Factors Contributing to Chondroplasia in Degenerate Rotator Cuff Disease

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Trinity 2010

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Submission for DPhil examination Trinity 2010

Chondroplasia, the development of cartilage-like characteristics in tendinous tissue, is a form of degeneration found in tendons including those of the rotator cuff. The molecular mechanism of its development is currently unknown. An examination of the features of the torn rotator cuff and the cartilage literature led to the identification of several potential drivers of chondroplasia including cell shape change/actin cytoskeleton and hypoxia. Lovastatin caused actin cytoskeleton disruption and promoted cartilage matrix deposition in the ATDC5 model. It was the most effective member of a panel of cytoskeletal inhibitors, increasing expression of the chondrocytic markers Sox5 and Sox9 and decreasing expression of COL1A1 and COL3A1 in primary human tenocytes. Its effects were dose dependent, reversible by mevalonate addition and long term treatment induced de novo expression of collagen II. Short term hypoxia upregulated VEGF-A and chondrocytic marker gene DEC1 expression but not other chondrocyte markers. Combination treatment with hypoxia did not enhance the effects of lovastatin. These data suggest that modulation of pathways that regulate the actin cytoskeleton and cell shape may alter tenocyte phenotype.

Acknowledgements

I thank the Furlong Research Charitable Foundation for funding this research. I thank Professor Carr and Dr Hulley for believing in me and for all their guidance and support during my studies. I thank Mr Andrew Price and Mr Raj Rout for provision of hamstring tendon. I thank Karolina Kliskey for assistance with the immunohistochemistry. I thank my fellow PhD students and the post-docs at the Botnar Research Centre and at Wolfson College for their support, friendship and much needed-reality checks. I thank my church fellowship group, my friends from the Graduate Christian Union and my prayer group, particularly Emilie Noteboom (I promise the computer will be turned off just as soon as I finish this!). I thank my family, especially my parents. I thank my Mum for emotional support and TLC. I thank my Dad for technical support and for being a great sounding board. Lastly I thank I AM for never giving up on us and for providing such a wonderful puzzle to investigate.

"Great are the works of the Lord;"

Psalm 111:2a (New International Version)

"All who are delighted with them want to understand them."

Psalm 111:2b (Good News Translation)

Table of Contents

ABBREVIATIONS I

CHAPTER 1 - INTRODUCTION 1

1.1 Tendinopathy and Chondroplasia 1

1.1.1 Tendinopathy 2

1.1.2 Theories of Tendon Degeneration 4

1.1.3 Chondroplasia 7

1.2 Normal Tendon Biology 8

1.2.1 Tendon Composition 8

1.3 A Description of Tendon and Cartilage 12

1.3.1 Developmental Origins 13

1.3.2 The Phenotype of Normal Tensional Tendon 15

1.4 Normal Cartilage and Fibrocartilage Biology 22

1.4.1 Introduction to Fibrocartilage 22

1.4.2 Hyaline Cartilage 22

1.4.3 A More Detailed Consideration of Fibrocartilage 29

1.5 A Comparison of Extracellular Matrices in Connective Tissues 30

1.5.1 ECM Mechanical Properties 30

1.6 Matrix Effects of Tendinopathy 47

1.6.1 Collagens in Degenerate Tendons 47

1.6.2 SLRPs in Degenerate Tendons 47

1.6.3 Lecticans in Degenerate Tendons 48

1.6.4 Glycoproteins in Degenerate Tendons 49

1.7 Rotator Cuff Tears 50

1.7.1 Histological Features of Chondroplasia 51

1.8 Potential Drivers of Chondrogenesis 53

1.8.1 Compression 54

1.8.2 Cell Shape and the Actin Cytoskeleton 56

1.8.3 Hypoxia 62

1.8.4 Combination 70

1.9 Aims of this Thesis 70

CHAPTER 2 - MATERIALS AND METHODS 71

2.1 Cell Culture 71

2.1.1 ATDC5 Culture 71

2.1.2 Tenocyte Culture 72

2.1.3 Monolayer Coverslip Culture 74

2.2 Biochemical Assays 74

2.2.1 Dimethylmethylene Blue (DMB) Assay 74

2.2.2 Picogreen Assay 75

2.3 Immunostaining 75

2.3.1 Staining on Coverslips 75

2.3.2 Histological Analysis 76

2.3.3 Microscopy and Imaging 79

2.4 Western Blotting 80

2.4.1 Sample Preparation 80

2.4.2 Western Blot Procedure 81

2.5 Molecular Biology 84

2.5.1 RNA Extraction and Reverse Transcription 84

2.5.2 RT-PCR 84

2.5.3 QPCR 85

2.6 Method Development 87

2.6.1 Mycoplasma Infection - Detection and Consequences 87

2.6.2 Housekeeper Gene Selection for QPCR 90

CHAPTER 3 - EFFECTS OF LOVASTATIN TREATMENT ON THE ATDC5 MODEL ..94

3.1 Introduction 94

3.1.1 Three Dimensional Culture and Chondrogenesis 94

3.1.2 Statins as Pro-chondrogenic Agents 96

3.2 Results 98

3.2.1 Lovastatin Disrupts ATDC5 Actin Cytoskeleton Arrangement 98

3.2.2 Lovastatin Inhibits Prenylation in ATDC5 Pellets 98

3.2.3 Effect of Lovastatin on Pellet Volume 101

3.2.4 Lovastatin Decreases Pellet DNA Content 103

3.2.5 Lovastatin Decreases Cell Proliferation 104

3.2.6 Effect of Lovastatin on Apoptosis 106

3.2.7 Lovastatin Increases Sulphated Glycosaminoglycan Concentration 109

3.2.8 Lovastatin Increases Collagen II Production 110

3.3 Discussion 111

3.3.1 Effectiveness of Pellet Culture as a Three Dimensional Culture System 112

3.3.2 Possible Alterations and Improvements to the Methods Undertaken in this Chapter 114

3.3.3 Effect of Statins on Cell Cycle 117

3.3.4 Effect of Statins on Matrix Production 121

3.3.5 Summary 122

CHAPTER 4 - TENOCYTE PHENOTYPING 123

4.1 Introduction 123

4.1.1 Phenotypic Marker Selection 123

4.1.2 The Panel of Tenocyte Markers used in this study 126

4.2 Results 127

4.2.1 Tenocyte Isolation 127

4.2.2 Tenocyte Phenotyping 128

4.2.3 Maintenance of Tenocyte Phenotype in Monolayer Culture 130

4.2.4 Further Characterisation of the Tenocytes with regard to Chondrocytic Markers 132

4.3 Discussion 133

4.3.1 Significance of Recent Studies 134

4.3.2 Recommended Established Markers 134

4.3.3 Criticism of Some Established Tenocyte Markers 137

4.3.4 Tenocyte Phenotype and Three Dimensional Culture 139

Summary 139

CHAPTER 5 - CELL SHAPE CHANGE AND TENOCYTE PHENOTYPE 140

5.1 Introduction 140

5.1.1 Chondroplasia and Cell Shape Change 140

5.1.2 Culture System Selection 140

5.1.3 Phenotypic Marker Selection 141

5.1.4 Proposed signalling link 142

5.1.5 The Panel of Inhibitors of Cytoskeletal Signalling 143

5.2 Results 146

5.2.1 Cytoskeletal Inhibitors cause Tenocyte Actin Reorganization 146

5.2.2 Short term treatment with Cytoskeletal Inhibitors Increases Sox9 and Sox5 mRNA Expression 148

5.2.3 Only Lovastatin Maintains the Upregulation of the Sox Genes Long Term and Long Term Lovastatin Treatment Induces Collagen II Expression 148

5.2.4 Lovastatin Dose-Dependently Regulates Expression of Chondrocytic and Tenocytic Markers 150

5.2.5 Lovastatin-mediated Regulation of Chondrocytic and Tenocytic Markers Correlates with Alterations in Prenylation Status 154

5.3 Discussion 156

5.3.1 Potential Future Work 160

CHAPTER 6 - HYPOXIA AND COMBINATION AND TENOCYTE PHENOTYPE 161

6.1 Introduction 161

6.1.1 Aims of this chapter 161

6.2 Results 162

6.2.1 Tenocytes show Increased Levels of HIF-1α, VEGF-A and FOXO3a in Hypoxic Conditions 162

6.2.2 Low Oxygen Concentration Stimulated VEGF and DEC1 Expression but did not alter other Chondrocytic and Tenocytic Marker Gene Expression over 3 days 163

6.2.3 Long Term Cobalt Chloride Treatment Upregulates Sox9 Expression 165

6.2.4 Hypoxia and Lovastatin do not Interact and Affect Chondrocytic and Tenocytic Marker Gene Expression over 3 Days of Co-culture 167

6.3 Discussion 170

6.3.1 Tenocyte Response to Hypoxia 170

6.3.2 Phenotypic Genes 171

6.3.3 The effect of Combination Treatments 174

6.3.4 The Possible Impact of Time upon Hypoxia as a Chondrogenic Driver 177

6.3.5 The Role of HIF-1α and HIF-2α 178

6.3.6 Potential New Chondrocytic Markers Induced by Hypoxia 187

6.3.7 Conclusion 189

CHAPTER 7 - DISCUSSION 190

7.1 Major Findings of this Thesis 190

7.1.1 Introduction 190

7.1.2 Restatement of Thesis Aims 191

7.1.3 The Prochondrogenic Effects of Lovastatin in the ATDC5 Chondrogenic Model. 191

7.1.4 Assessment of the Effects of Cytoskeletal Signalling Disruptors on Tenocyte Phenotype 192

7.1.5 Assessment of the Effect of Hypoxia on Tenocyte Phenotype 193

7.1.6 Assessment of the Effect of Combined Hypoxia and Cell Shape Alteration on Tenocyte Gene Expression. 193

7.2 Future Work 194

7.2.1 RhoA – A Phenotype Switch? 194

7.2.2 Tenascin-C and Chondroplasia 195

7.2.3 The Relationship of BMP-2 Expression to Sox Gene Expression 196

7.3 Weaknesses of this study 196

7.3.1 Disadvantages of Cell Culture - Mechanical 196

7.3.2 Disadvantages of Cell Culture - Matrix 197

7.3.3 Limitations of the Hypoxia Study 198

7.3.4 Disadvantages of QPCR 198

7.4 Challenges within this field 199

7.4.1 Chondroplasia and Transcription Factor Expression 199

7.4.2 Fibrocartilage and Chondroplasia 199

7.4.3 Non-Pathological Fibrocartilage Formation and Chondroplasia 200

7.4.4 Dedifferentiated Chondrocytes and the Tenocytic Phenotype 201

7.4.5 Summary 203

REFERENCES 204

APPENDIX A - PAPERS, PUBLICATIONS AND PRESENTATIONS OF THE WORK CONTAINED IN THIS THESIS 235

A.1 Papers 235

A.2 Presentations 235

A.3 Oral Posters and Posters 236

APPENDIX B - NOTES ADDED IN CORRECTIONS 237

B.1 Chapter 1 237

B.2 Pain and Tendinopathy 237

B.3 Chapter 2 237

B.4 Composition of Media for Cell Culture 237

B.5 Protein Quantification Techniques 241

B.6 Dimethylmethylene Blue Assay 243

B.7 Chapter 5 243

APPENDIX C - CORRESPONDENCE WITH EPITOMICS 245

Table of Figures

FIGURE 1.1 A SCHEMATIC OF A MULTI-UNIT HIERARCHICAL STRUCTURE OF THE TENDON 9

FIGURE 1.2 THE ORGANISATION OF TISSUES IN THE DISTAL PART OF THE RAT ACHILLES TENDON 12

FIGURE 1.3 LINEAGE PROGRAMMING OF MESENCHYMAL PROGENITORS: A WORKING MODEL 14

FIGURE 1.4 INTERACTION OF TENOCYTES WITH COLLAGEN BUNDLES 17

FIGURE 1.5 THE INTEGRIN RECEPTOR FAMILY 18

FIGURE 1.6 INTEGRIN SIGNALLING 19

FIGURE 1.7 ACTIN STRUCTURE IN A MOUSE CHONDROCYTE 24

FIGURE 1.8 EMBDEN-MEYERHOF-PARNAS GLYCOLYSIS PATHWAY 25

FIGURE 1.9 DIAGRAM OF THE ACTIONS OF SOX5, SOX6, AND SOX9 IN DIFFERENT STEPS OF CHONDROGENIC DIFFERENTIATION 27

FIGURE 1.10 TRANSCRIPTION FACTOR AND MATRIX COMPOSITIONAL CHANGES BETWEEN TENSIONAL TENDON, FIBROCARTILAGE AND HYALINE CARTILAGE 31

FIGURE 1.11 STRUCTURE OF AGGRECAN AND VERSICAN 35

FIGURE 1.12 A MODEL OF THE DIFFERENT ISOFORMS GENERATED BY ALTERNATIVE SPLICING OF THE MRNA TRANSCRIPT FOR VERSICAN. 36

FIGURE 1.13 SCHEMATIC REPRESENTATION OF CLEAVAGE SITES AND DEGRADATION FRAGMENTS OF VERSICAN OBSERVED IN FRESH TISSUE AND TENDON EXPLANT CULTURES. 39

FIGURE 1.14 SCHEMATIC DIAGRAM OF COLLAGEN FIBRIL FORMATION 46

FIGURE 1.15 SCHEMATIC OF THE 10 + 4 MICROFIBRIL STRUCTURE OF A THIN CARTILAGE COLLAGEN FIBRIL. 46

FIGURE 1.16 ALCIAN BLUE STAINING OF TORN ROTATOR CUFF TENDON 52

FIGURE 1.17 COLLAGEN II STAINING OF TORN ROTATOR CUFF TENDON 53

FIGURE 1.18 PUTATIVE MECHANISM FOR INDUCTION OF CELL SHAPE CHANGE 61

FIGURE 1.19 THE DOMAIN STRUCTURE OF HIF1α AND HIF1β/ARNT 64

FIGURE 1.20 STRUCTURE OF HIF-1α AND HIF-2α PROTEINS 65

FIGURE 1.21 DUAL REGULATION OF HIFA SUBUNITS BY PROLYL AND ASPARAGINYL HYDROXYLATION. 67

FIGURE 1.22 DIRECT TRANSCRIPTIONAL TARGETS OF HIF. 68

FIGURE 2.1 MYCOPLASMA CONTAMINATION OF HUMAN TENOCYTES. 88

FIGURE 2.2 COMPARISON OF THE RESPONSE OF MYCOPLASMA CONTAMINATED AND UNCONTAMINATED TENOCYTES TO LOVASTATIN 89
FIGURE 2.3 GAPDH EXPRESSION IN RESPONSE TO LOVASTATIN TREATMENT 91
FIGURE 2.4 COMPARISON OF GAPDH AND β-ACTIN AS HOUSEKEEPER GENES FOR COBALT CHLORIDE TREATMENT 91
FIGURE 2.5 COMPARISON OF GAPDH AND TBP AS HOUSEKEEPER GENES FOR LOVASTATIN TREATMENT AND HYPOXIA 92
FIGURE 2.6 COMPARISON OF GAPDH AND TBP AS HOUSEKEEPER GENES FOR COMBINATION TREATMENTS 93
FIGURE 3.1 CHOLESTEROL SYNTHESIS PATHWAY 97
FIGURE 3.2 LOVASTATIN TREATMENT CAUSES ACTIN CYTOSKELETON REARRANGEMENT 100
FIGURE 3.3 LOVASTATIN TREATMENT INHIBITS SMALL GTPASE PRENYLATION 101
FIGURE 3.4 EFFECT OF LOVASTATIN ON PELLET VOLUME 102
FIGURE 3.5 LOVASTATIN TREATMENT CAUSES STATISTICALLY SIGNIFICANT DECREASES IN DNA CONCENTRATION 103
FIGURE 3.6 CHONDROGENIC MEDIUM INCREASES CELL PROLIFERATION 105
FIGURE 3.7 LOVASTATIN TREATMENT DECREASES CELL PROLIFERATION 106
FIGURE 3.8 EFFECT OF LOVASTATIN ON CASPASE 3 108
FIGURE 3.9 EFFECTS OF LOVASTATIN TREATMENT ON PELLET SGAG CONCENTRATION 109
FIGURE 3.10 LOVASTATIN INCREASES COLLAGEN II PRODUCTION 111
FIGURE 4.1 EXPLANTED TENOCYTE POPULATION 128
FIGURE 4.2 SCLERAXIS IS EXPRESSED IN SERTOLI CELLS AND EXPLANTED TENOCYTES 130
FIGURE 4.3 STABILITY OF TENOCYTE PHENOTYPE 131
FIGURE 4.4 CULTURED TENOCYTES EXPRESS SOME CHONDROCYTE MARKERS BUT NOT COLLAGEN II 133
FIGURE 5.1 CYTOSKELETAL SIGNALLING DISRUPTORS 143
FIGURE 5.2 ACTIN ARRANGEMENT IN TENOCYTES TREATED WITH CYTOSKELETAL SIGNALLING DISRUPTORS 147
FIGURE 5.3 LONG TERM TREATMENT OF TENOCYTES WITH CYTOSKELETAL SIGNALLING DISRUPTORS 150

