodies to prevent an inflammatory response. However, their fate in cartilage is distinct, as the tissue does not contain phagocytic cells, and apoptotic bodies remain in the ECM and contribute to matrix degradation. Therefore, there is an association between cell death and matrix degradation. Investigations will need to consider to what extent inhibition of matrix degradation can prevent chondrocyte apoptosis and whether inhibition of chondrocyte apoptosis will ameliorate cartilage degradation enabling the development of OA to be attenuated. Studies to elucidate the regulatory mechanisms of chondrocyte apoptosis and the use of apoptosis inhibitors may help to improve cartilage wound healing (D’lima et al., 2001).

Therefore, any attempt to repair cartilage will have to rescue the cells from dying. Redman et al., (2004) have shown that using a sharp, precise instrument during wounding (surgical management of a wound) reduces cell death and promote matrix elaboration at the lesion edge in order to facilitate successful integration during articular cartilage repair. Treating the wound with enzymes has also been shown to increase cellular density at the wound margin and improve integration (Bos et al., 2002; Hunziker and Rosenberg, 1996).

The partial thickness tool was used to create wounds of a defined dimension, as wounds created using the drill bit were not reproducible. We did not use the popular trephine model to create a defect as this method penetrates the explant fully. The drill bit also caused extensive cell death, and in some cases shearing of the tissue was visualised. Noteworthy, however, is that the tool although sharp to begin with, did become blunt over time and so using the tool for further studies could jeopardise the comparison of results.

The significant cell rescue ability (and in some instances reduced matrix loss) of the chondroprogenitor cells could be due to extrinsic induction of cell-matrix interactions. Diffusible growth factors could be attributed to this, with the chondroprogenitor (CE)
pellet producing a molecular signal or inducing the release of a local factor within the wounded cartilage, thus, preventing an apoptotic response and maintaining matrix synthesis. However, the mechanism by which the homologous allografts influence the host chondrocytes is not characterised.

The mechanisms of cell death in cartilage injury and disease have been the targets of many studies. Tew et al., (2000) demonstrated cell death in response to wounding with a trephine to be necrotic immediately adjacent to the lesion edge, followed by a wave of apoptosis extending into the tissue. The type of cell death occurring within this study has not been clarified, as ethidium homodimer is a marker of general cell death and does not discriminate between apoptosis and necrosis.

The percentage of dead cells was determined in regions A & B at 0-100\textmu m and 100-200\textmu m from the wound edge representing superficial and deeper regions respectively. Within region A at both 0-100 and 100-200\textmu m the amount of cell death caused by the wound was significantly decreased after transplantation with a CE pellet. Within region B cell death was only significantly reduced at 0-100\textmu m from the wound edge.

One of the several strategies for tissue engineering that show promise for stimulating functional repair of articular cartilage defects is the transplantation of chondrocytes directly into a cartilage defect (Sams et al., 1995). In vivo cartilage repair studies have utilised cell transplantation either alone or in conjunction with other strategies, which include implanting scaffolds to aid migration and molecule infusion (e.g. growth factors), which recruit and regulate endogenous cells (Cuevas et al., 1988; Hunziker and Rosenberg, 1996). However, it is difficult to assess the role of potential regulators in vivo because chemical and physical milieu are complex and difficult to assess and control.

It was observed that some of the transplanted cells had adhered to the wound edge, with adjacent host matrix in some samples staining for PGs. The pellets themselves also contained matrix but weakly stained compared to the host matrix. It is possible that the entire pellet had initially adhered to the matrix but detached probably occurred during histological processing. The study was relatively short (7 days) and
therefore, further cultivation may have encouraged a stronger attachment to the host tissue. It is possible that the use of a preformed cartilage matrix plug such as a pellet may not aid integration and the use of cell suspension may improve adhesion to the host.

Current investigations in our laboratory are looking at the affects of placing high density micromass cultures of the CE cell population into the same wound model used in the present study (Samuel Webster, personal communication). Preliminary results demonstrate that chondroprogenitors readily adhere to partial-depth articular cartilage lesions, and synthesise a matrix implant containing proteoglycan and types I and II collagen. The density of labelling did not meet that of the underlying cartilage. The collagen matrix was oriented at 45 degrees to the surface and fibrils crossed perpendicularly. By 28 days, a small number of transplanted cells had differentiated to a point at which they synthesised alkaline phosphatase at the surface. Transplanted CE filled a wound more rapidly than transplanted full depth chondrocytes, which failed to completely fill a 500μm deep wound after 28 days in vitro. Cell tracking experiments have shown extremely limited cell migration between transplant and underlying explant matrices (Webster, 2004). The rationale for the above study was that during arthroscopic debridement the surface layer of chondrocytes within, and surrounding, the damaged area are removed. Surgical removal of articular tissue not only fails to have a beneficial biological effect, but it does not induce spontaneous repair and may in fact be detrimental. We have also identified a progenitor cell population within this layer and, therefore, their removal may hinder any attempt at repair. The return of chondroprogenitors to the injury site, may exploit their ‘stem-like’ properties.

The principle governing most repair methods is that introduced cells effect repair through the production of new ECM and the integration of new tissue with surrounding host matrix. The adherence of transplanted cells to the host has been enhanced with the use of enzymatic digestion, which removes proteoglycans from the surface (Chen et al., 1997, Hunziker et al., 1996). Proteoglycans such as decorin and biglycan render the surface anti-adhesive by masking the underlying adhesive molecules, e.g. fibronectin. The enzymatic digestion may release endogenous factors from the host tissue, culling the tissue’s own natural resources to repair.
A successful repair process may rely on initial seeding density, culture duration and rate of proliferation of transplanted pellets. Chen et al., (1997) demonstrated a defined linear relationship between biosynthetic activity and number of seeded chondrocytes, suggesting that the number of transplanted cells might affect the cell mediated repair process. It has been observed that mature bovine articular cartilage is more resistant to chondrocyte detachment with increased seeding times, and is dependent upon the formation of chondrocyte receptors for matrix components (Schinagl et al., 1999). Repeated applications of chondrocytes increased chondrocyte adhesion, demonstrating the loss of anti-adherent properties.

It is possible that increasing the cell density of the pellets may improve cell rescue and matrix loss. This would be followed by detailed analysis to reveal differences in quality and quantity of molecules synthesised by pellets itself. Tagging the pellets will enable discrimination between transplanted and host cells, which are imperative in assessing repair mechanisms. Examples include radiolabelling with tritiated thymidine, tagging with fluorescent tracers or inducing expression of specific proteins introduced into the DNA.

Recent repair strategies have also assessed the chondrogenic potential of bone marrow derived mesenchymal stem cells (MSCs). Kavalkovich et al., (2001) and Murphy et al., 2001) have demonstrated that direct injection of MSCs into OA joints resulted in their binding and subsequent elaboration of matrix. Injections into destabilised joints resulted in marked regeneration of the medial meniscus. They also revealed marked chondroprotective effects indicating therapeutic benefits. Improved cartilage repair is visualised by transplanting autologous MSCs with recombinant BMP-2 into a rabbit defect (Kavalkovich et al., 2002; Murphy et al., 2001). Thus, an exciting prospect would be to inject these isolated putative chondroprogenitor cells into a joint and visualise their effects. This procedure would be exploiting the fact that these cells have already decided to take the chondrocyte differentiation pathway. The in vivo environment should provide the signals required for the appropriate differentiation of the implanted cells. However, it is possible that the signals that are present during development and growth of tissues such as articular cartilage are no longer present in adults. The signals needed could be provided by various growth factors. However, articular cartilage developmental biology is poorly understood.
Whether theses putative chondroprogenitor cells will be used therapeutically within a pellet, seeded onto a matrix or in suspension will be considered following extensive *in vitro* studies and subsequent *in vivo* studies using animal models.
CHAPTER 8
General Discussion
8. GENERAL DISCUSSION

It is well documented that articular cartilage has an inherently poor repair capacity (Buckwalter and Mankin, 1998; Hunter, 1743). In order to repair cartilage, it is important to understand the lifespan of cartilage and how it changes. Post-natally, cartilage is found to have an anisotropic distribution of collagen fibrils. Within young adults, cell density is found to diminish greatly, and the matrix molecules are arranged in a highly organised, four layered structure each zone possessing differing mechanical properties. Within mature articular cartilage, chondrocytes rarely divide and, thus, their density diminishes dramatically from birth to age 65. The challenge for both basic scientists and clinicians is the restoration/repair of damaged joint articular surfaces by manipulating the microenvironment. Although numerous approaches have been attempted with various reports of success the literature is, in fact, confusing. The growth mechanisms of articular cartilage and the generation of its structure also remain to be elucidated. It may be argued that more closely to the native structure and mechanical function of articular cartilage is replicated, the more likely that the repair tissue will not deteriorate prematurely and essentially provides symptom-free function under normal physiologic stresses.

Adult articular cartilage has long been regarded as a post-mitotic tissue with virtually no cellular turnover due to its ‘lack of germinal layer’ which occurs in other tissues such as epithelia. Recently, it was proposed that articular cartilage grows by apposition from the articular surface and contains a population of slowly cycling cells suggestive of the existence of a chondroprogenitor cell population (Dowthwaite et al., 2004; Hayes et al., 2001).

Within this study, we have identified and confirmed the existence of a chondrocyte progenitor population by their rapid adhesion to fibronectin and their ability to form colonies of large cell number. Colony formation was confirmed following differential adhesion by surface zone chondrocytes isolated from 7-day, 18-month and over thirty-month-old bovine articular cartilage. The existence of a chondroprogenitor population within mature bovine tissue gives hope to its existence within mature human tissue. To date, cells having the ability to form colonies have been isolated from human articular cartilage from a 40-year-old donor (Sam Boyer, personnel communication).
Within this study, we also assessed the differentiation potential of clonally derived progenitor population by infecting them with a lineage label and injecting them into the proximal limb of stage 19-21 chick embryos and tracking them for 1 week in ovo. Overall, these results demonstrate that chondroprogenitor cells (derived from both early and late passaged cultures) as well as middle/deep derived chondrocytes have the ability to integrate into various tissues following their injection into the developing limb bud. This again indicates that chondrocytes are not irreversibly committed to the chondrocytic lineage and, therefore, may be quite plastic in their ability to adapt to their microenvironment. On the other hand, we did not carry out functional engraftment analyses, so cells may be ‘entrapped’ in a foreign tissue. Also, within this study we only injected progenitors isolated from 7-day old bovine articular cartilage into the developing limb bud. It will be interesting in future to inject progenitors isolated from more mature tissue into the developing limb bud to assess whether the progenitor population becomes more restricted with time and, therefore, less plastic.