FIGURE 5.4 EFFECT OF LOVASTATIN ON TENOCYTE VIABILITY.....152

FIGURE 5.5 EFFECT OF LOVASTATIN ON PHENOTYPIC MARKER MRNA LEVELS.....153

FIGURE 5.6 REVERSIBILITY OF LOVASTATIN EFFECTS WITH MEVALONATE 155

FIGURE 5.7 PROPOSED MODEL FOR CELL PHENOTYPE IN WHICH ACTIVATION STATUS OF RHOA FUNCTIONS AS A SWITCH BETWEEN TENOCYTIC AND CHONDROCYTIC PHENOTYPE. ..158

FIGURE 6.1 TENOCYTE RESPONSE TO HYPOXIA – NON-PHENOTYPIC MARKERS.....163

FIGURE 6.2 TENOCYTE RESPONSE TO GRADED HYPOXIA – PHENOTYPIC MARKERS.....165

FIGURE 6.3 RESPONSE OF TENOCYTES TO LONG TERM CHEMICALLY SIMULATED HYPOXIA 167

FIGURE 6.4 RESPONSE OF TENOCYTES TO A COMBINATION OF HYPOXIA AND LOVASTATIN TREATMENT 169

Table of Tables

TABLE 1.1 COMMON PRIMARY DISORDERS OF TENDONS CLASSIFIED BY ANATOMICAL AREA AND TENDONS MOST COMMONLY AFFECTED. 3

TABLE 1.2 INTEGRIN EXPRESSION IN TENOCYTES 20

TABLE 1.3 SMALL LEUCINE RICH PROTEOGLYCANS IN CARTILAGE, FIBROCARTILAGE AND TENDON ECM (CONTINUED OVERLEAF). 33

TABLE 1.4 VERSICAN ISOFORMS 36

TABLE 1.5 GLYCOPROTEINS IN CARTILAGE, FIBROCARTILAGE AND TENDON ECM. 41

TABLE 1.6 COLLAGENS IN CARTILAGE, FIBROCARTILAGE AND TENDON ECM (CONTINUED OVERLEAF). 43

TABLE 2.1 ANTIBODIES USED IN IMMUNOHISTOCHEMISTRY. 78

TABLE 2.2 ANTIBODIES USED IN WESTERN BLOTTING 83

TABLE 2.3 QIAGEN PRIMERS USED FOR QPCR 86

TABLE 2.4 SEQUENCES AND ANNEALING TEMPERATURE FOR NON-QIAGEN PRIMERS USED FOR QPCR 86

TABLE 4.1 TENOCYTE PHENOTYPE MARKERS AS POSITED IN SUMMER 2007. 125

TABLE 5.1 CELL PHENOTYPE MARKERS. 142

TABLE 6.1 TABLE SHOWING PUBLISHED EFFECTS IN CHONDROCYTES OF HYPOXIA, 3D CULTURE AND COMBINATION TREATMENT ON THE MARKERS USED IN THIS STUDY (AND COL1A2). 175

TABLE 6.2 SUMMARY OF CRITICAL REVIEW OF PAPERS CONCERNED WITH DETERMINING HIF-α SUBUNIT ROLE IN THE RESPONSE OF CHONDROCYTES TO HYPOXIA 185

Abbreviations

ACAN = aggrecan core protein

ACTB = β-actin

ADAMTS = A Disintegrin And Metalloproteinase with Thrombospondin Motifs

AHR = aryl-hydrocarbon receptor

ARNT = aryl hydrocarbon receptor nuclear translocator

bHLH = basic helix-loop-helix protein

BMP-2 = bone morphogenic protein 2

BNIP3 = BCL2/adenovirus E1B 19 kDa protein-interacting protein 3

CAD = C terminal activation domain (of HIF-α subunit)

CDET = common digital extensor tendon

CODDD = C terminal oxygen dependent degradation domain

COL1A1 = collagen, type I, alpha 1

COL1A2 = collagen, type I, alpha 2

COL2A1 = collagen, type IIB, alpha I

COL2A1va1 = collagen, type IIA, alpha I

COL3A1 = collagen, type III, alpha 1

COMP = cartilage oligomeric matrix protein

DEC1 = differentially expressed in chondrocytes 1

DMB = dimethylmethylene blue

ECM = extracellular matrix

EphA4 = ephrin type A receptor 4

EPAS1 = Endothelial PAS domain-containing protein 1

EPO = erythropoietin

ETS = E-twenty six

FACIT = Fibril Associated Collagen with Interrupted Triple helix

FCS = foetal calf serum

FOXO3a = forkhead box O3a

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

GGTase I = geranylgeranyl transferase I

HIF = hypoxia-inducible factor

HIF-α = hypoxia-inducible factor, alpha subunit

HIF-1α = hypoxia-inducible factor 1, alpha subunit

HIF-2α = hypoxia-inducible factor 2, alpha subunit

HIF-β = hypoxia-inducible factor, beta subunit

HRE = hypoxia response element

MIG6 = mitogen-inducible gene 6

MMP = matrix metallproteinase

MTJ = musculotendinous junction

MSC = mesenchymal stem cell

NAD = N terminal activation domain (of HIF-α subunit)

NODDD = N terminal oxygen dependent degradation domain

ODDD = oxygen dependent degradation domain

OTJ = osteotendinous junction

PAS = PER/ARNT/SIM

PCNA = proliferating cell nuclear antigen

PER = period circadian protein

PRELP = Proline/arginine-rich end leucine-rich repeat protein

QPCR = quantitative polymerase chain reaction (also known as Real Time polymerase chain reaction)

ROCK = Rho kinase

RT-PCR = reverse transcriptase polymerase chain reaction

SCX = scleraxis

SDFT = superficial digital flexor tendon

sGAG = sulphated glycosaminoglycan

SIM = single-minded protein

SLRP = small leucine rich-repeat protein

TBP = TATA box binding protein

VEGF-A = vascular endothelial growth factor A

Chapter 1 - Introduction

1.1 Tendinopathy and Chondroplasia

Tendinopathy (disorders primarily affecting the tendon including tendon rupture and chronic pain [1]) is a significant cause of pain and disability in the population. Studies among the elderly have found that rotator cuff disease was the most common cause of shoulder pain [2, 3]. Torn rotator cuff tendons may show a variety of degenerative changes including chondroplasia - the pathological development of a fibrocartilage-like phenotype in tensional tendon. This thesis examines the process of chondroplasia with special focus on factors which may promote its development.

This introduction will initially focus on the problem of tendinopathy before considering proposed theories of tendon degeneration and discussing chondroplasia as a specific form of tendon degeneration. In order to understand the disease process it is first necessary to describe the normal biology of tendons. This introduction will therefore focus on the macro-structure of tendons and their blood supply. Since little is known about the early stages of fibrocartilage development a related tissue, hyaline cartilage, will be used as a model for chondrogenesis. The phenotype of normal tensional tendon, fibrocartilage and hyaline cartilage will be discussed at both the extracellular matrix (ECM) and cell level before being compared and contrasted. A description of the matrix changes which occur during tendon degeneration will follow. Rotator cuff tears and chondroplasia will then be described in detail. Three possible drivers of

chondroplasia will be considered and a statement of specific aims for the thesis made.

1.1.1 Tendinopathy

Tendinopathy (disorders primarily affecting the tendon including tendon rupture and chronic pain [1]) is a significant cause of pain and disability in the population. Accurate figures for patients presenting with tendon disorders at UK health facilities are not available, however, up to 43% of new rheumatology patient referrals are for soft tissue problems [4]. Achilles tendinopathy has been estimated to affect 11% of runners [5]. Shoulder pain affects 16% of the general population [6]. Studies among the elderly have found that rotator cuff disease was the most common cause of shoulder pain [2, 3].

Whereas bones heal within 2-12 weeks, tendons do not heal quickly, taking 6-8 months for a full return to function. Tendons are relatively poorly vascularised compared to bones. This may contribute to their slow healing.

Little is known about how to promote and improve healing in tendons. This is due to a poor understanding of tendon biology. Historically tendons have been an under-researched tissue in comparison to other musculoskeletal tissues such as cartilage and bone.

Certain tendons appear to be more vulnerable to tendinopathy than others. Precisely what pre-disposes particular tendons to disease is presently unknown. Affected tendons include both those subject to high loads (such as the Achilles

and patellar) and those subject to smaller loads (such as the forearm extensor and supraspinatus). Commonly affected tendons are listed in Table 1.1.

Table 1.1 Common primary disorders of tendons classified by anatomical area and tendons most commonly affected.

Table contents removed for copyright reasons. See table 1 in J. D. Rees, A. M. Wilson, and R. L. Wolman, "Current concepts in the management of tendon disorders," Rheumatology (Oxford), vol. 45, pp. 508-21, May 2006. PMID = 16490749 doi = 10.1093/rheumatology/kel046

Different tendons appear to be subject to slightly different tendinopathies. Although the trigger(s) for these tendinopathies are not known for certain different causes have been proposed for different tendons. Achilles tendon, for example, is thought to be subject to both chronic "overuse" tendinopathy and to spontaneous ruptures [1]. In contrast, rotator cuff tear and lateral epicondylitis have both been associated with aging [8]. The frequency of occurrence of rotator cuff tear increases with age [9-11]. Brewer noted that degenerative changes occur in the rotator cuff tendon with aging [12], a finding confirmed by Riley et al. [8], although the authors noted that the relationship between tendon degeneration and age was not a simple one.

1.1.2 Theories of Tendon Degeneration

The aetiology of tendon rupture is not clear. Current theories propose that the tendon degenerates eventually resulting in rupture. The specific trigger(s) for this degeneration is unclear. Two theories have been proposed, one mechanical and one vascular. The mechanical theory proposes that repeated or prolonged stressing in the upper range of normal physiological strain causes microtrauma within the tissue. It is believed that either because of inherently low levels of matrix turnover (see Section 1.1.2.1.1) or because of an imbalance in the matrix maintaining enzymes (see Section 1.1.2.1.2) that this microtrauma is either not repaired or not repaired correctly. As damage and/or poor repair attempts accumulate, the ability of the ECM to withstand mechanical forces deteriorates and eventually the tendon ruptures. In contrast, the vascular theory proposes that a compromise of blood supply to the tendon results in cellular changes and death which result in degeneration of the tendon tissue. Once the tendon has ruptured the degenerative process is believed to continue.

1.1.2.1 Matrix Metalloproteinases and Tendinopathy

Matrix metalloproteinases (MMPs) are a family of enzymes whose members have the ability to cleave a wide variety of ECM components [13]. They are a normal component of healthy tendon, indeed Achilles tendon has been shown to express mRNA for wide variety of MMPs [14].

The mechanical theory of tendon degeneration holds that microtrauma induced damage is not properly repaired. This results in the accumulation of unrepaired or

poorly repaired tissue thus decreasing the ability of the tissue to withstand normal activity eventually leading to tendon rupture. MMPs form an essential component of the repair process by degrading damaged sections of matrix, allowing them to be replaced by newly synthesised sections. The potential role of this family of enzymes in tendinopathy is therefore of interest to tendon researchers. It is generally believed that the degradation process performed by MMPs which then allows the repair process to be fully effective is insufficient or defective in some form. However, views differ on the origin of this ineffective degradation. The source of this disagreement are observations made by two separate groups concerning the rate of matrix turnover in two pairs of adjacent tendons. In each tendon pair one tendon is frequently subject to tendinopathy (supraspinatus tendon in humans, superficial digital flexor tendon (SFDT) in horses) while the other is not (biceps brachii in humans, common digital extensor tendon (CDET) in horses). Both groups find that there are differences in the rate of collagen turnover (as determined by pentosidine accumulation and aspartic acid racemization) between the tendons frequently subject to tendinopathy and the adjacent usually unaffected tendon. However, the results of each group are mutually contradictory. Whether these differences are attributable to species differences, differences in tendon location or because one group is examining a tendon which might have been subject to extraordinarily high levels of use[1] remains to be seen.

[1] The horses used in the Birch group's studies [15, 16] were obtained from a commercial abattoir and could potentially have included horses used or trained for commercial purposes such as racing, jumping or eventing. Such commercial activities might place unusually high levels of strain on tendons within the leg of the animal. Additionally, such horses are usually subject to selective breeding introducing a possible confounding element to the study. It must also be noted, however, that both studies use a large number of individuals and thus the level of possible interference from subjects produced by the commercial horse industry will be dependent on the relative levels of commercial and non-commercial stock handled by the abattoir.

1.1.2.1.1 Birch group - Insufficient Matrix Metalloproteinase Activity

The Birch group found a lower rate of collagen matrix turnover in the SDFT (tendon more prone to tendinopathy) of horses than they did in the CDET (tendon less prone to tendinopathy) [15, 16]. They also noted that mRNA transcript levels for the collagenases MMP-1 and MMP-13 were lower in SDFT than in CDET [15]. There were lower levels of potential MMP-13 in the SDFT compared to the CDFT [15]. Additionally, there were higher levels of TIMP-3 mRNA in the SFDT [15].

These findings led this group to propose a model for tendon degeneration [15, 16] in which microdamage to the tendon ECM is not repaired and replaced with new tissue. This failure to replace damaged tissue results in compromised mechanical properties for the tendon overall increasing the likelihood of tearing.

1.1.2.1.2 Riley group - Imbalanced Matrix Metalloproteinases

In contrast to the Birch group, the Riley group found that the collagen component of human supraspinatus tendon (tendon more prone to tendinopathy) is subject to more turnover than the collagen component of the nearby biceps brachii tendon (tendon less prone to tendinopathy) [17] [18]). They have found higher levels of MMP-1, MMP-2 and MMP-3 activity in supraspinatus tendon compared to biceps brachii tendon [18].

Riley et al. instead suggest that imbalance in the relative levels and activities of MMP-1, MMP-13 and MMP-3 may result in the gradual degeneration of the

tendon ECM [1]. This theory is based on findings from painful and ruptured tendons. Ruptured supraspinatus tendons have increased levels of MMP-13 mRNA and decreased levels of MMP-3 transcript compared to control supraspinatus [19]. They also have increased levels of the active form of MMP-13 [19], increased MMP-1 activity and decreased MMP-3 activity compared to both control supraspinatus tendon and biceps brachii tendon [18, 19]. MMP-1 protein is expressed at the edge of ruptured supraspinatus tendon [20] and upregulated in patellar tendinosis [21]. MMP-3 mRNA levels are also decreased in painful and ruptured Achilles tendon [14, 22]. This suggests an increase in collagenase (MMP-1 and MMP-13) levels and activity with a simultaneous decrease in MMP-3 activity. Riley et al. have suggested that this disruption to the normal MMP balance may result in impaired repair of microtrauma or inadequate maintenance of the tendon tissue.

1.1.3 Chondroplasia

Torn tendons may show a variety of degenerative changes including calcifying tendinopathy, mucoid degeneration and chondroplasia [23]. Chondroplasia is the pathological development of a fibrocartilage-like phenotype in a region which normally exhibits a tensional morphology. Chondroplastic tissues are believed to have a poor capacity for repair. An understanding of the process of chondroid metaplasia could enable screening and early intervention. Chondroplasia has been reported in several tendinopathies including posterior tibial tendon insufficiency [24], lateral epicondylitis [8], calcific insertional tendinopathy of the Achilles tendon [25], trigger finger [26] and jumper's knee [27]. It has, however, received most scholarly attention in tears of the rotator cuff tendon, where it appears to

occur frequently [28]. The rotator cuff tendon will be used in this thesis as a key example of a tendon prone to chondroplastic degeneration.

1.2 Normal Tendon Biology

1.2.1 Tendon Composition

In order to understand tendon degeneration it is first necessary to consider the normal biology of tendons. This section therefore details various aspects of tendon macrobiology namely their macrostructure, their blood supply and examines the presence of fibrocartilaginous elements at various points in the normal tendon.