This study also analysed the differentiation of the chondroprogenitor cells towards the adipogenic, osteogenic and chondrogenic lineages in vitro. The clonally derived progenitors were only able to produce cartilaginous matrix when cultured at high density in chemically defined serum free media supplemented with 10ng/ml TGF-β1. Chondrocytes isolated from the various zones, however, were able to retain their zonal characteristics and continued to synthesise cartilaginous matrix when cultured in basic media containing only 10% FCS. Clonally derived progenitors, therefore, behave very differently to chondrocytes per se. Of interest were the differences in pellet morphology derived from the different clonal cultures, indicating differences within the clonal population themselves. Future studies would involve isolating a higher number of clonally derived chondroprogenitors followed by quantification of the amount of collagen and GAG produced together with a variety of differentiation markers both at the matrix and cell level.

Interestingly, although progenitor cells were found within the periosteum during the in ovo study, no osteogenic differentiation occurred in vitro following exposure to osteogenic inducing media. This result indicates that the progenitor cells’ ability to differentiate toward the osteogenic lineage may require cues that are only available
from the local environment \textit{in vivo}. These results highlight the importance of carrying out stem cell research within both \textit{in vivo} and \textit{in vitro} models since one cannot extrapolate the data between the two conditions.

Differentiation of the clonally derived chondroprogenitors' toward the adipogenic lineage was demonstrated as was the surprising differentiation towards the neuronal phenotype. Although the cultures demonstrated characteristic features of the specific phenotypes tested, future experiments would need to test for functionality as mentioned above. The induction of clones toward multiple mesodermal lineages could be analysed by the expression of several lineage specific genes and proteins as well as biochemical assays to confirm lineage specific metabolic activity.

We further characterised the progenitor population derived from the 7-day-old bovine articular cartilage by cloning single colonies and expanding them in culture. Following extensive sub-culturing we were able to demonstrate that the progenitors continued to express high-levels of Sox9. We were able to generate $10^{12}$ cells from a single progenitor that were still capable of chondrogenesis. Normal chondrocytes possess a limited mitotic potential of 30-35 population doublings (PD) and inevitably enter a state of replicative senescence in which cellular proliferation ceases (Adolphe et al., 1983; Allsopp et al., 1992; Evans and Georgescu, 1983; Kolettas et al., 1995; Martin and Buckwalter, 2002). The clonal progenitor cells have, to date reached a total of 50PDs, 15 more PDS than had been predicted for chondrocytes. The importance of these results are highlighted by the fact that in recent years, autologous chondrocyte implantation has become increasingly used to repair cartilage defects. The number of chondrocytes that can be harvested from a patient for this procedure is limited by biopsy size, and the age of the patient. Primary chondrocytes derived from full depth biopsies have been used in previous studies which, as well as having limited growth potential in culture, lose their unique cartilage-specific phenotype (this was also confirmed within the present study). We were able to reduce the progenitors doubling time by supplementing the media with various growth factors without compromising their ability to synthesise a cartilaginous matrix when cultured in the form of pellets. Therefore, the existence of a progenitor cell that could be potentially isolated from the articular cartilage by a minimally invasive procedure, expanded in culture and produce cartilaginous tissue is an exciting prospect to cartilage repair
research. Further investigation of regulatory factors to reduce expansion time and improve the composition of engineered pellets will be necessary.

**Further work:**

- To determine the degree of heterogeneity within the chondroprogenitor population that has been indicated in our results. An extensive molecular and biochemical characterisation will be needed. The expression of CD marker antigens similar to those observed in mesenchymal stem cells should be analysed using gene expression profiles and the use of FACS analysis. Current studies on skin cells have demonstrated distinct patterns of protein synthesis both *in vivo* and *in vitro* which have suggested the existence of ‘units’ which comprise the stem cell and its progeny. It is possible that articular cartilage is also comprised of these units which may incorporate into the appositional growth model during its development. Identification of specific markers for the progenitor cells will aid isolation procedures.

- The *in vitro* transplantation of chondroprogenitor cells demonstrated a decrease in cell death at the wound margin. An exciting prospect would be to inject clonally derived chondroprogenitor cells into a partially wounded *in vivo* model and visualise their effects. Tagging the pellets will enable discrimination between transplanted and host cells. This procedure will be exploiting the fact that these cells have already decided to pursue the chondrocyte lineage. The *in vivo* environment may provide the signals required for the appropriate differentiation of the implanted cells.

- It has been demonstrated in this study that the various growth factors tested have differing effects on chondroprogenitor behaviour. Future experiments should study the effects of various concentrations and combinations of growth factors at distinct time points during the cultivation of the chondroprogenitor population.

- Following the study using zonally derived chondrocytes and creating stratified pellets a similar procedure could be adapted by using clonally isolated
chondroprogenitor cells to produce engineered cartilage. The clonally derived progenitor cells could be expanded in monolayer and then cultured either alone or atop middle/deep derived chondrocytes. The system would then be cultured in media containing various growth factors in attempt recapitulate the zonal organisation of the native cartilage. The pellet system could also be used to elucidate the extensive developmental program. The control of gene expression during the regeneration process must be considered as it may depend on the stage-specific expression of transcriptional activities. For example, the basic helix-loop-helix factor scleraxis prefigures the appearance of cartilage in somites and limb buds of the embryo (Cserjesi et al., 1995). Further down the chondrogenic differentiation pathway, Sox9 is a major transcription factor implicated in the expression of the chondrocytic phenotype (Lefebvre et al., 1997).
LIST OF REFERENCES


338


Transcriptional suppression by interleukin-1 and interferon-gamma of type II 
cultured myoblasts and chondrocytes to fibroblast and epidermal growth 
48:140-8.
of experimentally produced defects in rabbit articular cartilage by autologous 
lesions in articular cartilage following chondrocyte transplantation. Anat Rec. 
218:142-8.
physicochemical determinants of the chondrocyte biosynthetic response. J 
Orthop Res. 6:777-92.
stabilize binding of sulfated glycosaminoglycans to dimethylmethylene blue. 
Grundke-Iqbal, I., K. Iqbal, M. Quinlan, Y.C. Tung, M.S. Zaidi, and H.M. 
Wisniewski. 1986. Microtubule-associated protein tau. A component of 
responsiveness of human articular chondrocytes in aging and development. 
articular chondrocytes: distinct profiles in primary chondrocytes, sub cultured 
Mechanical and biochemical changes in the superficial zone of articular 
Hall, A.C., J.P. Urban, and K.A. Gehl. 1991. The effects of hydrostatic pressure on 
Hamburger, V., and H. Hamilton. 1951. A series of normal stages in the development 
Hanada, K., J.E. Dennis, and A.L. Caplan. 1997. Stimulatory effects of basic fibroblast 
growth factor and bone morphogenetic protein-2 on osteogenic differentiation 
of rat bone marrow-derived mesenchymal stem cells. J Bone Miner Res. 
12:1606-14.


354


Shoemaker, R.S., A.L. Bertone, G.S. Martin, C.W. McIlwraith, E.D. Roberts, R.
Pechman, and M.T. Kearney. 1992. Effects of intra-articular administration of
methylprednisolone acetate on normal articular cartilage and on healing of
53:1446-53.

Shortkoff, S., L. Barone, H.P. Hsu, C. Wrenn, T. Gagne, T. Chi, H. Breinan, T.
Minas, C.B. Sledge, R. Tubo, and M. Spector. 1996. Healing of chondral and
osteochondral defects in a canine model: the role of cultured chondrocytes in
regeneration of articular cartilage. Biomaterials. 17:147-54.

Siczkowski, M., and F.M. Watt. 1990. Subpopulations of chondrocytes from different
zones of pig articular cartilage. Isolation, growth and proteoglycan synthesis in

1999. Injectable tissue-engineered cartilage using a fibrin glue polymer. Plast

Engineering of cartilage tissue using bioresorbable polymer carriers in

Smith, G.N., Jr., and K.D. Brandt. 1992. Hypothesis: can type IX collagen "glue"
together intersecting type II fibers in articular cartilage matrix? A proposed

with the pericellular (1 alpha, 2 alpha, 3 alpha) collagens of cartilage. J Biol

synovial fibroblasts is influenced by the nature of the hyaluronate in the

Sokoloff, L. 1983. Factors controlling growth and differentiation in the repair of


Sriramarao, P., M. Mendler, and M.A. Bourdon. 1993. Endothelial cell attachment
and spreading on human tenasin is mediated by alpha 2 beta 1 and alpha v

results of full thickness articular cartilage defects of the knee treated with
debridement and microfracture. In Linvatec Sports medicine conference, Vail,
Colorado.

Phenotypic stability of articular chondrocytes in vitro: the effects of culture
models, bone morphogenetic protein 2, and serum supplementation. J Bone
Miner Res. 15:166-74.

press.

101:753-63.


freeman, editor. Pitman Medical Publishing Co Ltd. 69-144.

360


361


Transforming growth factor-beta 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. Lab Invest. 71:279-90.


Webster, S.V. 2004. Towards improving the surgical lavage and debridement of articular cartilage with cartilage progenitor cells.


APPENDICES

APPENDIX I
Haematoxylin and Safranin O

Slides were:

1. Immersed in Mayer’s haematoxylin for 5 minutes
2. Washed and ‘blued’ in running tap water for 1 minute.
3. Counter-stain with Safranin O for 30 seconds
4. Washed for 30 seconds in water
5. Dehydrate by passing through a graded series of alcohols
6. Cleared in xylene
7. Mounted under a cover slip using distrene plasticizer xylene (DPX)

APPENDIX II
Oil red O

Culture dishes were:

1. Fixed in 60% isopropanol for 2 minutes
2. Incubated in oil red O solution (0.1% in 60% isopropanol) for 20 minutes.
3. Mounted with aquamount and coverslipped

APPENDIX III
Von Kossa

Culture dishes were:

1. Washed with PBS
2. Fixed with 10% neutral buffered formalin saline (NBFS) at room temperature
3. Washed with distilled water at least 5 times. Let last 2 rinses sit in water for 5 minutes
4. Placed in 5% silver nitrate (made fresh) onto culture dishes and placed under UV light for 20 minutes, until nodules within positive control were black
5. Washed with distilled water 5 times. Let last 2 rinses sit in water for 5 minutes
6. Differentiated with 5% sodium thiosulphate for 2 minutes
7. Washed with distilled water, 3 times.
8. Mounted in aquamount and coverslipped.
Sox 9 Ct - rRNA Ct

$y = 0.0475x + 11.573$
APPENDIX V

Publications arising from this work:


Bishop, J C; Smith, M; Webster, S V; Dowthwaite, G P; and Archer, C W. Differentiation and proliferative capacity of articular cartilage progenitor cells. Transactions of the 50th ORS 2004. Paper #761 (Poster presentation).


SOX9 Expression Is Maintained Within Long Term Cultures Of Chondroprogenitors. Bishop, J.C., Webster, S.V. and Archer, C.W.

**Study.** In recent years, autologous chondrocyte implantation (ACI) has been increasingly used to repair articular cartilage (AC) defects. The number of chondrocytes that can be harvested from a patient for this procedure is limited by biopsy size and the age of the patient. Previous studies demonstrate that primary chondrocytes, as well as having a limited growth potential in culture, lose their cartilage-specific phenotype

We have recently identified a progenitor cell population residing at the surface of AC. Here we report on the isolation and extensive expansion of these cells.

**Methods.** Bovine articular chondrocytes were isolated by pronase and collagenase digestion. Chondroprogenitor cells were isolated with a fibronectin adhesion assay and expanded in monolayer for 6 days. Chondrocyte progenitor cell colonies of >32 cells were isolated. Cells were transferred 35mm dish and cultured in DMEM+.

**In vitro expansion:** When cells approached confluence (P0), cells were trypsinised and transferred to 25cm² culture flasks (P1). Subsequent growth in 125cm² culture flasks was carried out by continual passage at a ratio of approx 1:6. At various passages aliquots of cells were removed for pellet culture. Cell numbers harvested and seeded at each stage were recorded throughout the process to allow the number of population doublings (PDs) at each stage to be calculated. Controls consisted of chondrocytes isolated from full thickness bovine articular cartilage.

**Gene expression in chondrocytes passaged in monolayer culture:** RNA was isolated from chondrocytes at various passages to build up a profile of gene expression during cell expansion using quantitative RT-PCR (Taqman). For this study we primarily looked at the transcription factor SOX9, a marker of chondrogenesis.

Results. Sox9 expression was maintained throughout the expansion of chondroprogenitor cells for 40 PDs in contrast to full depth cells, which showed significantly decreased Sox9 expression by 11 PDs.

**Discussion.** Present ACI repair strategies rely on amplifying a population of full depth chondrocytes taken from a biopsy. Our observations of continued expression of high levels of SOX9 within chondroprogenitor cultures, even with extensive sub-culturing, indicate advantages of amplification scale and chondrogenic phenotype maintenance over full depth chondrocytes. Also, it is of note that the chondroprogenitor’s differentiation pathway behaves and responds in a different manner to *in vitro* conditions than that of full depth isolated chondrocytes.

**References.**

DIFFERENTIATION AND PROLIFERATIVE CAPACITY OF ARTICULAR CARTILAGE PROGENITOR CELLS

*Bishop, J C; **Smith, M; *Webster, S V; *Dowdthwaite, G P; **Archer, C W
**Cardiff Institute of Tissue Engineering and Repair, Cardiff School of Biosciences, Cardiff, UK, +44 (0)2920875206, Fax: +44(0)2920874594
** Smith-Nephew, GRC, Heslington Park, York, UK

INTRODUCTION: In recent years, autologous chondrocyte implantation (ACI) has been increasingly used to repair articular cartilage defects. The number of chondrocytes that can be harvested from a patient for this procedure is limited by biopsy size, and the age of the patient. Previous studies demonstrate that primary chondrocytes as well as having a limited growth potential in culture lose their cartilage-specific phenotype. This is thought to be linked to a concomitant decrease in collagen II and aggrecan synthesis and a fibroblastic like morphology (1). The dedifferentiated phenotype can be recovered by culturing cells in an environment supporting a spherical morphology. We have identified a population of chondroprogenitor cells in the superficial zone of articular cartilage with an extended cell cycle time, a high affinity for fibronectin in cell adhesion assays and an ability to form colonies in vitro from a low seeding density (2). Here we report on the isolation and extensive expansion of these cells and their ability to redifferentiate when transferred into a 3D environment. The effect of different growth factors on expansion of cloned chondroprogenitor cells was also studied within a 2d monolayer culture to evaluate a procedure to quickly expand a small cell number.

MATERIALS AND METHODS: Chondrocyte Isolation: Surface zones (SZ) chondrocytes were isolated from the metacarpophalangeal joints of 7-day-old calves by careful dissection. Cartilage shavings were digested in 3.17 U/ml pronase in DMEM/F12 (1:1) containing 5% FCS at 37°C for 3 hours followed by 0.12 U/ml collagenase in DMEM/F12 + 5 % FCS at 37°C for 16 hours. Chondrocytes were centrifuged, washed in serum free DMEM/F12, counted and plated out as required.

Differential Adhesion: 35mm dishes were coated with 10 µg/ml fibronectin in PBS containing 1mM CaCl₂ and 1mM MgCl₂ (PBS+) overnight at 4°C. Fibronectin was removed and dishes washed with 1% BSA/PBS+ and 2.5 mM chondrocytes plated onto the dishes at 4000 cells in serum free DMEM/F12 and allowed to adhere to the dish for 20 minutes at 37°C. Media and non-adherent cells were removed and the remaining cells cultured for 6 days in DMEM/F12 + 10% FCS (DMEM+).

Cloning Chondrocyte Progenitor Cells: With the aid of an inverted microscope, 7 colonies of 32+ cells were identified and marked. Media was removed from the dishes; the cloning cylinder was then applied around the selected colony by pressing thicker edge (previously greased) lightly against the Petri dish. Two drops of trypsin were added to the cylinder and incubated at 37°C for 5 minutes. The cells were collected and transferred to a 25mm dish and cultured in DMEM+.

Expansion in culture: When cells in the 35mm dish approached confluence (P0), cells were trypsinised and transferred to 25cm² culture flasks (P1). Subsequent growth in 125cm² culture flasks was carried out by continual passage at a ratio of approx 1:6. At various passages aliquots of cells were removed for pellet culture. Cell numbers harvested and seeded at each stage were kept throughout the process to allow the number of population doublings at each stage to be calculated. Controls consisted of chondrocytes isolated from full thickness bovine articular cartilage. In addition another set of clones was cultured whereby throughout the expansion phase (P1 and P2) cells were cultured in DMEM+ (1% or 10% FCS) or DMEM+ supplemented with growth factors involved in cartilage metabolism. Factors tested included 5ng/ml fibroblast growth factor-2 (FGF-2), 10ng/ml epidermal growth factor (EGF), 10ng/ml platelet derived growth factor-bb (PDGFB), 1ng/ml of transforming growth factor-β1 (TGF-β), and a combination of 5ng/ml FGF-2 and 10ng/ml TGF-β or 10ng/ml Interleukin-18 (IL-18)

Gene expression in chondrocytes passaged in monolayer culture: RNA was isolated from chondrocytes at various passages to build up a profile of gene expression during cell expansion using RT-PCR (Tagman). For this study we primarily looked at the transcription factor SOX 9, a marker of chondrogenesis.

Pellet Culture: Aliquots of 250,000 cells were resuspended in chondrogenesis media (DMEM supplemented with gentamycin, ITS premix, ascorbate-2-phosphate (100µM) and TGF-β1 (10ng/ml)).

Histology and immunolabelling: Pellets were snap frozen in liquid nitrogen and sections of 10µm thickness were cut and stained with Safranin O/haematoxylin. For immunolabelling, sections were labelled with antibodies to collagen I and II and visualised using appropriate secondary Alexa Fluor 488 conjugated antibodies.

RESULTS: The growth kinetics of the 7 clones were investigated from the primary culture through 17 passages, corresponding to about 45 population doublings. P1 cultures reached confluence in about 2 weeks and an average 18 doublings, a doubling time of less than 24 hours. On replating the clones had slowed their proliferation rate, with a growth rate of about 3-4 days per population doubling. Cultures of cloned progenitor cells comprised a polygonal flat round-morphological appearance. Pellets cultures derived from early passaged chondroprogenitors and redifferentiated in chondrogenic media showed focal areas of glycosaminoglycans (GAG) deposition (Safranin O), were positive for type II collagen labelling and contained small rounded chondrocytes. Interestingly, although pellets derived from P17 stained for GAGs, the labelling for collagen type II was relatively weak. Control pellets derived from full depth isolated chondrocytes contained larger, vacuolated cells, separated by intensely stained matrix. However, pellets derived from full depth cultures at P3 although stained positively with Safranin O had very little collagen type II labelling.

Sox 9 expression was maintained throughout the expansion of chondroprogenitor cells in contrast to full depth cells which showed decreased Sox 9 expression by passage 4.

Not all the growth factors tested during chondroprogenitor expansion in monolayer induced a significant decrease in the doubling times of exponential growth. However, some growth factors were more effective at reducing doubling times than others. The growth factor which promoted the highest cell proliferation rates was FGF-2 significantly reducing the doubling time by 16%.

DISCUSSION: The data presented in this study indicate that, the population of expanded clones (32+ cells) maintained their ability to proliferate and redifferentiate into a hyaline cartilage like tissue. Following culture the continued expression of high levels of Sox 9 within chondroprogenitor cultures following extensive sub-culturing is an important observation as present repair strategies (ACT) rely on amplifying a population of full depth chondrocytes taken from a biopsy. This study has shown that this chondrogenic transcription factor is rapidly lost following the subculture of full depth cells. This is indicative that the chondroprogenitor’s phenotype and thus differentiation pathway behaves and responds in a different manner to that of full depth isolated chondrocytes when cultured. Expansion of cloned chondroprogenitor cells using FGF-2 would also allow faster amplification and to reduce the amount of donor tissue required during repair procedures.

Current investigations are looking at quickly expanding the bovine chondroprogenitors using FGF-2 and assessing their ability to maintain their chondrogenic potential in comparison to chondroprogenitors grown in standard media (DMEM+). It is hoped that the development of a two-phase culture system (monolayer culture followed by pellet culture) using growth factors and chondrogenic medium would exploit the chondroprogenitor’s already enhanced ability to form cartilage after extensive expansion in culture.

REFERENCES:
(1) Benya PD, Shaffer JD. Cell 1982;20:215-24
(2) Archer CW et al (2002) Trans ORS 009

50th Annual Meeting of the Orthopaedic Research Society
Poster No: 0761
The surface of articular cartilage contains a progenitor cell population

Gary P. Dowthwaite¹, Joanna C. Bishop¹, Samantha N. Redman¹, Ilyas M. Khan¹, Paul Rooney², Darrell J. R. Evans¹, Laura Haughton¹, Zubeyde Bayram³, Sam Boyer⁴, Brian Thomson⁴, Michael S. Wolfe⁵ and Charles W. Archer¹,²

¹Cardiff School of Biosciences and Cardiff Institute of Tissue Engineering and Repair, Cardiff University, PO Box 911, Museum Avenue, Cardiff CF10 3US, UK
²Tissue Services, National Blood Service, Langley Lane, Sheffield S5 7JN, UK
³Department of Histology and Embryology, Alkentz University, 070 Campus Antalya, Turkey
⁴Smith and Nephew Group Research Centre, York Science Park, Hesslington, York YO10 5DF, UK
⁵Centre for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA

Accepted 2 October 2003

Summary

It is becoming increasingly apparent that articular cartilage growth is achieved by apposition from the articular surface. For such a mechanism to occur, a population of stem/progenitor cells must reside within the articular cartilage to provide transit amplifying progeny for growth. Here, we report on the isolation of an articular cartilage progenitor cell from the surface zone of articular cartilage using differential adhesion to fibronectin. This population of cells exhibits high affinity for fibronectin, possesses a high colony-forming efficiency and expresses the cell fate selector gene Notch 1. Inhibition of Notch signalling abolishes colony forming ability whilst activated Notch rescues this inhibition. The progenitor population also exhibits phenotypic plasticity in its differentiation pathway in an embryonic chick tracking system, such that chondroprogenitors can engraft into a variety of connective tissue types including bone, tendon and periosteum. The identification of a chondrocyte subpopulation with progenitor-like characteristics will allow for advances in our understanding of both cartilage growth and maintenance as well as provide novel solutions to articular cartilage repair.