1.2.1.1 Tendon Structure

Tendon contains a sparse population of cells which are responsible for the maintenance of extracellular matrix (ECM), which is the primary component of the tissue. The composition of the ECM will be discussed at greater length in section 1.5, however for now it is sufficient to state that the major component is collagen I.

Overall tendon has a multi-unit hierarchical structure in which the components run parallel to the direction of strain (see Figure 1.1). Units of tropocollagen, a molecule consisting of three collagen polypeptide chains, align end to end to form a quarter-staggered array known as a fibril. The diameter of this structure can vary from 10 to 500nm, depending on species, age and location. Clusters of

fibrils surrounded by endotenon (a thin layer of connective tissue which contains blood and lymphatic vessels) form a structure known as a fibre. Bundles of fibres form fascicles. The tendon consists of bundles of fascicles surrounded by epitenon - a fine loose connective tissue sheath which contains blood and lymphatic vessels. Some tendons are additionally surrounded by a paratenon and/or synovial sheath but the rotator cuff lacks both of these structures [29].

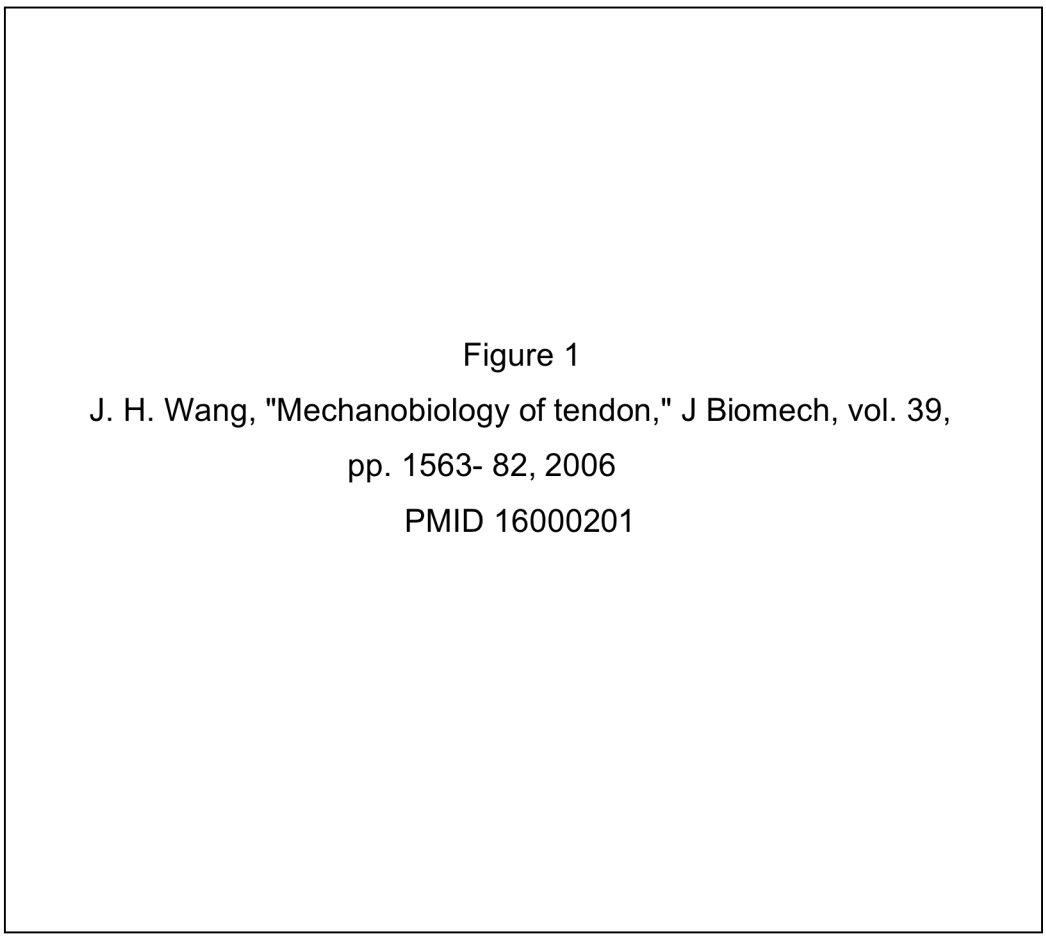


Figure 1.1 A Schematic of a Multi-Unit Hierarchical Structure of the Tendon

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1.2.1.2 Tendon blood supply

Broadly speaking, tendons receive their blood supply from both the musculotendinous junction (MTJ) and the osteotendinous junction (OTJ), the so-called intrinsic system, and from the paratenon and (if present) the synovial sheath, the extrinsic system. The relative contribution of each of these systems to the blood supply varies from tendon to tendon. At the MTJ vessels from the perimysium cross into the tendon and continue between the fascicles. These are likely to extend for, at most, a third of the tendon length. The tendon receives a blood supply from bone at the enthesis, although the fibrocartilaginous regions are avascular. The tendon midsubstance receives its blood supply from blood vessels which infiltrate the paratenon and form a transversely branching vascular network. Some of these arteries branch into the epitenon and into the endotenon where they form a complex mesh of vessels containing many anastomoses.

The rotator cuff tendon has an atypical blood supply. The bursal side of the tendon is lined with fibres from the coracohumeral ligament. This layer contains large arterioles [31]. The articular side of the cuff is lined with joint capsule, a sheet of continuous collagen fibrils. The tendon receives a blood supply from the periosteum, the MTJ and the OTJ [29].

Studies suggest that tendon vascularity is compromised at the enthesis and at sites of compressive loading. Some authors have suggested that there might a region of hypovascularity [32] or a region of vascularity which is subject to 'wringing' upon a particular form of loading in the supraspinatus [33]. This region is said to

coincide with the 'critical zone', an area of the rotator cuff in which most tears occur.

1.2.1.3 Anatomical Considerations

Tendons connect bone to muscle. However, for anatomical reasons, it is sometimes necessary for a tendon to loop around a bony prominence (for example, the fibularis brevis tendon bends around lateral malleolus). Such tendons are known as 'wrap-around' tendons. The region of tendon which is in contact with the bony prominence is fibrocartilaginous as is a portion of the enthesis, the region of tendon which blends in to the bone. The biology of these regions will be discussed in Section 1.4.1.

The insertion of the Achilles tendon into the calcaneus (Figure 1.2) contains examples of tensional tendon (here labelled as tendon midsubstance (MT)), enthesial fibrocartilage and sesamoid fibrocartilage (which is distinct from enthesial fibrocartilage and develops in a region of tendon which contacts the edge of the calcaneus). Tendons which do not wrap around a bony prominence are known as direct tendons.

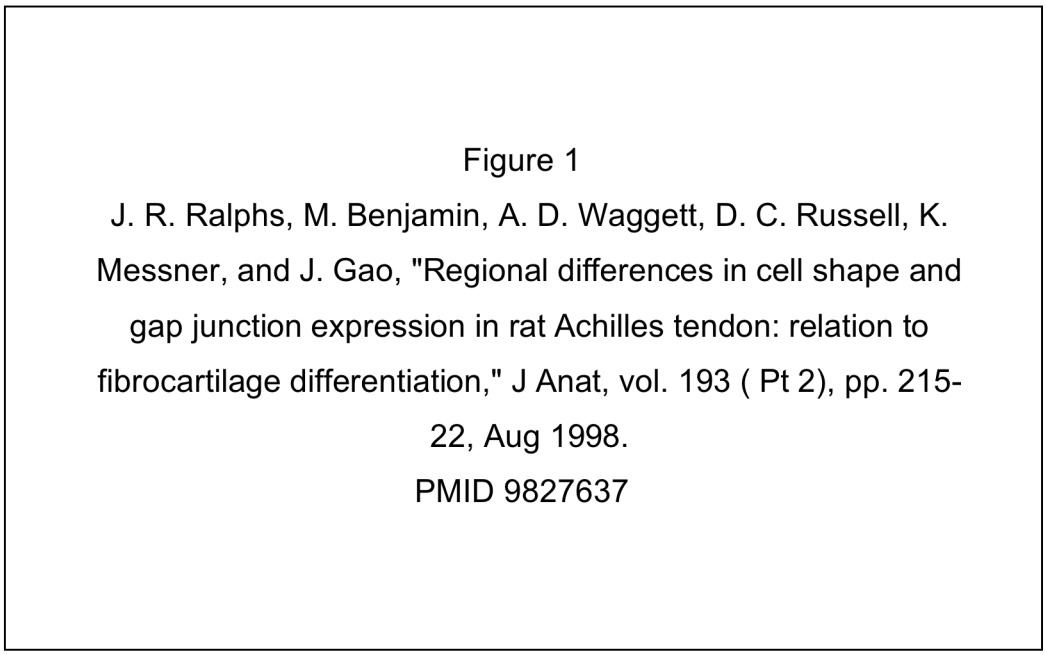


Figure 1.2 The Organisation of Tissues in the Distal Part of the Rat Achilles Tendon

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1.3 A Description of Tendon and Cartilage

Chondroplasia has been previously defined as the development of cartilage-like characteristics in tendinous tissue. In order to understand this process it is necessary to define tendon, fibrocartilage (the tissue which most closely corresponds to chondroplastic tissue) and also hyaline cartilage. The latter is to be employed as a model for the formation of the chondrocytic phenotype since little is known about normal fibrocartilaginous transformation.

This section will first consider the developmental origins of tendon and cartilage. It will then describe the phenotype of normal tensional tendon, fibrocartilage and hyaline cartilage at both the ECM and cellular level, considering matrix

composition, cell shape and phenotypic markers with particular reference to transcription factors.

1.3.1 Developmental Origins

Tenocytes and chondrocytes share a common origin, the mesenchymal stem cell (Figure 1.3). Expression of different master transcription factors results in different cell phenotypes. The differentiation of tendon is poorly understood, although cartilage differentiation is better defined.

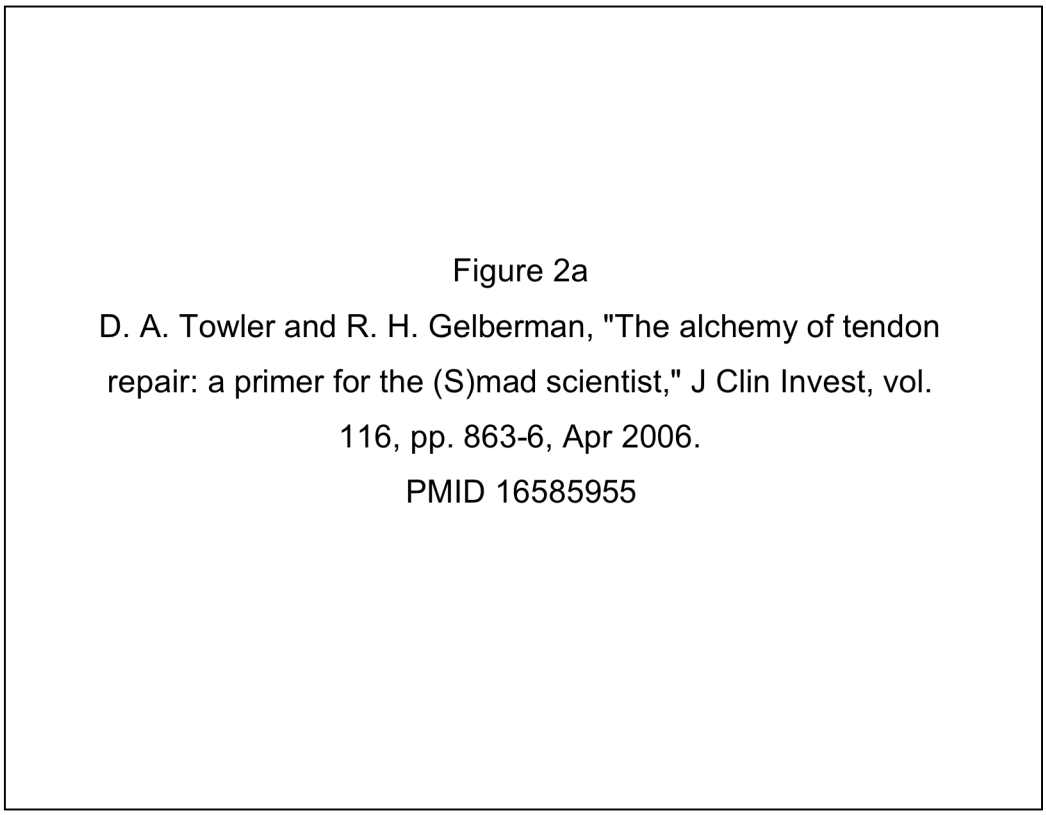


Figure 1.3 Lineage Programming of Mesenchymal Progenitors: a Working Model

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1.3.2 The Phenotype of Normal Tensional Tendon

1.3.2.1 Tendon Extracellular matrix

This section briefly details the nature of tensional tendon ECM, which will be discussed in greater depth in Section 1.5 where it will be contrasted with that of hyaline cartilage. Tensional tendon is predominantly composed of collagen with proteoglycans accounting for the remainder of the dry weight. Collagen I is the major component within tendon ECM, accounting for 66.6% of the dry weight of macroscopically normal cadaveric human supraspinatus tendons . It is arranged in fibrils which follow the lines of principal strain. Collagen III constitutes approximately 3% of total tendon collagen and is the next most abundant collagen species [37]. The ECM also contains small quantities of other collagens including collagen V and collagen VI [1].

Tendon also contains proteoglycans, however, these constitute a very small component of the ECM, less than 1% of the dry weight of adult bovine flexor tendon [38]. The relative proportions of each proteoglycan vary from tendon to tendon. The proteoglycan make-up contains members of the small leucine-rich proteoglycan (SLRP) and lectican families. In many tendons the SLRP decorin is the predominant proteoglycan, for example in adult bovine flexor tendons [38] and the distal section of the human biceps tendon [39]. Decorin, biglycan and the lectican aggrecan are part of the ECM of rotator cuff tendons [40]. Tendons also contain a small quantity of glycoproteins including cartilage oligometric protein (COMP).

1.3.2.2 Tenocytes

The resident cell population in tendons are referred to as tendon fibroblasts or tenocytes. They are specialised cells responsible for the production and maintenance of the ECM.

1.3.2.2.1 Tenocyte Morphology and Cytoskeletal Structure

Within the tendon tissue, tenocytes are arranged in short rows running parallel to the collagen bundles. Tenocytes are extremely long cells with a complicated three dimensional structure (see Figure 1.4). Sheet-like lateral processes extend from the cell body and wrap around neighbouring collagen bundles. Each tenocyte is in contact with 6-9 collagen bundles. In addition to interacting with the collagen bundles, the lateral processes also interact with similar processes extending from cells in adjacent rows. Cells in the middle of a row have a relatively short cell body maintaining contacts with their neighbours anteriorly and posteriorly. Cells at the end of the rows have long thin longitudinal processes which allow them to make contact with a cell process or cell body some distance away [41]. Actin stress fibres run the length of the cell following the lines of principal strain [42]. When cultured in vitro they assume a fibroblastic morphology and are morphologically indistinguishable from cells cultured from other loose connective tissues.