Key words: Cartilage, Progenitor cell, Notch

Introduction

Articular cartilage is an avascular, aneural tissue with a high matrix to cell volume ratio. The matrix comprises mainly type II collagen fibres and the high molecular weight aggregating proteoglycan aggrecan. The tissue is not, however, homogeneous with biochemical and morphological variations existing from the surface zone to the deeper calcified layer. The surface zone of the tissue is characterised by flattened, discoid cells that secrete surface zone proteoglycan (proteoglycan 4) (Schumacher et al., 1994). The mid zone of the tissue comprises rounded cells arranged in perpendicular columns and in addition to type II collagen and aggrecan, expresses cartilage intermediate layer protein (CILP) (Lorenzo et al., 1998). The deep zone and calcified zone chondrocytes express type X collagen and alkaline phosphatase (Schmid and Lienemayer, 1985), and in the deep zone the chondrocytes are considerably larger than in the other zones.

Clearly, the differentiation and proliferation events occurring during the development of articular cartilage must, therefore, be strictly controlled both temporally and spatially in order for the distinct zonal architecture of the tissue to be established. Various studies have shown that the surface zone of articular cartilage is centrally involved in the regulation of tissue development and growth. Not only does the surface of articular cartilage play a major role in the morphogenesis of the diarthrodial joint via differential matrix synthesis (Ward et al., 1999), but the expression of many growth factors and their receptors at the articular surface (Archer et al., 1994; Hayes et al., 2001) suggest that this region represents an important signalling centre. In addition, it has been shown in vivo that the surface zone of articular cartilage is responsible for the appositional growth of articular cartilage and from these studies we hypothesised that the surface zone of articular cartilage contains a progenitor/stem cell population that allows for the appositional growth of the tissue (Hayes et al., 2001). Identification of such cells holds exciting possibilities in the field of cartilage tissue engineering because the tissue has limited inherent reparative capacity after trauma (Hunziker, 1999). Here, we describe the isolation and partial characterisation of a specific articular cartilage progenitor cell using a previously described differential adhesion assay (Jones and Watt, 1993).

Materials and Methods

Cell isolation, differential adhesion assay and tissue culture

Petri dishes (35 mm) were coated with 10 µg ml⁻¹ bovine fibronectin (FN; Sigma, UK) in 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 1 mM MgCl₂ and 1 mM CaCl₂ (PBS⁺) overnight at 4°C. Dishes were blocked with 1% bovine serum albumin (BSA) in PBS⁺
before chordocytes were added. Control dishes were treated with PBS containing 1% BSA overnight at 4°C.

Previous studies have utilised differential adhesion to fibronectin in vitro to identify epithelial stem cells (Jones and Watt, 1993). Fibronectin is expressed in developing mammalian articular cartilage in addition to the classic fibronectin receptor integrin subunits α5 and β1 (Hynes, 1992). We therefore utilised fibronectin in an in vitro adhesion assay to identify and partially characterise articular cartilage progenitor cells. Chordocytes were isolated from the surface, middle and deep zones of articular cartilage of 7-day-old calves by sequential pronase/collagenase digestion as previously described (Archer et al., 1990). After isolation, chordocytes (4000 ml⁻¹) were seeded onto 35 mm plastic Petri dishes at 37°C for 20 minutes in 1:1 DMEM/F12 containing 0.1% Gentamycin (DMEM/F12-). After 20 minutes, media (and non-adherent cells) was removed and placed in a second dish for 40 minutes at 37°C before this media (and non-adherent cells) was removed and placed in a third dish. After removal of media at 20 and 40 minutes, fresh 1:1 DMEM/F12 containing 0.1% Gentamycin, 0.5 μg ml⁻¹ ascorbate, 1 μg ml⁻¹ glucose and 10% foetal calf serum (FCS; DMEM/F12+) was added to the remaining adherent cells which were maintained in culture for up to 10 days. In all experiments, six fibronectin-coated dishes and six untreated dishes were used for each point and for each zone of cartilage. Controls comprised cells subjected to differential adhesion on dishes coated with 1% BSA in PBS+-

For Notch 1 selection, magnetic tosyl-activated Dynal Beads (Dynal, UK) were coated with polyclonal anti-Notch 1 antibody (5 μg ml⁻¹; Santa Cruz, CA) following the manufacturer’s instructions. Freshly isolated chordocytes from the surface, middle and deep zone were incubated with antibody-coated beads for 30 minutes at 4°C and separated from Notch-negative cells using a powerful magnet. Non-magnetic cells were aspirated and Notch-positive cells washed three times in PBS before isolated cells were resuspended in DMEM/F12 and the purified cells counted using a haemocytometer. Notch 1-positive cells were then subjected to differential adhesion on fibronectin for 20 minutes (4000 cells ml⁻¹ in 35 mm dishes as described above), and initial adhesion and colony forming efficiency assayed up to 10 days as described below.

Within 3 hours of plating, initial chordocyte adhesion was assayed by counting the total number of cells adhering to the bottom of the dish using an inverted microscope equipped with phase contrast optics and expressed as a percentage of the initial seeding density. Colonies (defined as consisting of more than 32 chordocytes) were counted using the same microscope at 3, 6 and 10 days. Thirty-two cells were chosen as this represents a population of cells derived from more than 5 population doublings of a single cell, thereby discounting a transit amplifying cell (Jones and Watt 1993). Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. In some experiments (n=3), the number of cells per colony was counted to determine the average number of cells per colony. Results were analysed using the Student’s t-test. However, for comparative purposes, we also analysed the data in terms of colonies comprising more than 4 cells.

For γ-secretase inhibition studies, cells were isolated and subjected to differential adhesion to fibronectin as described above. Cells were maintained in media containing 50 nM N-[N-(3,5-difluorophenacyl)-L-alanyl]-5-phenylglycine t-butyl ester (DAPT) (Dovey et al., 2001) in 0.1% DMSO for 7 days with media changes every 48 hours. Initial adhesion and CFE were assayed as described.

For explant cultures, full-depth cartilage chips were removed and bisected. One half of each explant was cultured in the presence of 50 nM DAPT in DMEM/F12+ and 0.1% DMSO for 7 days, whereas the other half of the explant was cultured in DMEM/F12+ and 0.1% DMSO. Media was changed every 48 hours and samples fixed in 10% NBFS, was embedded and stained with toluidine blue. Sections were examined using brightfield optics and digital images obtained. A calibrated grid was then used to count the number of cells 0-100 μm and 101-200 μm from the articular surface. Results were analysed using the Student’s t-test.

In separate experiments, explants were excised and cut in half. Half of each explant was maintained in 50 nM DAPT and the other half was maintained in control media as described above for 7 days. On days 4, 5 and 6, the thymidine analogue bromodeoxyuridine (BrdU; final concentration 50 mM) was added to control and experimental media in order to identify s-phase chordocytes. Explants were removed after 24, 48 and 72 hours' incubation in BrdU, fixed in 10% formalin and wax embedded. Dewaxed sections were then immunolabelled with monoclonal anti-BrdU (5 μg ml⁻¹ in PBS) and localised using goat anti-mouse fluorescein-conjugated secondary antibody.

Immunocytochemistry

Chordocytes were labelled with antibodies raised against α5 and β1 integrin subunits after sequential pronase/collagenase digestion and at various time points after differential adhesion. Briefly, chordocytes (2x10⁶ cells ml⁻¹) were fixed in 95% ice-cold ethanol for 10 minutes and washed in PBS. The cells were incubated with primary antibodies diluted in PBS (2 μg ml⁻¹) for 1 hour at room temperature, washed three times in PBS and incubated with appropriate FITC-conjugated secondary antibodies (2 μg ml⁻¹) diluted in 20% heat inactivated foetal calf serum in PBS. Cells were washed in PBS and mounted in Vectashield containing 1.0 mg ml⁻¹ propidium iodide. Cells were then observed and photographed using a fluorescent microscope. To determine integrin, FN-extra domain A (EDA) and Notch 1 expression in vivo, full-depth articular cartilage was excised from 7-day-old bovine metacarpal-phalangeal joints and chilled by precipitation immobilization in n-hexane at ~80°C. Cryostat sections (10 μm) were cut on a Bright's cryostat and collected on APES (3-aminopropyltriethoxysilane)-coated slides and stored at ~20°C. Sections were defixed, post-fixed in ice-cold acetone for 5 minutes, washed in PBS and incubated with primary antibody diluted in PBS/0.01% Tween 20 (PBST) for 1 hour at room temperature. After washing in PBST, sections were incubated with relevant FITC-conjugated antibodies diluted in 20% heat-inactivated FCS in PBST for 1 hour before washing three times with PBST and mounting in Vectashield. Labelled sections were examined and photographed using either a Zeiss or an Olympus photomicroscope fitted with epifluorescent optics.

Flow cytometry

To assess integrin expression before differential adhesion, freshly isolated chordocytes were incubated in fresh DMEM/F12- at 37°C on a roller prior to labelling for FACS analysis. After differential adhesion, chordocytes were removed from dishes non-enzymatically (Sigma) and labelled for FACS analysis. All samples were counted and 2x10⁶ cells were incubated for 3 hours with antibodies to α5 and β1 integrin subunits and Notch 1 in sextuplicate at room temperature. Cells were centrifuged at 500 g, supernatants removed and cells washed three times in PBS with centrifugation between each wash. Cells were then incubated with relevant FITC-conjugated secondary antibodies for 1 hour at room temperature and washed three times in PBS as described above. Finally, labelled cell were re-suspended in 200 μl PBS and subjected to single channel FACS analysis.