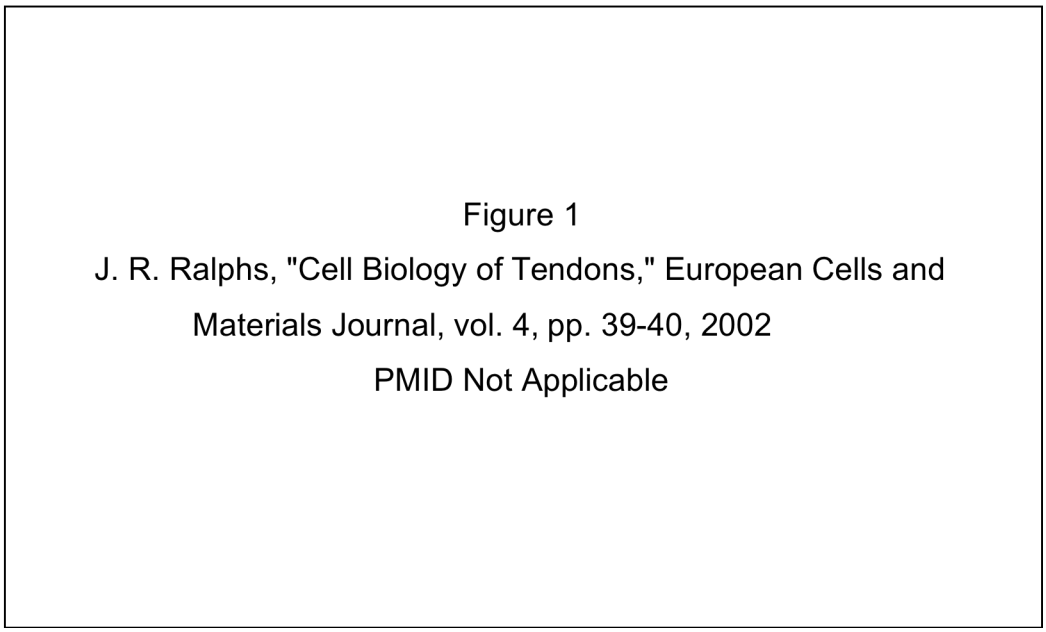


Figure 1.4 Interaction of Tenocytes with Collagen Bundles

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1.3.2.2.2 Mechanoresponsivity

Tenocytes are sensitive to mechanical signals. They express connexin 43 on both the lateral and longitudinal processes. In contrast connexin 32 is expressed on the lateral processes only [41]. These connexins form functional gap junctions [44] allowing the tenocytes to act as a functional syncitium. Tenocytes have primary cilia [45], which are orientated parallel to the collagen fibres and along the long axis of the tendon [46]. Primary cilia are believed to play a role in mechanosensing. They also express voltage gated ion channels [47].

Tenocytes are intimately linked to their ECM in part by expression of a range of integrins (see Table 1.2). Integrins are heterodimers comprised of an α and a β subunit [48]. Currently some 18 α subunits and 8 β subunits are known [48].

These bidirectional transmembrane receptors are capable of binding a wide range of ECM molecules including a range of collagens, fibronectin and matrilin-1 on their extracellular portion [48](see also Figure 1.5). The intracellular portion is able to bind a large number of signalling proteins including Src, thus providing a signalling link between the ECM and the cell nucleus [48]. Integrin signalling can affect a wide variety of cell processes including the cell cycle and the actin cytoskeleton (see Figure 1.6)

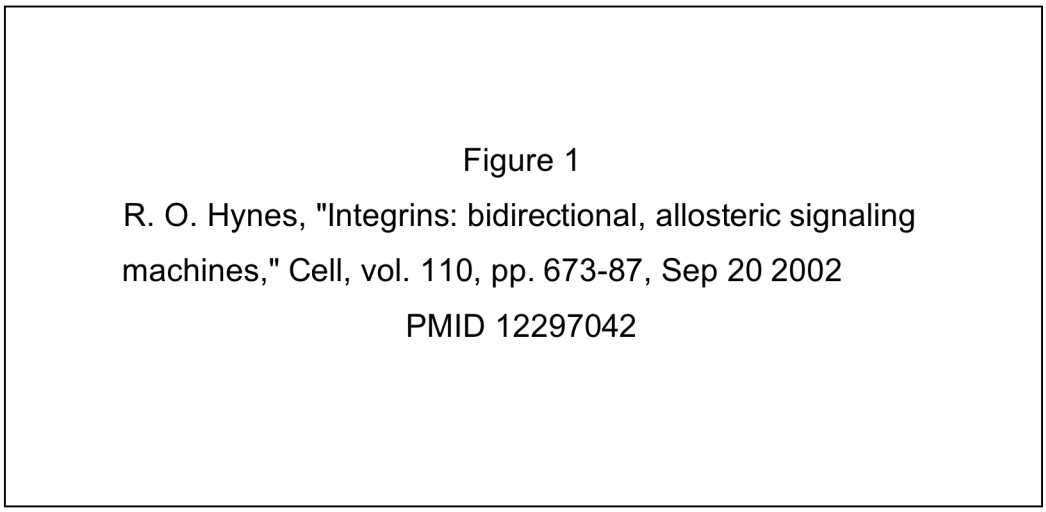


Figure 1.5 The Integrin Receptor Family

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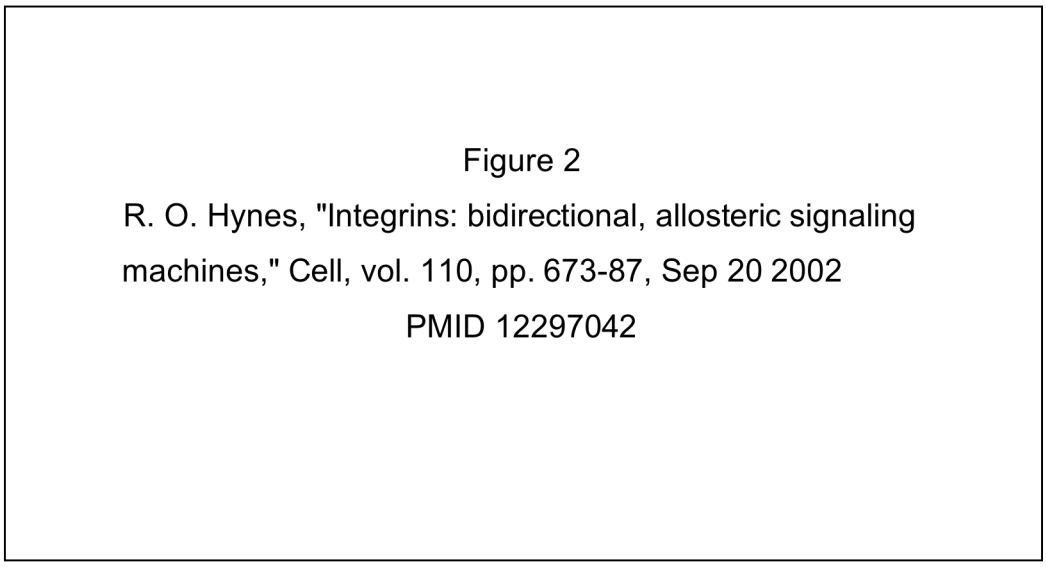


Figure 1.6 Integrin Signalling

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The integrin $\alpha_5\beta_1$ is known to be a major mechanoreceptor in articular chondrocytes (reviewed in [49]). It is known to mediate cyclical compressive loading induced upregulation of COMP [50] and proteoglycans [51]. Human tenocytes are documented to express both the α_5 [52] and β_1 [53] subunits and the $\alpha_5\beta_1$ heterodimer is reported in both dog [54, 55] and rabbit [56] tenocytes. This raises the possibility of similar signalling pathways to those observed in chondrocytes occurring in tenocytes.

Both embryonic mouse and adult rat tendon expresses the integrin α_{11} subunit [57] [58]. Chondrocytes are known to express the integrin α_{10} subunit [59, 60]. A recent study has shown a relationship between the ratio of COL1A1:COL2A1 and the ratio of mRNA for α_{10}: α_{11} in bovine articular chondrocytes [61].

Integrin	Species
α_2 subunit	Mouse [57]
α_5 subunit	Human [52]
β_1 subunit	Dog [62], Human [53]
$\alpha_5\beta_1$	Dog [54, 55], Rabbit [56]
$\alpha_{11}\beta_1$	Mouse [57], Rat [58]
$\alpha_v\beta_3$	Dog [54, 55]

Table 1.2 Integrin Expression in Tenocytes

1.3.2.2.3 Tenocyte Metabolism

Tenocytes generate ATP via both aerobic and anaerobic pathways [63], [64]. Previous studies have shown these cells to be sensitive to decreased oxygen levels, with total hypoxia reported to cause apoptotic cell death [65].

1.3.2.2.4 Tenocyte Molecular Markers

In common with other connective tissue cells, tenocytes differentiate from multipotent mesenchymal precursors. The differentiation process is initiated by

the expression of various master transcription factors, of which the only known regulator for tenocytes to date is scleraxis. The question of specific molecular markers for tenocytes will be discussed in greater detail in Chapter 4.

1.3.2.2.4.1 Scleraxis

Scleraxis is a class II bHLH which is expressed in tendon and cartilage. Originally identified as a developmental marker [66], its expression is maintained in adult tissue [67]. Knockout of scleraxis produces marked but not total tendon defects [68] suggesting that this transcription factor is a key regulator of tendon phenotype.

Basic helix-loop-helix (bHLH) proteins are transcription factors involved in cell patterning. They are separated into two groups, class I and class II, according to expression. Class I bHLHs, including ITF2, HEB and the two products of the E2A gene, E12 and E47, are ubiquitously expressed whereas class II bHLHs, such as scleraxis and MyoD, are cell specific. Class I bHLHs heterodimerise with class II bHLHs and bind to a consensus sequence known as an E-box resulting in the expression of phenotype specific genes. Scleraxis is known to be capable of heterodimerising with ITF2 [69], E12 [66] and E47 [70], although it is likely that this list is not exhaustive. Binding of the scleraxis/E47 heterodimer is necessary for the transcription of the alpha 1 chain of collagen I in tenocytes [71]. Scleraxis also appears capable of regulating the transcription of the alpha 2 chain of collagen I [72].

1.3.2.2.4.2 Tenomodulin

Tenomodulin is a transmembrane glycoprotein, which was originally identified due to its homology with the anti-angiogenic molecule chondromodulin [73]. It is positively regulated by scleraxis [74] and is necessary for tenocyte proliferation and tendon maturation [75]. It has been suggested as a marker of mature tenocytes for use in both developmental biology [74] and cell culture [76]. Its use in the latter capacity will be discussed further in Chapter 4.

1.4 Normal Cartilage and Fibrocartilage Biology

1.4.1 Introduction to Fibrocartilage

Fibrocartilage has been described as a "transitional tissue" since it combines features of cartilage with features of dense, collagenous connective tissue, blending seamlessly into either tendon or bone. Little is known about the molecular development of fibrocartilage. However, it may share some aspects of the development of hyaline cartilage and this similarity has been used to generate hypotheses used in this work.

1.4.2 Hyaline Cartilage

1.4.2.1 Cartilage Extracellular Matrix

Cartilage matrix consists of networks of fibrils mainly composed of collagen II, collagen IV and aggrecan. In contrast to the highly ordered alignment of collagen fibrils in tendon cartilage collagen fibrils form a random, loose network [77]. The fibrils are composed of collagen II and collagen XI. The outside is decorated by

collagen IX. The fibrillar appearance of the collagen differs slightly between interterritorial and pericellular matrix. Fibrils become progressively coarser and more obviously banded (seen under transmission electron microscopy) with increasing distance from the chondrocytes themselves. The highest concentrations of collagen IX and collagen XI are seen in the chondron, an area of specialised matrix which directly surrounds the chondrocyte. The primary functional property of the network of fibrils based on collagen II is to provide tensional stability.

Collagen VI forms an apparently separate network of fibrils which are thinner than those of the collagen II/IX/XI network. The function of this network is currently unknown, however it is known to interact with collagen II and with aggrecan.

The collagen fibres are linked together by a number of different proteoglycans and glycoproteins. These appear to regulate fibrillogenesis by preventing the addition of new collagen molecules to a fibril. Aggrecan, the most abundant proteoglycan in cartilage, draws water into the cartilage creating a large osmotic swelling pressure.

1.4.2.2 Chondrocytes

1.4.2.2.1 Chondrocyte Morphology and Cytoskeletal Structure

Chondrocytes are spherical with a cortical actin structure (Figure 1.7). This shape and actin structure is believed to be of importance to the chondrocyte phenotype,

both the initial differentiation of these cells and the subsequent maintenance of the chondrocyte phenotype. This concept will be discussed further in Section 1.8.2.

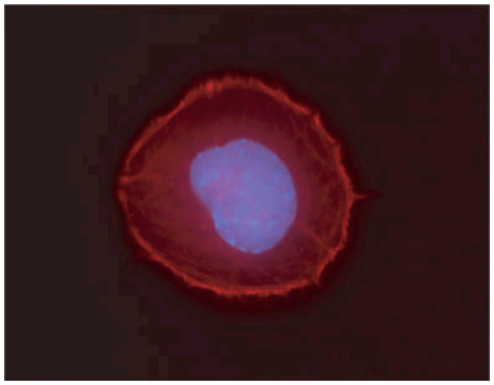


Figure 1.7 Actin structure in a mouse chondrocyte

Red = actin, blue = DAPI. From Bursell et al. [78]. Image covered by CC BY 2.0 .

1.4.2.2.2 Chondrocyte Metabolism

Chondrocytes, the resident hyaline cartilage cell population, are highly adapted to hypoxia, generating ATP via the non-oxygen consuming Embden-Meyerhof-Parnas pathway [79], [80] (Figure 1.8). The relative contribution of anaerobic and aerobic respiration to the generation of ATP in fibrochondrocytes is not known, however, fibrocartilage is avascular, which is suggestive of a stronger contribution from anaerobic pathways.

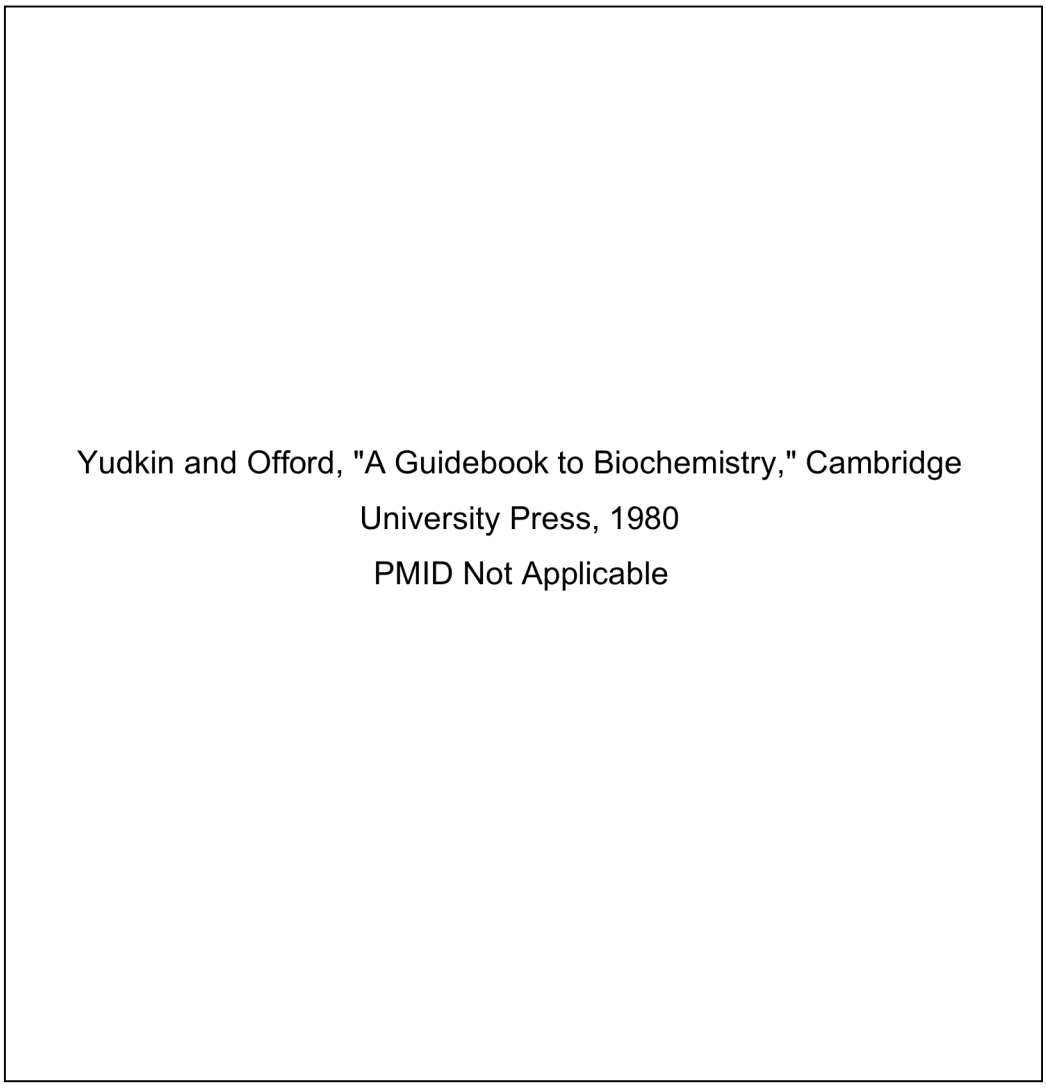


Figure 1.8 Embden-Meyerhof-Parnas glycolysis pathway

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1.4.2.2.3 Chondrocyte Molecular Markers

1.4.2.2.3.1 Sox9

Sox9, a member of the Sox family of transcription factors, is a 'master regulator' of chondrogenesis. In the developing mouse embryo it is expressed at high levels at all sites where chondrogenesis occurs [82]. Expression in the mesenchymal condensations begins prior to overt chondrogenesis and continues throughout cartilage deposition. Mutations in or around the Sox9 gene that interfere with or

abolish the normal function of the protein cause campomelic dysplasia in humans [83-86]. The symptoms of this disease include severe anomalies in multiple skeletal elements including a deformed pelvis and spine, a small thoracic cage and shortened and bowed long bones [87, 88].