Notch intracellular domain (NICD) transfection

Activated Notch constructs (Notch ICV) were obtained from Raphael Kopan (University of Washington) (Schroeter et al., 1999). Surface zone chordocytes were subjected to differential adhesion to fibronectin and grown in the presence of 50 nM DAPT for 3 days. Excess (1 μg) plasmid DNA was transiently transfected into surface zone chordocytes 3 days after differential adhesion using Effectene
Fig. 1. Frozen sections (A,B) and isolated chondrocytes (C-F) from 7-day bovine articular cartilage immunolabelled for α5 (A,C,E) and β1 (B,D,F) integrin subunits. α5 and β1 integrin subunits are localised throughout the depth of the articular cartilage (A,B) although not every chondrocyte is labelled. Isolated chondrocytes from surface zone immediately after sequential pronase/collagenase isolation labelled with antibody to alpha α5 (C) and β1 (D) subunits. Labelling for α5 (E) and β1 (F) is also present 72 hours after differential adhesion to fibronectin. Fibronectin-EDA was localised in frozen tissue sections to the surface 2-3 cell layers (G). Integrin α5 and β1 subunit expression was assessed by flow cytometry after sequential pronase/collagenase digestion (4 hours) and 72 hours after differential adhesion assay to fibronectin (H). At 4 hours and 72 hours, there was no difference in integrin subunit expression between surface zone chondrocytes (P=0.05), although during this time period the overall expression of α5 and β1 subunits was significantly decreased (P<0.01). At 4 hours, middle zone chondrocytes had a higher expression of β5 subunits relative to β1 subunits (P<0.01), although there was no difference in expression after 72 hours (P=0.05).

reagent (Qiagen), and colonies consisting of more than 32 cells were counted 10 days after differential adhesion and colony forming efficiency calculated.

Culture and transient transfection of 293GP packaging cells

293GP cells expressing the gag and pol proteins (Burns et al., 1993) were cultured to 70-80% confluency in DMEM/F12 containing 0.5 mg ml⁻¹ Gentamycin and 10% FCS. Cells were then transfected with 1 μg plasmid DNA encoding VSV-G and lacZ using the Quaigen Effectene kit following the manufacturer’s instructions. Briefly, plasmid DNA (1 μg VSV-G and 1 μg lacZ) was resuspended in 148 μl condensation buffer and vortexed, 16 μl of enhancer reagent was added, mixed and incubated at room temperature for 5 minutes. Following incubation, 50 μl of Effectene reagent was added to the solution, mixed and after 10 minutes’ incubation at room temperature, 1 ml of DMEM/F12+ was added. The solution was then mixed and the suspension added drop-wise to 293GP cells. Transfected cells were cultured for 3 days in DMEM/F12+ and viral supernatants collected after 3 days and frozen at −80°C.

Chondroprogenitor cell isolation and infection

Surface and deep zone chondrocytes were isolated from 7-day-old bovine articular cartilage using sequential pronase/collagenase digestion and 5×10⁶ cells subjected to differential adhesion to FN (10 μg ml⁻¹ in PBS+) in 60 mm dishes for 20 minutes. Non-adherent cells were aspirated and chondrocytes were cultured for up to 5 days prior to infection. Chondrocytes were infected with pseudotyped retrovirus-conditioned media (5×10⁶ CFU ml⁻¹) containing 10 μg ml⁻¹ polybrene for 24 hours prior to infection. Media was removed and cells washed in DMEM containing no additives, trypsinised, centrifuged and resuspended at 5×10⁶ cells 10 μl⁻¹.

In ovo injections and tissue processing

After harvesting, 10 μl aliquots of cell suspension containing 1×10⁶ cells (both surface and deep zone derived) were immediately injected into the proximal or distal wing bud of 3-day-old (Stage 12-14) (Hamburger and Hamilton, 1951) chick embryos which had been previously windowed. Eggs were sealed with adhesive tape and re-incubated for various times up to day 10 (Stage 36-37). Embryos were killed by cervical dislocation, a note of their developmental stage taken and embryos washed in 0.1 M PBS (pH 7.4). After washing, embryos were fixed in 2.5% paraformaldehyde in 0.1 M PBS (pH 7.4) for 1 hour at room temperature followed by 3×20 minute washes in 0.1 M PBS containing 2 mM MgCl₂, 0.01% deoxycholic acid and 0.02% igpal ([Octylphenoxypy]polyoxyethanol, pH 7.4). Embryos were then reacted at 37°C overnight for lacZ in 0.1 M PBS containing 2 mM MgCl₂, 0.01% deoxycholic acid, 0.02% igpal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM spermidine trihydrochloride and 1 μg ml⁻¹ X-gal previously solubilised in DMSO. Embryos were washed extensively in 0.1 M PBS, post-fixed in 10% NBBS overnight and wax embedded. Serial wax sections (8-10 μm) were taken onto 3-aminopropyl triethoxy silane-coated slides air-dried overnight, dewaxed, stained with 1% eosin for 15 seconds and examined under bright field microscopy after coverslipping. In separate experiments, immunocytochemistry using antibody to bacterial gene product was performed as a control against endogenous β-galactosidase activity and also to co-localise bovine-specific type I collagen within engrafted tissues.

Results

Initially, we examined integrin and fibronectin expression in 7-day-old bovine articular cartilage using immunocytochemistry and flow cytometry (Fig. 1). Both α5 and β1 integrin subunits were expressed in the majority of chondrocytes at the surface.
of the tissue with decreasing label intensity and decreasing numbers of chondrocytes labelled in the deeper zones of the cartilage (Fig. 1A,B). Using chondrocytes immediately after isolation and at various times after differential adhesion, surface, middle and deep zone chondrocytes were shown to express α5 and β1 subunits at all time points analysed regardless of substrate although differences in labelling intensity and the number of labelled cells were noted (Fig. 1C-F and data not shown). FN-EDA was localised pericellularly within the surface 2-3 cell layers of the articular cartilage (Fig. 1G). Using flow cytometry immediately after isolation and 4 hours after differential adhesion (Fig. 1H), β1 subunits were shown to be preferentially expressed by surface zone chondrocytes (88±4.8) compared with middle (67±2.1) and deep (62±3.7) zone chondrocytes. Substantially more cells in the surface zone expressed α5 subunits (79±4.8) compared with middle (52±2.1) and deep (2.5±1.7) zone chondrocytes.

Next, we assessed the degree of chondrocyte adhesion to fibronectin (Table 1). Surface and middle zone chondrocytes were more adherent to fibronectin than the other cohorts examined at 20 minutes. Initial adhesion ranged between 3.5% and 14.5% of the original cell number. Significant differences in adhesion were evident between surface zone chondrocytes plated on fibronectin for 20 minutes and those plated on fibronectin for 40 minutes (P<0.001) and cells cultured on BSA-coated dishes for 20 minutes (P<0.001). Middle zone chondrocytes were significantly more adhesive at 20 minutes than at 40 minutes when plated on fibronectin-coated dishes (P<0.001). In addition, middle zone chondrocytes were more adhesive to fibronectin at both time points compared with BSA-coated dishes (P<0.001). No differences in adhesion were observed between deep zone chondrocytes regardless of substrate or time point (P>0.05 in all cases).

If these cells with a high affinity for fibronectin are a population of chondroprogenitor cells then they should have the ability to form large numbers of colonies from an initially low seeding density, as is the case in other tissues with a clearly defined stem cell population (Jones and Watt 1993). To determine the clonality of the adhesive chondrocytes, we counted the number and size of colonies of chondrocytes subjected to differential adhesion to fibronectin (Fig. 2A). Differences in the initial adhesion of surface zone cells were reflected in CFE at 6 and 10 days that was not matched by the CFE of middle zone cells (Fig. 2B). The CFE of surface zone chondrocytes initially cultured on fibronectin for 20 minutes was greater than that of all other samples (P<0.01 at 6 days and P<0.001 at 10 days) when we applied the definition of a colony as being more than 32 cells. Indeed, using the criteria of 32 cells as indicative of a colony, no colonies were present in any other cohort besides surface zone cells initially plated on fibronectin. Using 4 cells as being indicative of a colony for comparative purposes, the same trend is apparent with surface zone cells subjected to differential adhesion to fibronectin for 20 minutes having a significantly enhanced CFE at both 6 and 10 days relative to all other cohorts (Fig. 2C-D). In addition, the average number of cells per colony was greater in surface zone cells initially grown on fibronectin for 20 minutes at both 6 (Fig. 2E) (P<0.05) and 10 (Fig. 2F) (P<0.01) days compared with all other samples.

These results suggest that a subpopulation of cells within the surface zone have the properties of a progenitor cell. Other studies in our laboratory using BALBc mice had identified Notch family members within the surface zone of developing articular cartilage and that Delta was widely distributed through the remainder of the tissue (Hayes et al., 2003). These studies suggested that Notch 1 was a suitable marker for the chondroprogenitor population and immunolabelling of bovine cartilage with a panel of antibodies to Notch family members revealed the presence of Notch 1 in the surface 2-3 cell layers of 7-day bovine articular cartilage (Fig. 3A), although not all cells within this layer were labelled. Another cohort of Notch 1-positive cells was also observed in the mid and deep zone of the tissue. Using flow cytometry, we showed that 86% of the surface zone cells isolated by pronase/collagenase digestion were Notch 1-positive compared with 10% and 34% from the middle and deep zone, respectively (Fig. 3B). Using Dynal Bead capture of Notch 1-positive surface zone chondrocytes, we showed that a Notch 1-enriched population of chondrocytes had both an increased adhesion to fibronectin (P<0.01) (Fig. 3C) and an increased CFE (P<0.05) (Fig. 3D) relative to unselected cells. These data suggest that the Delta/Notch signalling pathway may have a major influence in controlling both chondrocyte colony forming efficiency and differentiation.

To determine the role of Notch signalling in the colony forming ability of surface zone chondrocytes we cultured surface zone cells in the presence of a γ-secretase inhibitor, DAPT (Dovey et al., 2001), which is known to bind to the active site of presenilin in the γ-secretase complex and not interfere with β-catenin-mediated signalling (Kornilova et al., 2003). The γ-secretases are responsible for the cleavage of the amyloid precursor protein during the progression of Alzheimer's disease and are also responsible for the intramembranous cleavage of Notch receptors (Berezovska et al., 2000). Because of their role in Alzheimer's disease progression, much interest has focused on the development of γ-secretase inhibitors (Dovey et al., 2001), which not only prevent the accumulation of amyloid plaques but also prevent Notch family signalling (Berezovska et al., 2001). Treatment
with DAPT did not affect the initial adhesion of chondrocytes to fibronectin (Fig. 4A), but abolished clonality at both 6 and 10 days compared with controls when the 32 cell definition was applied (Fig. 4B) such that CFE was equal to that of deep zone chondrocytes. NICD was able to rescue colony abolition when added to DAPT-treated cultures after 3 days (P<0.05) (Fig. 4C) but NICD transfection did not increase colony forming efficiency compared with controls (P>0.05) (Fig. 4C). Culture of cartilage explants in the presence of 50 nM DAPT for 7 days produced a region of hypocellular, weakly stained matrix immediately beneath the surface zone (Fig. 4D,E). The region 101-200 μm from the articular surface contained fewer cells in DAPT-treated samples compared with controls (P<0.05) (Fig. 4D-F), whereas there was no difference in cell number 0-100 μm from the articular surface (P>0.05) (Fig. 4D-F). In addition, it was shown that incubation of explants in 50 nM DAPT prevented cell proliferation as there was no evidence of BrdU incorporation in any of the treated samples examined (n=24) (Fig. 4G,H).