The binding of Sox9 to a sequence within the chondrocyte-specific enhancer region in the promoter of COL2A1 is necessary for collagen II expression in chondrocytes [89, 90]. Sox9 binding also enhances the expression of aggrecan core protein transcription [91]. Hyaline articular cartilage contains approximately ten times as much Sox9 transcript as tensional tendon [67].

1.4.2.2.3.2 Sox5 and Sox6

Sox5 and Sox6 are members of the D group of Sox proteins [92]. They bind to regions of DNA as either as homodimers or as a heterodimer. Unlike Sox9 they do not contain a transactivation domain, but Lefebvre has hypothesized that Sox5 and Sox6 dimer binding may affect transcription by recruiting additional proteins to a transcriptional complex and/or by bending the DNA thus allowing Sox9 to transactivate more efficiently [93].

Sox5 and Sox6 mRNA is expressed at every site of chondrogenesis in the developing mouse embryo [94]. It is also expressed at moderate levels in adult human articular cartilage [95]. Many cartilage ECM genes contain Sox5 and Sox6 binding sites, including COL2A1 [94] and aggrecan [96]. Knock-out studies indicate that Sox5 and Sox6 do not play an important part in the initial stage of developmental chondrogenesis, namely the condensation of mesenchymal

precursor cells. They do play an important part in the emergence of chondroblasts (Figure 1.9), acting mostly as enhancers of chondrogenesis [97]. Sox5 and Sox6 appear to have redundant functions in chondrogenesis. $Sox5^{-/-}$ and $Sox6^{-/-}$ mice have a mild skeletal phenotype only, while $Sox5^{-/-}Sox6^{-/-}$ mice display a severe chondrodysplasia.

Although the role that Sox5 and Sox6 play follows on from the function of Sox9, it is not known whether Sox9 regulates Sox5 or Sox6. Indeed, little is known of the regulation of either Sox5 or Sox6 at a transcriptional, post-transcriptional or post-translational level [92].

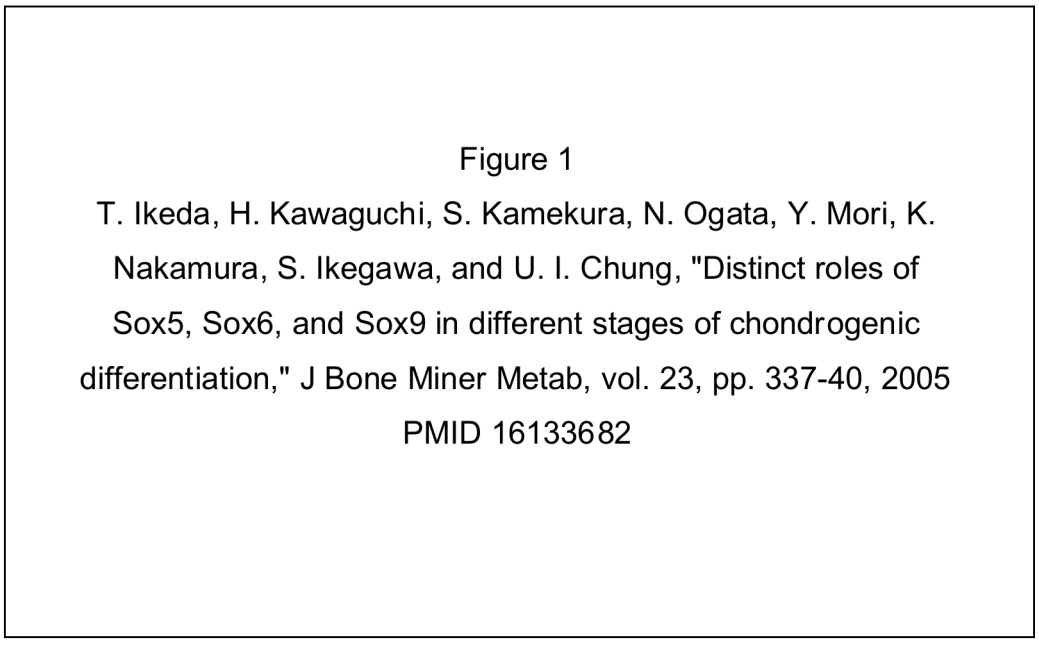


Figure 1.9 Diagram of the actions of Sox5, Sox6, and Sox9 in different steps of chondrogenic differentiation

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1.4.2.2.3.3 Differentially Expressed in Chondrocytes 1 and 2

Differentially expressed in chondrocytes 1 (DEC1) and differentially expressed in chondrocytes 2 (DEC2) are class II basic helix loop helix (bHLH) proteins that are

hypothesised to have a role in chondrocyte differentiation. Both DEC1 and DEC2 are expressed in cartilage [98, 99]. DEC1 is known to be upregulated during chondrogenesis [100][2]. Overexpression of DEC1 in mesenchymal stem cells promotes chondrogenic differentiation [100] and inhibits adipogenesis [101]. Overexpression of DEC2 is reported to inhibit myogenesis [102]. DEC1 and DEC2 have been shown, in other cell types, to act as transcriptional repressors [65]. The exact mechanism of this repression seems to vary but it may include the direct binding of an E-box in the promoter of a target gene followed by the recruitment of corepressor components including histone deacetylase 1 or the formation of heterodimers with a non-class I bHLH preventing the non-class I bHLH from binding the E-box and transactivating its target genes [103]. The mechanism of action of DEC1 during chondrogenesis has therefore been hypothesised to be that of repression of differentiation towards alternative lineages.

DEC1 and DEC2 contain a hypoxia response element (HRE) within their promoter region [104]. In common with Sox9, DEC1 and DEC2 mRNA is upregulated in response to hypoxia in both ATDC5 cells [104] and growth plate chondrocytes.

Unlike the Sox genes DEC1and DEC2 do not appear commonly in cartilage studies. However, their role in chondrogenesis and their character as transcription factors, thus forming a potential convergence point for multiple regulatory pathways, made them an interesting target for this study. Due to primer

[2] Studies have yet to examine the expression of DEC2 during chondrogenesis.

difficulties only DEC1 was eventually studied. Neither DEC1 or nor DEC2 have been studied in tendon or in fibrocartilage to date.

1.4.3 A More Detailed Consideration of Fibrocartilage

1.4.3.1 The Role of the Mechanical Environment in the Formation of Fibrocartilage

Within the tendon fibrocartilage usually occurs at the entheses or in regions where the tendon passes over a bony pulley (sometimes termed "wrap-around regions") [105]. The mechanical microenvironment within these regions alters significantly over a short physical distance. The role of mechanical forces in the development of fibrocartilage is discussed further in Section 1.8.1. Although the precise origin of the fibrochondrocytes in the tissue is not known, Vogel et al have hypothesised that the phenotype of the native cell population is plastic allowing for metaplastic adaptation to the local environment [106].

1.4.3.2 An Overview of Fibrocartilage

In keeping with its role as a "transitional tissue", there is a continuous gradation of fibrocartilage phenotypes ranging from an almost tensional phenotype to one that is close to hyaline cartilage [107]. The precise characteristics of a given region depend upon both age [108] and the magnitude of the compressive force to which the region is exposed [107]. However, some generalisations may be made.

1.4.3.3 Fibrochondrocytes

The resident cell population, sometimes referred to as fibrochondrocytes, are spherical with visible cytoplasm, a morphology reminiscent of chondrocytes in hyaline cartilage. In common with chondrocytes, they reside in lacunae within an amorphous pericellular matrix which stains positively with toluidine blue. The territorial matrix of fibrocartilage contains aggrecan and often a small quantity of collagen II. Unlike hyaline cartilage, however, the major collagenous component is collagen I [105]. The collagen fibres are often less regularly arranged than in tendon and may be woven among the cells [107]. Fibrochondrocytes stain positively for actin [109]. Chondrocytes have a cortical arrangement of actin filaments, which maintains the rounded cell shape no filaments. The arrangement of actin filaments in fibrochondrocytes has not been described although it seems likely that they will also have a cortical distribution .

The relative contributions of anaerobic and aerobic respiration to the generation of ATP in fibrochondrocytes is not known, however, fibrocartilage is avascular, which is suggestive of a stronger contribution from anaerobic pathways.

1.5 A Comparison of Extracellular Matrices in Connective Tissues

1.5.1 ECM Mechanical Properties

The function of tendon, fibrocartilage and hyaline cartilage is to withstand the mechanical forces placed upon them. The forces placed upon each tissue are different, with tendon being subjected to predominantly tensile forces, cartilage to

compressive forces and fibrocartilage to a combination of the two. In order to deal with the mechanical forces placed upon them, the resident cell populations secrete and maintain a specialised ECM. These ECMs have many components in common, however the proportions of a given component may differ between tissues. The absolute list of components present, their relative amounts and their spatial organisation create the mechanical properties of the ECM.

This section will discuss the composition of healthy tendon and cartilage ECMs before addressing the effect of tendon degeneration on tendon matrix composition and organisation. Fibrocartilage will not be covered extensively, simply because less is known about the make-up of this tissue. Additionally, as has previously been stated, fibrocartilage is a transition tissue, and as such it is difficult to assign it an absolute matrix composition. It is probably better viewed as a continuum of matrix (see Figure 1.10), especially enthesial fibrocartilage which blends from tendon ECM into bone in a manner not dissimilar to hyaline cartilage.

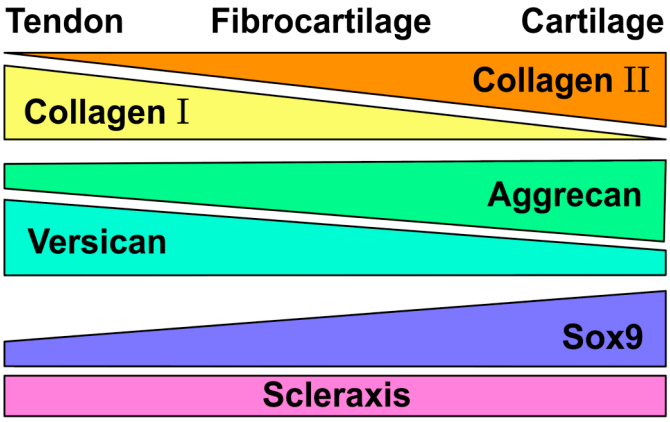


Figure 1.10 Transcription Factor and Matrix Compositional Changes between Tensional Tendon, Fibrocartilage and Hyaline Cartilage

Diagram showing a simplified and, in some cases, extrapolated version of transcription factor and matrix compositional changes between tensional tendon, fibrocartilage and hyaline cartilage.

1.5.1.1 Small Leucine Rich Proteoglycans

See also Table 1.3.

Small leucine rich proteoglycans (SLRPs) are biologically active components of the ECM which belong to the leucine rich repeat superfamily of proteins. The SLRP family contains 17 proteins. These are currently classified into 5 families on the basis of conservation and homology at the protein and genomic levels [110]. SLRPs have a central leucine rich repeat region flanked by conserved cysteine repeats. Several members of this family may be post-translationally modified by the addition of one or more sGAG chains (including chondroitin sulphate, dermatan sulphate and keratan sulphate) although the presence or absence of the sGAG chain(s) can vary between tissues for the same SLRP. Other post translational modifications of SLRPs include glycosylation and tyrosine sulphation [111].

The SLRPs are believed to control collagen fibrillogenesis, with knock-out animals often showing a tendon phenotype. In vitro experiments suggest that SLRPs are able to interact with collagen at specific binding sites allowing the regulation of fibril size or the delay of fibril formation [112]. They are also hypothesised to protect against degradation of collagen by matrix metalloproteinases [113] and to contribute to matrix integrity by interacting with both collagens and other matrix components [112].

Protein	SLRP Class	Tensional Tendon	Fibrocartilage	Hyaline Cartilage	Notes
Biglycan	I	Present [40, 122, 124, 125]	Present [122]	Present [117]	Transcription increased by compressive loading [114, 115] Binds collagen, affects collagen-fibril formation, binds growth factors [116]
Decorin	I	Present - predominant small proteoglycan [123, 125]	Present [123]	Present - most abundant SLRP mRNA [118]	Binds collagen, affects collagen-fibril formation, binds growth factors [116]
Asporin	I	?	Present (meniscus) [119]	Present [119]	-
Fibromodulin	II	Present in unsulphated form [40] - minor component [122]	Present [122]	Present [120, 121]	Binds collagen, affects collagen-fibril formation [116]

Table 1.3 Small leucine rich proteoglycans in cartilage, fibrocartilage and tendon ECM (continued overleaf).

Protein	SLRP Class	Tensional Tendon	Fibrocartilage	Hyaline Cartilage	Notes
Keratocan	II	Present [126, 129] More mRNA than hyaline cartilage [76]	Present [126]	Present [126, 129]. Less mRNA than tensional tendon [76]	In tendon and hyaline cartilage core protein either does not contain keratan sulphate, or contains poorly or non-sulphated keratan [126]
Lumican	II	Present [122]	Present [122]	Present [118, 127]	Binds collagen, affects collagen-fibril formation, binds growth factors [116]. In contrast to juvenile cartilage, adult cartilage form lacks keratan sulphate chains, existing as a proteoglycan only [127]
PRELP	II	Absent in human patellar tendon [128]. Present in bovine deep flexor tendon at the same concentrations as average cartilage [120, 131]	Low concentrations in meniscus [128]	High concentrations in adult tissue [128]	Cartilage core protein often does not contain keratan sulphate [120, 128].
Chondroadherin	IV	mRNA present in rat tail tendon. Protein undetectable by IHC [130]	?	Present in large quantities [130]	-

Table 1.3 (continued) Small leucine rich proteoglycans in cartilage, fibrocartilage and tendon ECM.

1.5.1.2 Lectican

Aggrecan and versican are proteoglycans of the lectican family. They have a central protein core which is post-translationally modified by the addition of various glycosaminoglycans. Both proteoglycans contain chondroitin sulphate side chains. Versican additionally contains dermatan sulphate, while aggrecan additionally contains keratan sulphate. Both aggrecan and versican have been described as having anti-adhesive properties [132]. Aggrecan and versican show opposing levels of expression. Tissues appear to express high levels of one or the other but not both equally. In normal midsubstance Achilles tendon, mRNA levels were found to be higher for versican than for aggrecan [133] while the reverse was found in articular cartilage [118].

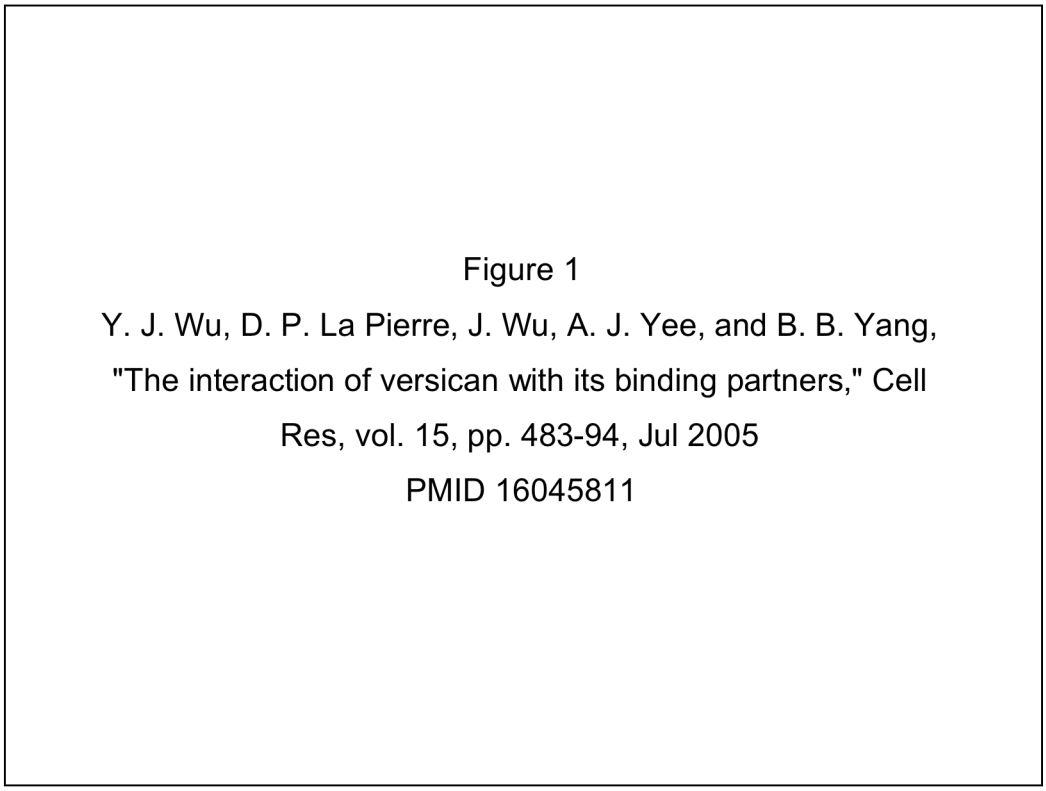


Figure 1.11 Structure of Aggrecan and Versican

doi = 10.1038/sj.cr.7290318

Isoform	GAG Domains		Estimated Number of GAG chains	Predicted Core Protein	Observed* Core Protein
	α	β		Molecular Weight/kDa	Molecular Weight/kDa
V_0	√	√	17-23	370	550
V_1	X	√	12-15	262	500
V_2	√	X	5-8	180	400
V_3	X	X	0	72	?

Table 1.4 Versican isoforms

* = Observed values from SDS-PAGE where samples are first subject to chondroitinase ABC treatment [134].

Figure 1
T. N. Wight, "Versican: a versatile extracellular matrix proteoglycan in cell biology," Curr Opin Cell Biol, vol. 14, pp. 617-23, Oct 2002
PMID 12231358

Figure 1.12 A model of the different isoforms generated by alternative splicing of the mRNA transcript for versican.

doi = 10.1016/S0955-0674(02)00375-7

1.5.1.2.1 Versican

Versican is expressed in many tissues of the body [96]. It has many functions including adhesion, migration, angiogenesis and apoptosis [96, 136]. The precise function of versican in any given situation is highly context dependent, leading to sometimes contradictory reports of its effects [96].

The transcript from the versican gene (CSPG2) is subject to alternative splicing of exons 7 and 8 resulting in four different versican isoforms known as V_0, V_1, V_2 [134] and V_3 [137] (see Table 1.4). These are predicted to carry varying numbers of sGAG chains from 17-23 for V_0 [134] to 0 for V_3 [137]. Intriguingly, the splice variants expressed in tendon and cartilage appear to vary slightly, with tensional tendon expressing mRNA for all versican isoforms [138] while adult cartilage and intervertebral disc express only the V_1 isoform [139][3]. The V_0 and V_1 isoforms are thought to be the most abundantly expressed isoforms in tendon.

A recent study has found that 98% of versican fragments in bovine deep flexor tendon lack the G1 domain, although they appear to have retained the G3 domain which the authors speculate is important to their retention in the ECM [125]. Most of the versican fragments appear to be generated from V_0 and V_1 isoforms [125]. Cleavage appears to occur mostly at three sites in the GAG-β domain between D^{1329}-S^{1330}, E^{2187}-S^{2188} and E^{2628}-S^{2629} (amino acid numbering taken from V_0 isoform)(see also Figure 1.13) [125]. Cleavage at the first site is believed to be attributable to cysteine peptidases such as cathepsin K, L and S [125]. Cleavage

[3] Although foetal cartilage additionally expresses mRNA for the V_0 and V_3 isoforms. Disappointingly the authors fail to show an image which confirms that the primers for V_2 isoform are capable of successfully detecting this mRNA.

at the latter two sites, in contrast, appears to be caused by aggrecanases [125]. Recently members of the A Disintegrin And Metalloproteinase with Thrombospondin Motifs (ADAMTS) family were identified as aggrecanases, specifically ADAMTS -1 [140], ADAMTS-4 [141], ADAMTS-5 [142, 143], ADAMTS-9 [144] and ADAMTS-15 [145], although it is possible that the family contains additional, as yet unidentified members with aggrecanase activity. Human Achilles tendon is known to express mRNA for a wide range of members of the ADAMTS family [14]. Strangely, although ADAMTS cleavage sites have been reported in human versican isoforms V_1 [146] and V_2 [147], the cleavage sites reported in this study do not correspond to the bovine equivalent of the human sites [125].

Versican mRNA is upregulated in response to compressional loading in foetal bovine tendon [115]. However, versican mRNA levels were not significantly affected by either compressional or tensional loading regimes in excised adult rabbit medial collateral ligament [148]. Dynamic compression did not result in statistically significant differences in versican mRNA levels in adult bovine intervertebral disc organ culture [149]. Whether mechanical loading affects versican transcription levels in cartilage is currently not known.

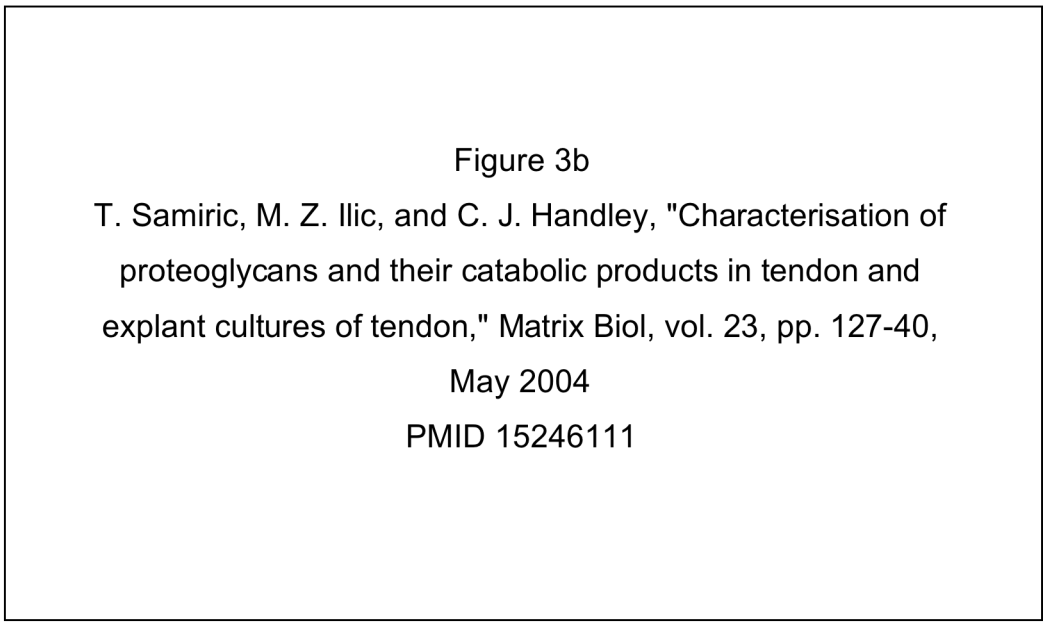


Figure 1.13 Schematic representation of cleavage sites and degradation fragments of versican observed in fresh tissue and tendon explant cultures.

doi = 10.1016/j.matbio.2004.03.004

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1.5.1.2.2 Aggrecan

In contrast aggrecan is strongly expressed in cartilage but only weakly expressed in tensional tendon [150], although a study of human Achilles tendon reports that expression is restricted to the fibrocartilaginous regions only [122]. Studies of the tensional region of the bovine deep flexor tendon indicate that approximately 65% of the aggrecan molecules in this region lack the G1 domain which mediates binding to hyaluronan [125, 150]. These molecules also appear to lack keratan sulphate chains [150].

1.5.1.3 Glycoproteins

Both tendon and cartilage contain small quantities of glycoproteins in addition to collagenous and SLRP components. See Table 1.5.

Glycoprotein	Tensional Tendon	Fibrocartilage	Cartilage	Notes
COMP	Present. Accounts for ~3% of the dry weight [153]	Present [153]	Present. Less than tendon [153].	Involved in the control of collagen fibrillogenesis [151]
Tenascin C	Present [160]	Present - increased amounts compared to tensional tendon [156, 157]. Particularly associated with rounded fibrochondrocytes [158].	Present [154]	Upregulated by tensile strain. This is dependent on ROCK activity. [152]
Elastin?	Present ~ 2% dry mass of tendon [161]	Absent [159]	Present in elastic cartilage only	-
Fibronectin	Present in low levels [162, 163]	?	Present in low levels. 50-80% of chains in articular cartilage are unique (V + C)− form [155].	-

Table 1.5 Glycoproteins in cartilage, fibrocartilage and tendon ECM.

1.5.1.4 Collagens

Collagen accounts for two thirds of the dry weight of adult articular cartilage [164] and between 60 and 80% of the dry weight of tendon [1]. However, hyaline cartilage is mostly collagen II while tendon is mostly collagen I. See Table 1.6.

Over 20 different collagen proteins have been described. They are divided in to 7 major groups according to structure [165]. There are 3 groups of particular relevance to tendon and cartilage. Fibrillar collagens such as collagens I, II and III form the major component in connective tissues. Fibril Associated Collagens with Interrupted Triple helices (FACIT collagens) such as collagen IX in cartilage contain short triple helix regions which are interrupted by short non-collagenous domains, although the N-terminal non-collagenous region can be quite sizeable. They are not covalently polymerised in the matrix but are believed to physically bind to the surface of fibrillar collagens. They form a minor component in percentage terms of connective tissues. Network forming collagens include collagen X in hypertrophic cartilage.

Structurally, collagen I and II form different macromolecular complexes with different matrix partners required for assembly. Collagen I is a heterotrimer of two α1(I) chains and one α2(I) chain, while collagen II is a homotrimer of α1(II) chains and collagen III is also a homotrimer of α1(III). Collagen II requires collagens IX and XI to form fibrils, while collagen I requires collagen V [166] and fibronectin (Figure 1.14). Typically collagen II fibres are thin, around 20-200nm, while collagen I is much thicker at 80-1000nm [167].

Collagen	Type	Tensional Tendon	Fibrocartilage	Cartilage	Notes
I	Fibrillar	Present ~ 60% of dry mass 95% of collagen [1]	Present [105]	Absent	-
II	Fibrillar	Absent	Present [105]	Present > 90% of total collagen [169]	-
III	Fibrillar	Present ~ 3% collagen in tendon [1]	Present [105]. More than tensional canine rotator cuff tendon [171]	Present > 10% of total collagen [169]	-
V	Fibrillar	Present [173]	Present in meniscus and intervertebral disc [172]	α1(V) chain present [170] in pericellular matrix	Regulates fibrillogenesis [168]
VI	Beaded filament forming	Present in PCM [174]	Present around fibrochondrocytes [122, 131]	Present in chondrons [164] <1% of total collagen [169]	Cell associated
IX	FACIT	-	Present [109]	Present [164] 1% of total collagen [169]	-

Table 1.6 Collagens in cartilage, fibrocartilage and tendon ECM (continued overleaf).

Collagen	Type	Tensional Tendon	Fibrocartilage	Cartilage	Notes
X	Network forming	Absent [184]	Present [158, 181] in the mineralized region only [109]	Present in hypertrophic regions [164]	-
XI	Fibrillar	α1(XI) accounts for 30% of α chains in type V collagen [173]	Present [182]	Present [164] 3% of total collagen [169]	Regulates fibrillogenesis [168]
XII	FACIT	Present in adult tendon XIIB-1 type mRNA [176]	Present [105]	Present in adult cartilage XIIB-2 type mRNA [176]	Upregulated by stretch [175]. May be involved in promoting the alignment of collagen fibres [176].
XIII	Transmembrane	Absent (in foetal tendons, probably also in adult, mRNA data) [179]	?	Present [179]	-
XIV	FACIT	Present [180, 185]	Present [183]	Present [180]	Important for developmental fibrillogenesis [177, 178]

Table 1.6 continued Collagens in cartilage, fibrocartilage and tendon ECM.

Collagen	Type	Tensional Tendon	Fibrocartilage	Cartilage	Notes
XVI	FACIT	?	?	Present in the territorial matrix [188]	-
XVIII	Endostatin	Absent (as endostatin) [186]	Present around fibrochondrocytes (as endostatin) [186]	Present (as endostatin) [189]	Non-collagenous domain 1 may become cleaved - this is known as endostatin. Endostatin is anti-angiogenic. Upregulated by hydrostatic pressure [186].
XX	FACIT	mRNA present in day 17 chick embryos [190]	?	mRNA present in day 14.5 chick embryos [190]	Minor ECM component.
XXIII	Transmembrane	Present (mRNA from mouse sternal cartilage) [179]	?	Absent (mRNA from mouse sternal cartilage) [179]	mRNA relatively low abundance, may be present at lower concentrations than COL5A1 [179]
XXVII	Fibrillar	Absent (no mRNA expression in mouse tail) [192]	?	Present particularly concentrated around hypertrophic chondrocytes [191]	Promoter of COL27A1 has a Sox9 responsive enhancer [187]

Table 1.6 continued Collagens in cartilage, fibrocartilage and tendon ECM.

Figure 10a

R. J. Wenstrup, J. B. Florer, E. W. Brunskill, S. M. Bell, I. Chervoneva, and D. E. Birk, "Type V collagen controls the initiation of collagen fibril assembly," J Biol Chem, vol. 279, pp. 53331-7, Dec 17 2004

PMID 15383546

Figure 1.14 **Schematic diagram of collagen fibril formation**

doi = 10.1074/jbc.M409622200

Figure 1

K. E. Kadler, A. Hill, and E. G. Canty-Laird, "Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators," Curr Opin Cell Biol, vol. 20, pp. 495-501, Oct 2008

PMID 18640274

Figure 1.15 Schematic of the 10 + 4 microfibril structure of a thin cartilage collagen fibril.

doi = 10.1016/j.ceb.2008.06.008

1.6 Matrix Effects of Tendinopathy

Torn tendons frequently have a matrix which is not only disordered in its structure but also has an altered biochemical composition. It is unclear how much of this altered matrix composition is attributable to pathogenic degeneration and how much is attributable to a healing response. This section describes the changes in matrix molecules which are observed in tendinopathic samples.

1.6.1 Collagens in Degenerate Tendons

Degenerate [22] and torn tendons [193, 194] show increases in collagen I transcription consistent with an attempt at repair. Similar increases in collagen III transcription are also seen in degenerate [22] and torn tendons [194, 195]. However, it is likely that the cell response is unbalanced in favour of collagen III, since this protein is reported to have increased proportions in torn rotator cuff [37]. This is significant since collagen III tends to produce smaller, less organised fibrils [1].

Gigante et al. report, and staining undertaken during this thesis has confirmed, the presence of collagen II in some torn rotator cuff tendons [184]. Since this constitutes a characteristic of chondroplasia it will be discussed at greater length in Section 1.7.1.

1.6.2 SLRPs in Degenerate Tendons

Most reports find a decrease in decorin transcription in ruptured tendon samples [133, 194]. This could be interpreted as a healing response since decorin has been

associated with cell cycle arrest [111, 196] and the inhibition of cell migration [197]. Corps et al reported increased biglycan transcription in painful tendinopathy but unchanged levels in ruptured Achilles tendons [133]. Similarly, Samiric et al. reported increased deposition of biglycan in tendinopathic tissue and greater fragmentation of biglycan in pathological tendons, suggesting active remodelling [198]. Increased levels of intact fibromodulin have been reported in tendinopathic tendons [198] however there was a downward trend in fibromodulin transcription in this study.

1.6.3 Lecticans in Degenerate Tendons

1.6.3.1 Versican in Degenerate Tendon

Versican levels have not been reported for torn rotator cuff tendon. Reports for expression in ruptured Achilles tendon vary. Although an initial study found decreased mRNA levels [138] the authors subsequently found that the housekeeper that was used was affected by the disease process [133]. A later study published by the same group found no change in versican mRNA levels in diseased tendon compared to healthy controls [133].

To date only a single study has examined versican protein distribution. This study was undertaken in patellar tendon. The authors found increased levels of versican in regions of chondroplasia with expression particularly concentrated in the matrix surrounding the fibrochondrocytic cells [27]. A similar pattern of expression, although much more pericellular in nature, has been reported in elastic cartilage but not in hyaline cartilage [199]. The exact significance of the apparent

upregulation of versican in chondroplastic regions is uncertain, particularly since versican has already been reported to be present in the pericellular matrix of tenocytes [174].