In order to assess the differentiation potential of the progenitor population, we infected a lineage label into the cells and injected them into the proximal limb of stage 22 chick embryos and tracked them for 1 week in ovo. Twenty-four hours after injection, β-galactosidase-positive cells were present in positions corresponding to the original injection site (Fig. 5A,B). Examination of embryos injected with labelled deep zone cells gave variable results. Labelled cells were either absent suggesting that the cells could not survive in the chick
Fig. 3. Seven-day bovine articular cartilage labelled with antibody to Notch 1 (A) and counterstained with propidium iodide. Chondrocytes within the uppermost 2-3 cell layers of the surface zone (arrows) label strongly for Notch 1. Chondrocytes were labelled with anti-notch 1 antibody and subjected to single-channel FACS analysis immediately after isolation (B). 86% of surface zone cells label positively for N1 compared with 10% and 34% from middle and deep zone, respectively. (*p<0.001 compared with middle and deep.) Chondrocytes were selected immunomagnetically and subjected to differential adhesion and initial adhesion and CFE assessed. Notch 1-selected surface zone cells (SFN N1) were more adherent than N1-selected middle (MFN N1) and deep zone (DFN N1) cells and unselected cells (SFN 20, MFN 20, DFN 20) (C). In addition, the CFE of surface zone cells selected for N1 was greater than notch-positive middle and deep zone cells and unselected cells (D). ***p<0.001 compared with middle and deep, **p<0.01 compared with N1 selected and unselected middle and deep cells, ***p<0.01 compared with unselected surface zone cells, ****p<0.01 compared with selected and unselected middle and deep zone cells. Abbreviations as in Table 1.

embryos, or if cells were present they were seen as masses of labelled cells in loose connective tissue not integrated into surrounding host tissue (Fig. 5K).

Examination of embryos incubated to 10 days (Stage 36) revealed β-galactosidase-positive cells in numerous tissue types, including cartilage, bone, tendon and muscle connective tissues (Fig. 5C-F). The sites of these positive cells corresponded with the sites of injection such that proximal injections gave β-galactosidase-positive cells in proximal tissues and distal injections revealed β-galactosidase-positive cells in distal structures. Furthermore, if cells were injected into the central proximal region of the limb bud, cells engrafted into the humerus. More lateral injections engrafted into tendons and perimysium. In order to test for functional engraftment, we used an antibody specific for bovine type I collagen. We found that in tendon, parallel arrays of fibrillar collagen ran along the tendon length (Fig. 5H) contrasting with dense immunofluorescence in the subperiosteal bone (Fig. 5J). In addition, both the perichondrium and articular fibrocartilage (Fig. 5G) and the perimysium (Fig. 5H) labelled with antiboine type I collagen antibody.

Discussion

Using differential adhesion to serum fibronectin, we have described the isolation and partial characterisation of a subpopulation of articular cartilage chondrocytes, with properties akin to those of a progenitor cell and that are able to engraft into a variety of tissue types, albeit of the connective tissue lineage. These cells reside within the surface zone of articular cartilage, where the EDA isofrom of fibronectin is differentially expressed and the cells have an extended cell cycle time (Hayes et al., 2001). This sub-population of surface zone cells has a high affinity for serum fibronectin but not other ligands, e.g. collagen types I, II and IV, laminin and tenascin (J.C.B., G.P.D. and C.W.A., unpublished results), and were capable of forming large numbers of colonies from an initially low seeding density, unlike cells isolated from the middle zone which also have high fibronectin affinity. The initial adhesion of surface zone chondrocytes to fibronectin can be explained by their high expression of α5β1 integrin subunits, the 'classical' fibronectin receptor (Hyne, 1992). This high level of α5β1 expression and affinity for fibronectin does not, however, provide a marker of the cells' colony forming ability. Middle zone chondrocytes exhibit higher affinity for fibronectin than surface zone cells (~15% middle compared with ~10% surface) (Table 1), but lack the ability to form colonies (Fig. 2) and may represent a transit amplifying population. In addition, the percentage of cells that possess a high colony forming efficiency within the surface zone (approximately 1-2% of the initial number adhered) is only a fraction of the number of cells expressing α5β1 subunits (approximately 75%), for this reason we could not use α5β1 integrin expression as a chondroprogenitor marker.

Previous studies have documented the expression of Notch family members during articular cartilage and growth plate development (Hayes et al., 2003; Crowe et al., 1999). Of particular interest was the specific expression of Notch 1 at the developing articular surface of mouse knee joints (Hayes et al., 2003). This specific expression in the surface zone of articular cartilage suggested that Notch 1 may provide a marker for colony forming cells in the bovine model used in the present
Fig. 4. Treatment with DAPT did not affect the adhesion of surface and deep zone chondrocytes to fibronectin (A) but abolished the CFE of surface zone cells at 6 and 10 (B) days. Indeed, the CFE of DAPT-incubated cells was not different from that of deep zone cells at either time point. Transfection with NICD rescued this abolition of CFE (C). NICD transfection did not increase CFE in cells not treated with DAPT (P>0.05). Cartilage explants were removed from 7-day bovine articular cartilage and cultured in the presence (D) or absence (E) of 50 nM DAPT for 7 days as described in Materials and Methods. Note that in the presence of DAPT, an acellular weakly stained band is present beneath the surface zone (arrows). These images represent a selection from 3 separate experiments each containing 6 explants per treatment. Note that the image in D is the other half of the explant from that shown in E. Using a graduated grid, the number of cells 0-100 and 101-200 μm from the articular surface was counted and the region 101-200 μm from the articular surface was shown to contain fewer cells in treated samples relative to controls (F). Explants were treated with DAPT for 7 days with the addition of BrdU on days 4, 5 and 6. Localisation of BrdU in controls (G) reveals cell proliferation, whereas there was no BrdU localisation in DAPT-treated samples (H). ns, P>0.05 compared with surf control; *P<0.01 compared with DAPT treated; **P<0.05 compared with all other cohorts, DMSO; 0.1%, dimethyl sulfoxide, Mock; no plasmid, PBS; fibronectin only.

study. Indeed, Notch 1 expression in immature bovine articular cartilage matches that in developing mouse articular cartilage, such that in both species, Notch 1 is present in the chondrocytes of the surface zone articular cartilage to a depth of 2-3 cells (see Fig. 3) (Hayes et al., 2003). Flow cytometry of freshly isolated chondrocytes revealed that Notch 1 expression was significantly increased in surface zone chondrocytes and these high levels of Notch 1 expression were maintained in surface zone cells after differential adhesion, relative to middle and deep zone chondrocytes. Using magnetic immunoselection, Notch 1-
positive cells were isolated from bovine articular cartilage and subjected to differential adhesion. Surface zone cells thus isolated had a higher affinity for fibronectin than N1-selected middle and deep zone cells and unselected cells (Fig. 3). These

NI-selected surface zone cells also had an increased colony forming efficiency compared with unselected cells. These results suggest that Notch 1 plays a significant role in the signalling mechanisms controlling the clonality of surface zone chondrocytes, although given that approximately 75% of surface zone cells express Notch 1 and only 1-2% of these selected cells form colonies, Notch 1 expression per se is not a specific marker of progenitor chondrocytes. The precise role of Notch in the promotion of clonality or maintenance of progenitor status remains unclear, although our own studies have shown the expression of several Notch ligands (Jagged and Delta) in articular cartilage, although their expression is not specific to the articular surface (Hayes et al., 2003). Notch 1 signalling may play one of two roles in the surface zone of articular cartilage; it may function to maintain cells in a proliferative state, i.e. maintain clonality, or it may promote chondrocyte differentiation and hence cartilage growth. In skin, activation of Notch by Delta promotes terminal differentiation, i.e. prevents proliferation (Lowell et al., 2000); however the high CFE of Notch 1-selected chondrocytes and the reduction in CFE by Notch signal inhibition would suggest that Notch 1 signalling within articular cartilage maintains clonality and proliferation. These inhibitory effects are negated by activated Notch but activated Notch does not increase colony forming ability, suggesting that clonality is dependent upon rate limiting factors downstream of Notch signalling.

Cartilage explants cultured with DAPT contain a hypocellular zone beneath the articular surface and BrdU immunolabelling highlights the lack of proliferation in DAPT-treated samples. These results would indicate that Notch inhibition via DAPT inactivation of presenilin prevents chondroprogenitor proliferation, thus depleting the number of daughter cells capable of differentiating and contributing to articular cartilage growth.

At present, we cannot state which member of the Notch family controls chondrocyte proliferation/differentiation, but the results of the immunolabelling for Notch 1 in both bovine and mouse (Hayes et al., 2003) and the enhanced clonality of Notch 1-selected chondrocytes suggests that this family member is central to cartilage growth and differentiation.

The engraftment of bovine surface zone-derived cells and their tissue-specific matrix synthesis in ovo highlights the plasticity of this cell population. This plasticity further supports our argument that these cells represent a progenitor population as plasticity is a key marker of a stem cell population (Morrison et al., 1997).

We conclude that immature articular cartilage contains a
population of progenitor cells (which as yet has no definitive marker) that is responsible for the appositional growth of the tissue and that this population of cells exhibits a significant degree of plasticity in its differentiation pathway. The existence of a progenitor population within the surface zone of articular cartilage opens up the possibility of using this population to engineer cartilage in vitro. Because these cells are undifferentiated, they should have the capability to reproduce the structural and hence biomechanical properties of normal articular cartilage and thus integrate more fully into articular cartilage lesions.

The authors thank Isabelle Le Roux (ICRF) and R. Kopan (University of Washington) for provision of materials used in this study. This work was funded by Arthritis and Rheumatism Campaign UK, EPSRC and BBSRC.

References


SURFACE ZONE ARTICULAR CHONDROCYTES INHIBIT TRAUMA INDUCED CELL DEATH AND MATRIX LOSS IN WOUNDED CARTILAGE

*Bishop, J (A-BBSCC); **Archer, C W; **Thompson, B (A-Smith and Nephew); *Dowthwaite, G (A-Arthritis and Rheumatism Campaign)
*School of Biosciences, Cardiff University, Cardiff, Wales, UK. +44(0)29 29875206, Fax: +44(0)29 29874594, archer@cardiff.ac.uk

Introduction: Previous studies have shown that cartilage repair grows by apposition from the articular surface that is driven by proliferation of surface zone cells (1) and that these cells have an extended cell cycle time (2), both suggestive of the presence of a population of chondroprogenitor cells resident within the surface zone of articular cartilage. In recent years, autologous chondrocyte transplantation has been increasingly used to repair articular cartilage defects (3). These procedures inevitably lead to the formation of a fibrocartilaginous plug of repair tissue within the wound that lacks the distinct structural architecture of articular cartilage proper. This lack of a defined structure eventually leads to repair failure due to the altered biomechanical properties of the repair tissue compared with articular cartilage itself. The reasons for both fibrocartilaginous plug formation and its eventual failure are unclear but they may include the poor intrinsic reparative ability of cartilage itself, the fact that full depth cartilage is used which contains chondrocytes at different stages in the differentiation pathway and a lack of integration at the wound margin; the so-called zone of necrosis.