1.6.3.2 Aggrecan in Degenerate Tendon

Aggrecan core protein (ACAN) mRNA levels were found to be increased in the margins of torn rotator cuff tendon compared to cadaveric controls [194]. They were found to be similarly increased in painful Achilles tendinopathy, although they were not elevated in samples of ruptured Achilles tendon [133].

1.6.4 Glycoproteins in Degenerate Tendons

An increase in staining for fibronectin has been noted in ruptured tendons in both the Achilles [200] and the rotator cuff tendons [201]. Tillander et al. [201] regard the presence of fibronectin as an indicator of a stalled healing process in ruptured tendons.

In degenerate rotator cuff tendons there is an alteration both in the location and the isoform of the glycoprotein tenascin-C. In healthy supraspinatus tenascin-C is associated with the fibrous regions of the tendon matrix, whereas in chondroplastic regions it is strongly associated with the rounded fibrochondrocytes [160, 184]. Tenascin-C is reported to have anti-adhesive properties, suggesting it may play a role in tenocyte rounding and therefore in the pathogenesis of chondroplasia [160]. Gigante et al. regard the localisation of

expression of tenascin-C to the immediate area of the cell as an early step in their proposed mechanism of chondroplastic degeneration [184].

1.7 Rotator Cuff Tears

Perhaps the most common degenerate tendinopathy is rotator cuff tear. A recent meta-analysis has suggested a prevalence of 30.3% for rotator cuff tears within the cadaveric population [202]. The cause of these tears is unknown and they are difficult to treat effectively. These tendons undergo a slow series of degenerative changes, including the generation of fibrocartilage. The precise role of chondroplasia in cuff tear is uncertain, particularly since it is not clear whether this process begins prior to the initiation of the tear or whether it develops as a consequence of the tear. Chondroplasia is, however, likely to alter the mechanical properties of the cuff and to impede the healing of the tendon.

Matthews et al. [28] compared rotator cuff specimens from control samples and tears graded according to size using the system of Post et al. [203] for degenerative changes including chondroplasia. Fibrocartilaginous metaplasia, as determined by cell morphology and toluidine blue staining, was found in 73% of the tear samples, with specimens from medium, large and massive tears showing considerably more evidence of chondroplasia than those from small tears. Since small tears are believed to become larger tears this suggests a possible progression of disease. Small tears are believed to have more potential for healing, while the larger the tear becomes the more chondroplastic it becomes and the more the potential for successful repair becomes compromised. An understanding of the

process of chondroid metaplasia could therefore enable screening and early intervention.

Most groups that have investigated chondroplasia have viewed it as one of a number of degenerative processes that occur within the tendon tissue prior to tearing [184]. However, we believe that the differential distribution of chondroplasia across the tear size series suggests a post-tear origin for the metaplasia. This post-tear origin hypothesis has accordingly influenced the reasoning for the selection of the potential drivers investigated in this thesis.

1.7.1 Histological Features of Chondroplasia

In regions of chondroplasia the cells take on a more chondrocytic morphology becoming rounded lying within lacunae. The surrounding collagen fibres are disorientated. The ECM of chondroplastic regions contains large quantities of sulphated glycosaminoglycans (as determined by metachromatic staining) [28] (Figure 1.16).

Additionally Gigante et al. have reported positive staining for chondrocytic markers including collagen II. Staining of rotator cuff tendons carried out during this thesis confirmed these findings (Figure 1.17). Although there is some diffuse staining throughout much of the chondroplastic tissue, regions of strong staining are present around the lacunae containing the fibrochondrocyte-like cells, suggesting that the cells have begun a de novo production of a cartilage-like matrix. Significantly, these regions of strong positive staining are only seen in the context of rounded fibrochondrocyte-like cells, not in the presence of elongated

tenocytes, which suggests that cell shape change and production of collagen II are closely linked. Indeed it is possible to see rounded fibrochondrocyte-like cells lying within lacunae without collagen II but not strong collagen II staining without rounded fibrochondrocyte-like cells, suggesting that cell rounding may precede collagen II secretion.

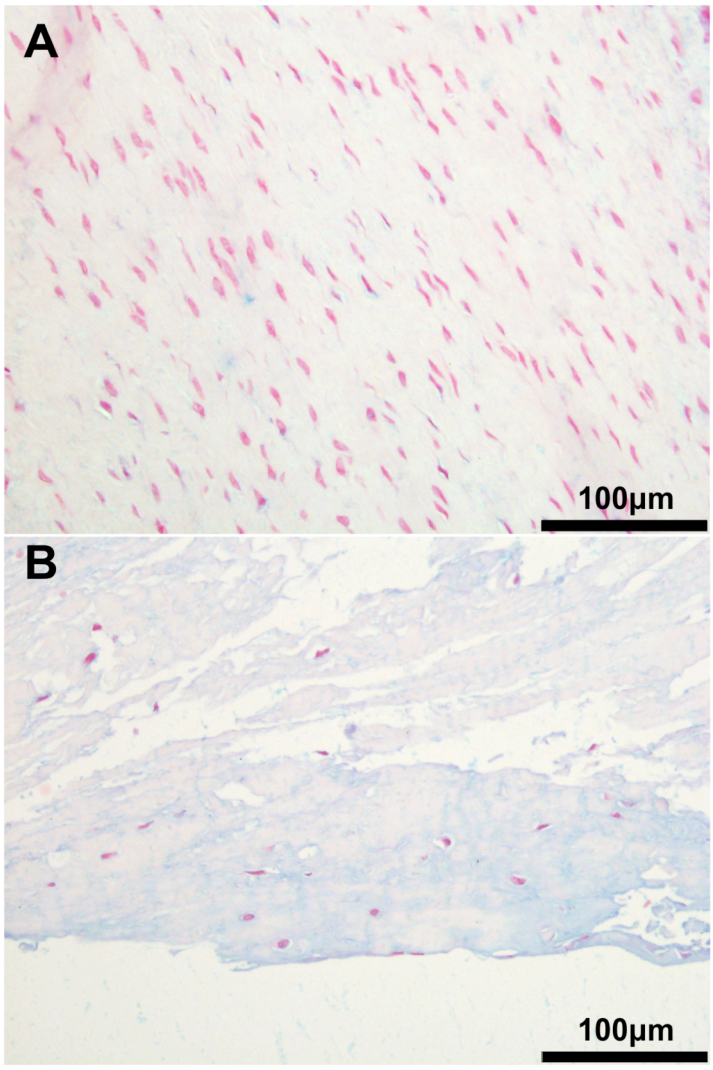


Figure 1.16 Alcian Blue Staining of Torn Rotator Cuff Tendon

3µm wax embedded sections of torn rotator cuff tendon from were stained with Alcian blue and counterstained with nuclear fast red (Staining performed by Mr Matthew Burgess. Images taken by the author). A = small tear with tensional morphology, B = massive tear showing chondroplasia.

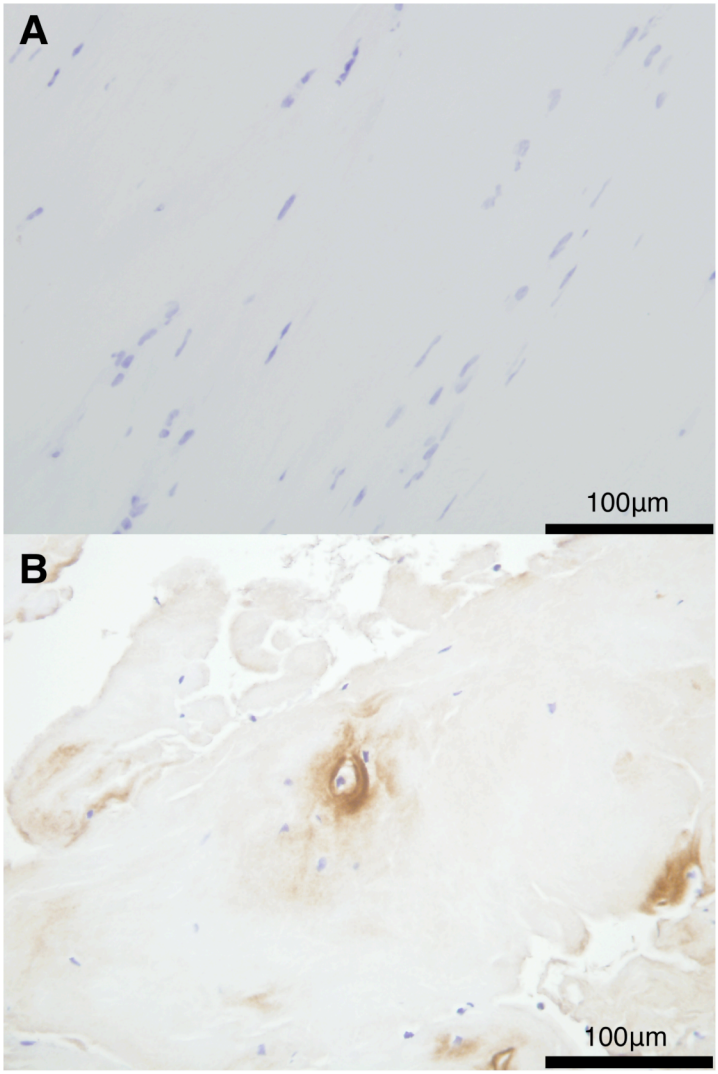


Figure 1.17 Collagen II Staining of Torn Rotator Cuff Tendon

3µm wax embedded sections of torn rotator cuff tendon from were stained with anti-collagen II and counterstained with haematoxylin. A = small tear showing tensional morphology, B = massive tear showing chondroplasia.

1.8 Potential Drivers of Chondrogenesis

The environment of the degenerating rotator cuff is altered compared to that of normal cuff tendon. Successive repairs of microtears may have already changed not only the gross mechanical properties of the tissue but also the mechanical

microenvironment surrounding many of the tenocytes. Upon partial or full-thickness tearing the tendon becomes subject to hypoxia due to the severing of the blood vessels which feed the tendon. There is also a loss of tensile loading when the tendon is torn, with consequent alterations of cell shape and cytoskeletal rearrangement. Additionally, the tissue may be subject to compression due to swelling of the torn cuff or the bursa. These are serious insults to a mechanically sensitive tissue which normally respires aerobically. This thesis hypothesises that the tenocytes respond to the profoundly altered environment of the chronically torn rotator cuff by transdifferentiating into fibrochondrocytes. There are three major environmental pressures which may induce this phenotypic shift: compression, cell shape change induced by a lack of tensile loading or by an altered ECM and hypoxia. These will be discussed in turn.

1.8.1 Compression

Perturbation of the mechanical environment of the shoulder, particularly the abnormal application of compressive forces to the tendon tissue, has been suggested as a driver of chondroplasia by some authors [184]. Compressive forces are implicated in the development of non-pathological regions of fibrocartilage within tendons. The fibrocartilaginous region of the Achilles tendon which is in contact with the calcaneus does not appear until after birth, when the tendon becomes heavy load bearing for the first time [204]. The fibrocartilage continues to develop as the organism ages [108]. As noted previously, non-pathological fibrocartilage is found in regions of tendons which are normally in contact with a bony pulley. The degree of fibrocartilaginous phenotype appears to increase with increasing compressive forces [107]. Surgical

rerouting of tendons which normally wrap around a bony pulley to eliminate the compressive loading caused by contact with the bone results in a decrease in the levels of sGAG, link protein and collagen II with an almost complete loss of fibrocartilage after four weeks [205, 206]. Adult and foetal bovine fibrocartilage explants subjected to in vitro compression show increased expression of the fibrocartilaginous proteoglycans aggrecan and biglycan and decreased expression of decorin, a proteoglycan typically expressed in the tensional region of tendons [114, 207]. However, there is no consensus in the literature as to whether the rotator cuff tendons are subject to compressive force, and if so at which stage of the disease this compression occurs and which particular bone(s) are responsible.

Potential sources of compression in the shoulder include the acromion and associated bony prominences known as enthesophytes. Neer et al. hypothesised that contact with the underside of the acromion could contribute to tendon degeneration [208]. This could occur in two ways, by an alteration to acromial shape or by an alteration in the physical location of the rotator cuff tendon in relation to acromion.

Bigliani et al. have classified phenotypic variations in the shape of the acromion and have suggested that patients with a shape known as Type III (hooked morphology) have a higher frequency of rotator cuff tears [209]. This shape of acromion also seems most prone to the development of enthesophytes which may cause further compression and friction [210]. On the basis of this evidence they proposed a role for such structures in the initiation of rotator cuff tear. This view has been criticised in recent years, with authors suggesting that the Type III

acromion is produced by ossification of the coracoacromial ligament and that it is a secondary consequence of rotator cuff tear rather than the cause [10]. However, this does not preclude its potential importance in the development of chondroplasia, which our group views as another secondary consequence of rotator cuff tear. No data currently exist as to the relationship between acromion shape, the presence of enthesophytes and chondroplasia.

Alternatively, the rotator cuff tendon itself may shift position within the joint relative to the acromion. As the tear size increases the shoulder joint may become increasingly unstable, since the force of the supraspinatus is unable to be transmitted due to a hole in the tendon. This may result in the muscles of the shoulder joint pulling the head of the humerus into the acromion, a phenomenon known as superior humeral subluxation. Once again the relationship between superior humeral subluxation or, indeed, the acromiohumeral interval and chondroplasia is unknown.

However, it should be noted that chondroplasia is reported to occur initially on the articular surface (furthest away from the acromion) rather than the bursal surface [211], a feature which would suggest that compression (at least from the acromion) is not a significant feature of the aetiology of this condition.

1.8.2 Cell Shape and the Actin Cytoskeleton

This section first considers the relationship between cell shape, particularly that induced by the arrangement of the actin cytoskeleton and chondrocyte phenotype. It will then discuss the relationship between the RhoA signalling pathway, actin

structure and mesenchymal stem cell differentiation with special reference to chondrocytes. Finally it will detail cell shape changes in the chondroplastic rotator cuff tendon considering the role of mechanical signalling potentially transmitted via RhoA to actin structure, cell shape and cell phenotype.

1.8.2.1 Cell Shape, the Actin Cytoskeleton and the Chondrocytic Phenotype

1.8.2.1.1.1 Actin Cytoskeletal Structure is Important to Chondrocyte Differentiation

Cell shape as mediated by the actin cytoskeleton is thought to play an important role both in the differentiation of chondrocytes from mesenchymal stem cells and in the maintenance of this phenotype. [212]. During differentiation cells alter their morphology from fibroblastic to rounded. Concurrently the actin cytoskeleton undergoes a rearrangement into a cortical structure. The cells begin to express the chondrocytic transcription factors Sox5, Sox6 and Sox9. [213]. This is followed by upregulation of chondrocyte genes including the matrix proteins aggrecan and collagen II. [212].

1.8.2.1.1.2 Actin Structure is Important to Maintenance of Chondrocyte Phenotype

Chondrocytes cultured in monolayer dedifferentiate after a small number of passages, altering their morphology from round or polygonal to fibroblastic [214]. The physiological cortical arrangement of actin is lost and stress fibres form. The cells downregulate expression of classical chondrocyte genes Sox5, Sox6, Sox9 and collagen II [94]. Expression of fibroblast-associated genes including collagen

I and collagen III is upregulated. Culture in a matrix which supports cell rounding, such as alginate beads, results in a partial re-differentiation of these cells with the upregulation of chondrocytic marker expression and the downregulation of fibroblast-associated genes [215]. The same effect can be achieved with chemicals, such as cytochalasin D, which cause actin depolymerisation suggesting that the actin cytoskeleton plays a crucial role in the maintenance of the chondrocyte phenotype [216].

1.8.2.2 RhoA Activity and Mesenchymal Cell Phenotype

1.8.2.2.1.1 RhoA Signalling can Determine Eventual Phenotype in MSC Differentiation

RhoA is a member of a protein family known as the small GTPases which control many aspects of cell biology including the organisation of the actin cytoskeleton, cell cycle progression and gene expression. RhoA signalling has been shown to be important in the commitment of mesenchymal stem cells (MSC) to a particular lineage. Work by McBeath et al. suggests that increasing or decreasing the activity of RhoA can alter MSC commitment to either adipocyte or osteoblast phenotypes, even in the absence of soluble factors [217], with more active RhoA increasing osteogenesis and less active RhoA increasing adipogenesis. This function was thought to be mediated through the downstream effectors Rho kinase (ROCK) 1 and/or ROCK2.