We have recently devised a method of isolating articular cartilage progenitor cells from surface zone articular cartilage using differential adhesion to fibronectin. The cells thus isolated have a high colony forming efficiency from an initially low seeding density. Our aim was to use progenitor chondrocytes isolated by differential adhesion in a pellet culture system and characterise the pellets derived from surface middle and deep zone articular cartilage. Additionally, pellets from surface, middle and deep zone cartilage were used in an in vitro model of articular cartilage wounding to assess their effects on wound responses.

Materials and Methods: Chondrocyte isolation: Surface (SZ), middle (MZ) and deep (DZ) zone chondrocytes were isolated from the metacarpal phalangeal joints of 7-day old calves by careful dissection. Cartilage shavings were digested in 3.17 IU ml⁻¹ pronase in DMEM/F12 (1:1) containing 5% FCS at 37°C for 3 hours followed by 0.12 IU ml⁻¹ collagenase in DMEM/F12 + 5% FCS at 37°C for 16 hours. Chondrocytes were centrifuged, washed in serum free DMEM/F12, counted and plated out as required. Differential adhesion and pellet culture: 60mm dishes were coated with 10μg ml⁻¹ fibronectin in PBS containing 1mM CaCl₂ and 1mM MgCl₂ (PBS+) overnight at 4°C. Fibronectin was removed and dishes washed with 1% BSA/PBS+ and SZ, MZ and DZ chondrocytes plated onto the dishes at 1 x 10⁵ cells ml⁻¹ in serum free DMEM/F12 and allowed to adhere to the dish for 20 minutes at 37°C. Media and nonadherent cells were removed and the remaining cells cultured for 4 days in DMEM/F12 + 10% FCS. After 4 days, chondrocytes were trypsinised, pooled into SZ, MZ and DZ fractions and counted. Cells were resuspended in DMEM/F12 +10% FCS containing 1% HEPES buffer at 2 x 10⁶ cells ml⁻¹ and 500μl aliquots placed in 1.5 ml microtubes. Tubes were centrifuged at 2000 rpm for 5 minutes to pellet the cells and the pellets maintained for up to 10 days with media changes every other day. Pellets were either used in transplantation experiments at 3 days or fixed in 10% NBFS and processed for routine histology and immunolabelling using monoclonal antibodies to chondroitin 4 and 6 sulphate, dermatan sulphate, link protein and type II collagen. Cell death was assessed by incubating explants with 2μM ethidium homodimer for 90 minutes prior to fixation and wax embedding.

Results: Pellet culture: Little evidence of matrix staining with Safranin O was apparent in pellets derived from SZ, MZ and DZ after 3 and 7 days in culture and the chondrocytes were tightly packed. By 10 days, distinct differences in pellet morphology were apparent between the three zones. SZ pellets were highly cellular and stained weakly with Safranin O. MZ pellets contained fewer cells but were more intensely stained with Safranin O whilst DZ pellets contained large cells in an intensely Safranin O stained matrix. Immunolabelling revealed the presence of type4, 6, 10 and link protein at all time points and from all cartilage zones whilst type II collagen was only present at 10 days in pellets derived from all zones. Wounding and pellet transplantation: Wounded explants (both drill and microscapel) containing no pellet exhibited loss of Safranin O staining around the full depth of the wound margin to a depth of 10 cell diameters and this loss of stain also extended along the unwounded surface of the cartilage. The application of a pellet to the wounds reduced the extent of matrix loss (as shown by Safranin O) to varying degrees depending on the source of the pellet. Little if any loss of staining was apparent in explants containing a SZ derived pellet, whilst the loss of stain in the explants containing MZ and DZ derived pellets was reduced to 5-6 cell diameters compared with the control wounded explants. Cell death analysis using ethidium homodimer revealed extensive cell death around the wound margin and along the articular surface of control explants containing no pellet to a depth of 10 – 12 cell diameters. Label for cell death was present to a depth of 5-6 cell diameters the wound margin and along the articular surface of explants containing DZ and MZ derived pellets. In comparison, few if any cells were labelled with ethidium homodimer in explants containing SZ derived pellets.

Discussion: Cells derived from various regions of articular cartilage and subjected to differential adhesion to fibronectin and cultured as pellets show distinct morphological differences over 10 days in culture suggesting that these subpopulations of cells maintain their “positional phenotype” in vitro. Additionally, transplantation of undifferentiated (3 day) pellets into either a scalpel or drill wound abrogates cell death and matrix loss associated with such wounding. The level of abrogation of cell death and matrix loss depends on the origin of the cells such that wounds containing a SZ pellet exhibit little if any cell death and matrix loss whilst MZ and DZ pellets reduce cell death and matrix loss relative to controls, although not abrogating these factors completely. The prevention of cell death and matrix loss around cartilage wounds will prove to be important in the full integration of cartilage repair tissue after surgical intervention and the use of autologous cells separated by differential adhesion may aid in full recovery.

References

**Smith and Nephew, York., UK.

48th Annual Meeting of the Orthopaedic Research Society
Poster No: 0418
Introduction: There are two major problems which affect current strategies in cartilage repair. One problem is tissue integration between host and reparative tissue. The second problem is the generation of a repair tissue with the structural characteristics of articular cartilage. Using the marsupial *Monodelphis domestica* as a model system, it has been shown that articular cartilage grows by apposition from the articular surface towards the subchondral bone and that this growth is driven by the proliferation of surface zone cells (1, 2). Additionally, a population of cells with an increased cell cycle time was identified within the surface zone; a property typical of many progenitor cell populations (2). The aim of our research is to identify and characterise a chondroprogenitor population from articular cartilage to enable the rapid culture of undifferentiated chondrocytes in vitro for future clinical use. Here we describe the isolation and partial characterisation of a cell population from the articular surface which exhibits differential adhesion to fibronectin, differential integrin expression and the ability to form large numbers of colonies from an initially small seeding density; properties that are common to known progenitor cell populations of other tissues. Additionally we report on the presence of the cell surface signalling molecule Notch 1 (N1) in a subpopulation of surface zone chondrocytes and that this N1-expressing subpopulation has an enhanced ability to form large numbers of colonies from an initially low seeding density.

Materials and Methods: Tissue culture and differential adhesion assay: Cartilage slices were isolated from the surface (SZ), middle (MZ) and deep (DZ) zones of 7 day old bovine metacarpal-phalangeal joints by fine dissection. Slices were then incubated in pronase (0.1% in DMEM/35%FCS) for 3 hours at 37°C followed by collagenase (0.04% in DMEM/5%FCS) for 16 hours at 37°C. Chondrocytes were counted and seeded onto fibronectin (10μg/ml)-coated or PBS/1% BSA-coated 35 mm dishes at 4,000 cells/ml in serum free DMEM (DMEM-). For 20 minutes. After 20 minutes, media and non-adherent cells were removed and placed in similarly treated dishes for a further 40 minutes before this media and nonadherent cells were placed in a third dish. After removal of media at 20 and 40 minutes, fresh DMEM- was added to the remaining cells which were cultured for up to 10 days. In all experiments 6 fibronectin and 6 uncoated dishes were used for each zone of cartilage. Fibronectin was used as a ligand in the experiments since it is known to be differentially expressed at the articular surface during mammalian development (3). Within three hours of plating, chondrocyte adhesion was assayed by counting the total number of cells per dish using phase contrast microscopy and expressed as a percentage of the initial seeding density. Additionally, colonies of chondrocytes consisting of 32 or more cells were counted at 0, 3, 6, 9 and 10 days after differential adhesion (n = 6 experiments). Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. In some experiments (n = 3) the number of cells per colony was counted to determine the average number of cells per colony. Results were analysed using the Students t test. Flow cytometry: Four hours after differential adhesion, chondrocytes were removed from dishes non-enzymatically and 2 x 10^6 cells were incubated for 3 hours with antibodies to α5 (AB1928) and β1 (MAB1951) integrin subunits and anti-N1 (SC 6014) at room temperature. Cells were centrifuged at 3,000 rpm, supernatants removed and cells washed three times in PBS with centrifugation between each wash. Cells were then incubated with relevant FITC conjugated secondary antibodies for 1 hour at room temperature and washed three times in PBS and subjected to flow cytometry. Notch 1 Immunolabelling and Immunomagnetic Isolation: Frozen sections of 7 day bovine full depth articular cartilage were immunolabelled with anti-N1 antibody and localised with the appropriate secondary FITC conjugated secondary antibody. Chondrocytes were isolated by sequential pronase/collagenase digestion from surface middle and deep zone articular cartilage and incubated with M450 toyol-activated Dynal beads conjugated to goat anti-human N1 antibody for 4 hours at 4°C. N1 selected cells were counted and 4,000 cells/ml were subjected to differential adhesion to fibronectin for 20 minutes. Initial adhesion and CFE were assessed as described above. In all experiments, results were analysed using Students t test. Results: Initial adhesion ranged between 3.5% and 14.5% of the original cell number. Significant differences in adhesion were evident between surface zone chondrocytes seeded on fibronectin for 20 minutes (9.05% +/- 0.44) and those seeded on PBS coated dishes for 20 minutes (3.83% +/- 0.27; p < 0.001) and also with those seeded on fibronectin for 40 minutes (4.89% +/- 0.43; p < 0.001). Middle zone chondrocytes were significantly more adherent at 20 minutes (14.53% +/- 0.86) than at 40 minutes (10.58% +/- 0.51) when seeded onto fibronectin-coated dishes (p < 0.01). Additionally, middle zone chondrocytes were more adhesive to fibronectin at both time points compared with PBS-coated dishes (p < 0.001). No differences in adhesion were observed between deep zone chondrocytes regardless of substrate or time point (p > 0.05 in all cases).

At days 0 and 3, no colonies containing 32 or more cells were present in any sample. At 6 and 10 days, the CFE of surface zone chondrocytes initially cultured on fibronectin for 20 minutes was greater than that of the other samples (p < 0.01 at 6 days, p < 0.001 at 10 days). In addition, the CFE of surface zone cells initially cultured for 20 minutes on fibronectin was greater at 10 days compared with that at 6 days (p < 0.05). No change in CFE was evident between 6 and 10 days for any other sample (p > 0.05 in all cases). The average number of cells per colony was greater in surface zone cells, initially grown on fibronectin for 20 minutes at both 6 (p < 0.05) and 10 (p < 0.01) days compared with all other samples. FACS analysis showed elevated levels of both α5 and β1 integrin subunits in surface zone cells compared with middle and deep zone cells (p > 0.05).

N1 immunolabelling revealed occasional N1 positive cells within uppermost 2-3 cell layers of the articular cartilage. When surface zone cells were isolated and analysed for N1 using FACS over 84% of the surface zone population were N1 positive and this result was reflected in the cell counts obtained after N1 selection. Adhesion assays performed using N1 selected chondrocytes revealed that the N1 positive cells were more adherent than either negative cells or unselected cells (p > 0.05) and that the CFE of N1 selected cells was increased 4 fold relative to negative cells and unselected cells (p > 0.001).

Discussion: The ability of a population of cells to form large numbers of chondrocyte colonies from a low seeding density, differences in α5β1 integrin subunit expression and differential N1 expression when taken together with previous results demonstrating the prolonged cell cycle time at the articular surface (2), strongly suggest that a subpopulation of progenitor chondrocytes resides in the articular surface. Additionally, the prolonged adhesiveness of mid zone cells, their restricted ability to form large numbers of colonies and their relatively short cell cycle (2) strongly indicates the presence of transit amplifying cells within this zone. Furthermore, the use of N1 selection increases the CFE of surface zone cells seeded on fibronectin fourfold relative to unselected cells suggesting that N1 will be a useful marker in the further purification of chondroprogenitor cells. The eventual isolation and purification of such a progenitor population will prove to be vital in advancing strategies for cartilage repair.

This work was funded by The Arthritis and Rheumatism Campaign, UK.

**Smith and Nephew, York, England.**
Articular Chondroprogenitor Cells Exhibit Plasticity in their Differentiation Pathway
Archer, CW* (Archer@cardiff.ac.uk), Bishop JC*, Redman SN*, Thomson B* and Dowthwaite GP*.
+Cardiff School of Biosciences, Cardiff University, Museum Avenue, PO Box 911, Cardiff CF10 3US, Wales
+44(0)29 20875206.
#Smith and Nephew GRC, York Science Park, Haslington, York, YO1 5DF, UK

Introduction: There are two major problems afflicting current cartilage repair strategies. One problem is tissue integration between host and reparative tissue. The second problem is the generation of a repair tissue with the structural characteristics of articular cartilage. Previous studies have shown that articular cartilage grows by apposition from the articular surface towards the subchondral bone and that this growth is driven by the proliferation of surface zone cells (1, 2). Additionally, a population of cells with an increased cell cycle time was identified within the surface zone, a property typical of many stem cell populations (2). Here, we describe the isolation and partial characterisation of a cell population from the articular surface that has many properties common to known stem cells of other tissues.

Materials and Methods: Tissue culture and differential adhesion assay: Cartilage slices were isolated from the surface (SZ), middle (MZ) and deep (DZ) zones of 7 day old bovine metacarpal-phalangeal joints by fine dissection and incubated in pronase (0.1% in DMEM/5%FCS) for 3 hours at 37°C followed by collagenase (0.04% in DMEM/5%FCS) for 16 hours at 37°C. Chondrocytes were counted and seeded onto fibronectin (10μg ml⁻¹)-coated or PBS/1% BSA-coated 35 mm dishes at 4,000 cells ml⁻¹ in serum free DMEM (DMEM-) for 20 minutes. After 20 minutes, media and non-adherent cells were removed and placed in similarly treated dishes for a further 40 minutes before this media and nonadherent cells were placed in a third dish. After removal of media at 20 and 40 minutes, fresh DMEM- was added to the remaining cells, which were cultured for up to 10 days. In all experiments 6 fibronectin and 6 uncoated dishes were used for each zone of cartilage. Fibronectin was used as a ligand in the experiments since it is known to be differentially expressed in cartilage during mammalian development (3). Within three hours of plating, chondrocyte adhesion was assayed by counting the total number of cells per dish using phase contrast microscopy and expressed as a percentage of the initial seeding density. Additionally, colonies of chondrocytes consisting of more than 32 cells were counted at 0, 3, 6 and 10 days after differential adhesion (n = 6 experiments). Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. In some experiments (n = 3) the number of cells per colony were counted to determine the average number of cells per colony. Results were analysed using the Students t-test.

Chondroprogenitor plasticity: Colony forming cells were isolated as described above. Twenty four hours after differential adhesion, chondrocytes were transfected with pseudotyped retrovirus encoding lac z for 24 hours. Transfected cells were incubated for up to 5 days before being lifted from the dishes, resuspended at 1 x 10⁵ cells 10μl⁻¹ in additive free DMEM- and injected into limb buds of stage 23 chick embryos. Embryos were incubated for up to 7 days, sacrificed and the limbs reacted histochemically for β-galactosidase activity prior to wax embedding and serial sectioning Controls comprised transfected deep zone cells isolated in tandem with the surface zone cells and injected into embryos on the same day.

Results: Colony forming efficiency: At days 0 and 3, no colonies containing more than 32 cells were present in any sample. At 6 and 10 days, the CFE of surface zone chondrocytes initially cultured on fibronectin for 20 minutes was greater than that of the other samples (p < 0.01 at 6 days, p < 0.001 at 10 days). In addition, the CFE of surface zone cells initially cultured for 20 minutes on fibronectin was greater at 10 days compared with that at 6 days (p < 0.05). No change in CFE was evident between 6 and 10 days for any other sample (p > 0.05 in all cases). Additionally, the average number of cells per colony was greater in surface zone cells initially grown on fibronectin for 20 minutes at both 6 (p < 0.05) and 10 (p < 0.01) days compared with all other samples.

Chondroprogenitor plasticity: Examination of serial sections of stage 36 chick embryos reacted for β-galactosidase activity revealed labelled surface zone cells in a variety of tissues including: cartilage, muscle, tendon and bone. Sections from embryos injected with deep zone cells revealed either no labelled cells or very few cells incorporated into loose connective tissue.

Conclusion: The ability of a population of cells to form large numbers of chondrocyte colonies from a low seeding density taken together with previous results demonstrating the prolonged cell cycle time at the articular surface (2) strongly suggest that a subpopulation of progenitor chondrocytes resides in the articular surface. This conclusion is strengthened by the fact that bovine surface zone cells subjected to differential adhesion exhibit plasticity in their differentiation pathway and can engraft into various avian tissues.

This work was funded by The Arthritis and Rheumatism Campaign, UK and Smith + Nephew plc.
Introduction: Articular cartilage (AC) has an inherently poor reparative capacity. Recently, the use of autologous chondrocytes has gained popularity in the repair of AC defects. These strategies often do not lead to full integration with host tissue at the wound margin (zone of necrosis); the repair tissue invariably is fibrocartilaginous and lacks the distinct structural architecture of AC proper. Repair failure ensues due to the altered biochemical properties of the fibrocartilaginous plug as full depth cartilage containing both differentiated and undifferentiated chondrocytes are transplanted. It is known that AC grows by apposition from the articular surface and that AC contains slowly cycling cells suggesting the existence of a chondroprogenitor cell population. We have recently devised a method of isolating AC progenitor cells from the surface zone (SZ) using differential adhesion to fibronectin. Our aim was to use a 3-D pellet culture system to characterize the isolated progenitor chondrocytes and compare with middle, deep zone AC. Additionally, pellets from surface, middle, deep zone and full depth cartilage were used in an in vitro model of AC wounding to assess their effects on wound responses.

Experimental Method: Chondrocyte isolation: SZ, MZ, DZ chondrocytes were isolated from the metacarpal phalangeal (MCP) joints of 7-day-old calves by careful dissection. Cartilage shavings were sequentially digested in pronase followed by collagenase. Chondrocytes were centrifuged, washed in DMEM/F12, counted and plated out as required. Differential adhesion and pellet culture: Sixty mm dishes were either coated with 10μg ml⁻¹ fibronectin in PBS containing 1mM CaCl₂ and 1mM MgCl₂ (PBS+) or PBS only overnight at 4°C. Fibronectin/PBS+ was removed and dishes were washed with 1% BSA/PBS+ and SZ, MZ and DZ chondrocytes plated onto the dishes at 1 x 10⁵ cells ml⁻¹ in DMEM/F12 (SZ) or DMEM/F12 +10% (DMEM+) (MZ, DZ & FD) and allowed to adhere to the dishes for 20 minutes at 37°C (SZ only). Media and non-adherent SZ cells were removed and the remaining cells (including MZ & DZ) cultured for 4 days in DMEM+. After 4 days, chondrocytes were trypsinised, pooled into SZ, MZ, and DZ fractions and counted. Cells were resuspended in DMEM+ containing 1% HEPES buffer at 2 x 10⁵ cells ml⁻¹ and 500μl aliquots placed in 1.5ml microtubes. Tubes were centrifuged at 2000 rpm x g for 5 minutes to pellet the cells and maintained for up to 10 days. Pellets were either used in transplantation experiments at 3 days or fixed in 10% MBFS and processed for routine histology and immunolabelling for various extracellular matrix proteins at days 3, 7 and 10. Wounding and pellet transplantation: Full depth AC explants were removed from MCP joints of 7-day-old calves and partial thickness wounds were made using partial thickness defect tool (as described Hunziker et al. 1996) 3 day cultured SZ, MZ and DZ pellets were placed in the defects and the explants maintained in DMEM+ for 7 days. Controls comprised wounded explants containing no pellet or pellets, which had been, freeze thawed. Incubating explants with 2μm ethidium homodimer (EIHHD) for 90 minutes prior to fixing and wax embedding assessed cell death.

Results: Pellet culture: Following 3-7 days in culture pellets derived from SZ, MZ and DZ contained tightly packed chondrocytes with little evidence of matrix staining. By 10 days, distinct differences in pellet morphology were apparent between the three zones. SZ pellets were highly cellular and stained weakly with safranin O (Safo). MZ pellets contained fewer cells but were more intensely stained with Safo whilst DZ pellets contained large cells in an intensely Safo stained matrix. Immunolabelling revealed the presence of type I, II, III, III, V, VI type collagen and type II collagen was only present at 10 days in pellets derived from all zones. These results suggest that these subpopulations of cells maintain their 'positional phenotype' in vitro. Wounding and pellet transplantation: Wounded explants containing no pellet exhibited loss of Safo staining around the full depth of the wound margin, which extended along the unwounded surface, which complemented cell death analysis using EIHHD. The application of a pellet to the wounds reduced the extent of matrix loss to varying degrees depending on the source of the pellet. Wounds containing a SZ pellet exhibited little if any cell death and matrix loss whilst MZ and DZ pellets reduced cell death and matrix loss relative to controls, although not abrogating these factors completely.

Conclusion: These experiments demonstrate that SZ, MZ and DZ chondrocytes maintain their positional phenotype in a pellet culture system. Furthermore, SZ pellets abrogate cell death and matrix loss in a cartilage wound model suggesting that SZ chondroprogenitor will prove useful in repair of AC during surgical intervention.