1.8.2.2.1.2 RhoA Signalling is Important in Chondrocyte Differentiation

Osteoblasts and adipocytes are not the only mesenchymal cell types whose phenotype is affected by RhoA signalling. Recent work by the Beier group suggests that RhoA and its downstream effectors ROCK1 and ROCK2 may also play a part not only in the commitment of MSC to a cartilage lineage [218] but also to the progression of chondrogenesis itself [219]. Activated RhoA acts through ROCK1 and/or ROCK2 to maintain cells in a 'mesenchymal' phenotype, maintaining the expression of stress fibres, cell proliferation and inhibiting sulphated glycosaminoglycan (sGAG) synthesis. Inhibition of ROCK signalling results in increased sGAG synthesis and elevated Sox9 mRNA expression. Cell rounding and cortical actin organisation are also observed [218].

1.8.2.2.1.3 RhoA Signalling is Important in Maintaining Chondrogenic Phenotype

It appears that RhoA signalling also plays a role in the ongoing maintenance of the chondrogenic phenotype. Tew et al. have reported that the inhibition of ROCK1 and ROCK2 in dedifferentiated adult articular chondrocytes resulted in the upregulation of Sox9 mRNA expression [220]. Additionally, our group [221] and others [222] have found that statins, inhibitors of protein prenylation which disturb the intracellular location and function of small GTPases such as Rho, have a similar effect.

1.8.2.3 Cell Shape Change, Loss of Tensile Loading and Rotator Cuff Tear

It is not known precisely what initiates the cell shape change seen in chondroplastic regions. Previous studies have suggested alterations in the ECM including alterations in the location of the anti-adhesive protein tenascin-C [184]. There is, however, a possible link between the loss of tensile loading experienced by torn tendon tissue and signalling which could lead to chondroid metaplasia. These two possibilities will be discussed and a putative mechanism for cell shape change via actin cytoskeletal signalling presented.

1.8.2.4 Tensile Loading and Tenocyte Phenotype

Little work has been done on the effects of the loss of tensile loading on tenocytes. However, Hannafin et al. noted that after four weeks of ex vivo organ culture the tenocytes in unstretched canine flexor digitorum profundus tendon were more rounded than their stretched counterparts [223]. Work by Lavagnino et al. [224] has demonstrated a downregulation of COL1A1 mRNA expression upon release of tension from tenocytes in 3D culture. Interestingly, a concurrent loss of actin cytoskeletal organisation was noted. Treatment of tenocytes subject to tension with cytochalasin D resulted in a similar downregulation of COL1A1 possibly indicating a shared signalling pathway.

1.8.2.5 Tendon ECM and Tenocyte Phenotype

Tenocytes reside in a specialised ECM to which they are attached through various proteins including integrins. The extracellular portion of these receptors binds to the ECM components while the intracellular portion is able to recruit various signalling molecules, some of which are able to signal through RhoA to the actin cytoskeleton.

Our previous work and those of other authors have demonstrated that the ECM of degenerate tendon is profoundly altered [28, 184, 225-227]. Given the mechanical inter-relationship between tenocytes and the ECM it is likely that an altered ECM would result in altered integrin signalling potentially leading to alterations in actin filament structure.

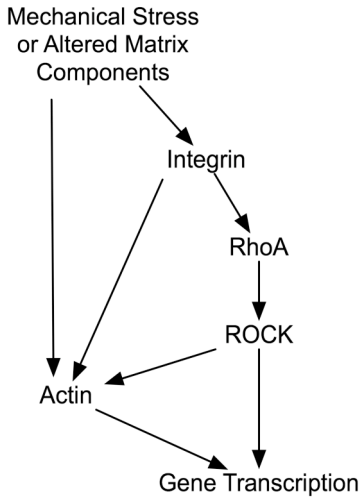


Figure 1.18 Putative Mechanism for Induction of Cell Shape Change

1.8.3 Hypoxia

In 1931 Codman identified a region of the supraspinatus tendon that he believed was relatively avascular [32]. Since this region co-incides with a region of the tendon in which tears frequently occur it has been hypothesised that the potentially hypoxic conditions of such a region could contribute to tendon degeneration. Over the years there has been much debate as to whether the supraspinatus tendon does have a compromised blood supply [228] or whether the blood supply could be intermittently compromised by normal mechanical actions. Some authors have suggested that normal adduction of the arm with neutral rotation (a "resting" position with the arms held beside the body) has the effect of "wringing out" the blood vessels thus rendering a portion of the supraspinatus tendon hypoxic [33]. Movement of the arm would return blood to the vessels potentially creating conditions not unlike those seen in reperfusion injury. No work appears to have been done at a cellular level to examine the effects of reperfusion on tenocytes. This thesis will also not examine these effects since it is concerned, not with degeneration in all its forms, but with chondroplasia specifically. Reperfusion does not seem, a priori, to be a good model for the induction of chondroplasia since reperfusion is not a phenomenon believed to occur during embryonic chondrogenesis, the normal development of fibrocartilage or during the initial stages of fracture healing.

Sustained hypoxia may occur in the rotator cuff either as a result of an avascular zone or as the result of tendon tearing. Any rupture, whether microtear or complete, will damage blood supply in addition to damage to the tendon matrix. This vascular compromise will result in a localised decrease in oxygenation in the

tissue which may last for weeks or months due to the slow repair speed of tendon. During this time tenocytes must make metabolic adaptations or die.

This section concentrates first on hypoxia inducible factor (HIF), a component one of the mechanisms by which oxygen concentration is sensed by the cell. It then moves on to detail the role of hypoxia in chondrogenesis before presenting evidence for hypoxic conditions within the torn rotator cuff.

1.8.3.1 Mechanisms of Oxygen Sensing in Cells

1.8.3.1.1 Hypoxia Inducible Factor

Hypoxia inducible factor (HIF) is a heterodimeric transcription factor, whose subunits belong to the basic-helix-loop-helix (bHLH) PER/ARNT/SIM (PAS) protein family. In the active form it is a dimer comprised of an alpha subunit and a beta subunit (also called aryl hydrocarbon receptor nuclear translocator (ARNT)). The two subunits interact with each other via their PAS domains and bind to a specific DNA sequence known as the hypoxia response element (HRE) via their bHLH domains (Figure 1.19). The α-subunit additionally contains a C terminal activation domain (CAD), which interacts with the transcriptional co-factors CBP/p300, and an N terminal activation domain (NAD), which is believed to be able to interact with other transcriptional co-factors. It also contains two oxygen dependent degradation domains (ODDD) that are the means by which HIF is regulated.

Figure 2

C. J. Schofield and P. J. Ratcliffe, "Oxygen sensing by HIF hydroxylases," Nat Rev Mol Cell Biol, vol. 5, pp. 343-54, May 2004

PMID 15122348

Figure 1.19 The domain structure of HIF1α and HIF1β/ARNT

doi = 10.1038/nrm1366

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Three different alpha subunits have described to date, HIF-1α, HIF-2α (also called EPAS-1 (Endothelial PAS domain-containing protein)) and HIF-3α. Little is known about HIF-3α but it is believed to act as a repressor of HIF signalling [230]. HIF-1α and HIF-2α are strongly homologous, especially in the bHLH and PAS domains (Figure 1.20). There is, however, less homology between the two isoforms in the NAD. Both HIF-1α and HIF-2α are subject to the same post-translational control mechanisms.

Evidence from knock-out mice suggests that despite their similarities HIF-1α and HIF-2α, at least, are non-redundant isoforms [231]. Some studies have suggested

that there may be differences in distribution between the two isoforms [231]. Other studies have suggested that some genes may exhibit a preference for a particular isoform [231]. Indeed this has recently been suggested for Sox9 [232]. The evidence for this position and the implications of these findings for chondroid metaplasia will discussed further in Section 6.3.5.

Figure 1
C. J. Hu, A. Sataur, L. Wang, H. Chen, and M. C. Simon, "The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha," Mol Biol Cell, vol. 18, pp. 4528-42, Nov 2007
PMID 17804822

Figure 1.20 Structure of HIF-1α and HIF-2α proteins.

doi = 10.1091/mbc.E06-05-0419

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1.8.3.1.2 Post Translational Regulation of HIF-α

HIF-α and HIF-β are constitutively produced by the cell. However, unlike HIF-β, HIF-α subunits are subject to post-translational regulation by hydroxylation of conserved amino acid residues by two groups of enzymes.

In normoxic conditions two prolines in the NAD are hydroxylated by prolyl-4-hydroxylases. This increases the affinity of the HIF-α subunit for the von Hippel-Lindau protein complex which targets the protein for degradation by the ubiquitin-proteasome pathway. Prolyl-4-hydroxylases require both oxygen and iron as co-factors.

In normoxic conditions an asparagine in the CAD is hydroxylated by factor inhibiting HIF (FIH). This prevents the binding of the transcriptional co-factor CBP/p300 and results in the inactivation of HIF-α subunit transcriptional activity. FIH also requires oxygen as a co-factor.

Under hypoxic conditions neither the prolyl-4-hydroxylases nor FIH can function, since both groups of enzymes require oxygen as a co-factor. The HIF-α subunits are therefore left un-hydroxylated. They do not become targets for the pVHL complex and are able to translocate to the nucleus. They can associate with CBP/p300, form a heterodimer with HIF-β, bind to HREs and activate gene transcription (Figure 1.21).

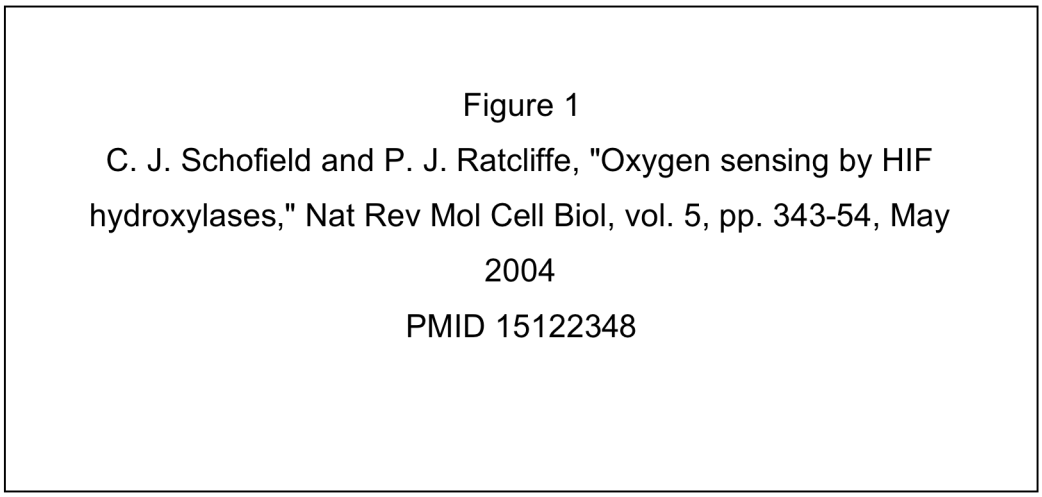


Figure 1.21 Dual regulation of HIFα subunits by prolyl and asparaginyl hydroxylation.

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1.8.3.1.3 Hypoxia Regulated Genes

Certain genes are often upregulated by hypoxia, regardless of the phenotype of the cell concerned. These genes are known as the classical hypoxia response genes and include pro-survival genes such as VEGF-A and anti-survival genes such as BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3). Hypoxia can upregulate a wide variety of response genes.

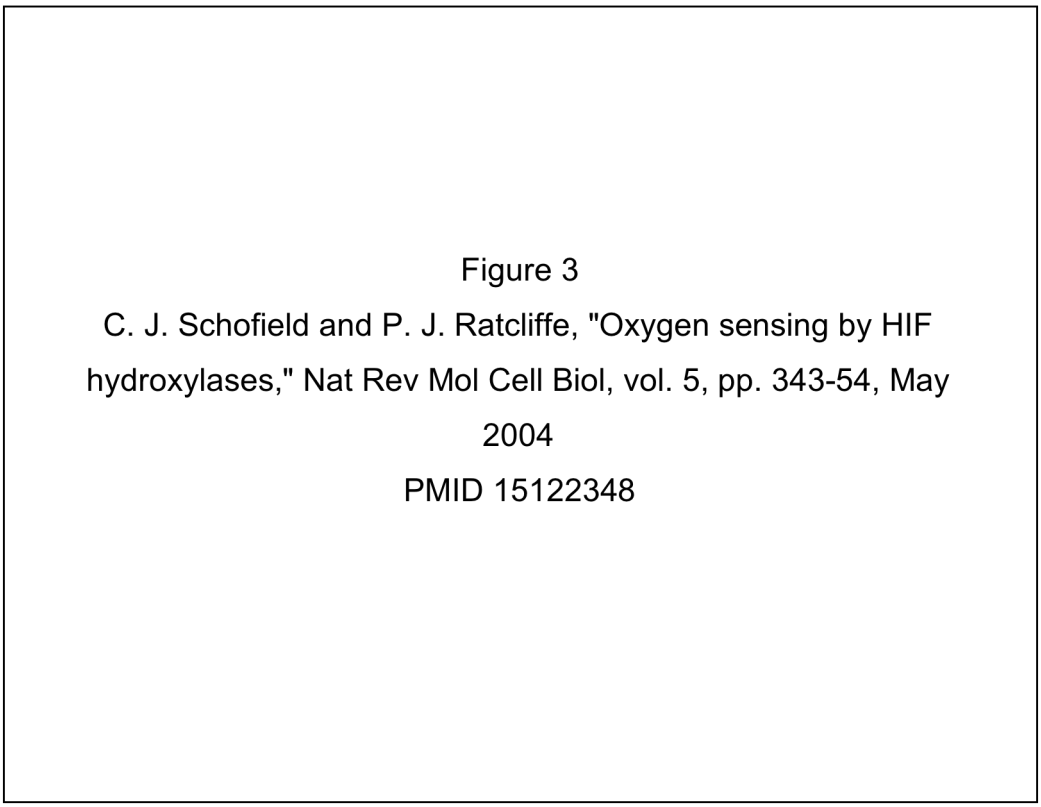


Figure 1.22 Direct transcriptional targets of HIF.

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1.8.3.2 Hypoxia and Chondrogenesis

Recent work has highlighted the role of hypoxia in chondrogenesis and maintenance of the chondrocytic phenotype. Chondrocytes are normally subject to hypoxia in vivo, both developmentally, in the cartilage anlagen, and in mature articular cartilage. Hypoxia is capable of promoting chondrocytic marker expression in both developmental models and in mature chondrocytes.

In the ST2 and ATDC5 cell line models of differentiation culture in 1% oxygen for 4 days is reported to moderately upregulate the expression of collagen II and aggrecan mRNA [234-236]. In models of chondrocyte differentiation from mesenchymal precursors, reduced oxygen enhances the expression of chondrocytic markers induced by growth factors [234, 237, 238].

Of most probable significance for tenocyte studies, however, are the findings with mature adult chondrocytes. Culture for as little as 1 day in 1% oxygen is sufficient to elevate Sox9, collagen II and aggrecan mRNA levels in chondrocytes dedifferentiated through serial passage in monolayer [232].

1.8.3.3 Tendon Rupture and Hypoxia

Upon tendon rupture the blood supply to the affected area is compromised, suggesting localised hypoxia. Matthews et al [28] and Benson et al [239] have recently reported changes associated with hypoxia in degenerate rotator cuff samples. Blood vessel number increases in small and medium size tears, however, there is a decrease in vessel content as the tear size increases. Massive tears, the site of the most pronounced chondroplasia, are relatively avascular [28]. Stabilised HIF-1α has been demonstrated in samples of torn rotator cuff tendon [239]. Samples from all tear sizes also showed expression of VEGF [240], a pro-angiogenic cytokine classically upregulated by low oxygen concentration [241]. Additionally, the hypoxia-induced death gene, BNIP3 is upregulated increasingly as tear size progresses, associated with increasing levels of tenocyte apoptosis [239]. Indeed Liang et al. have found that tenocytes cultured in 0.1% oxygen rapidly accumulate HIF-1α. When cultured in 0.1% oxygen for twenty-four hours

the tenocytes showed upregulation of the hypoxia responsive pro-apoptotic proteins BNIP3 and Nix followed by overt apoptosis from 48h onwards [242]. The secretion of VEGF-A was also increased. The response of tenocytes to less extreme oxygen conditions is currently unknown. Physiological oxygen concentrations within the normal rotator cuff have yet to be determined by any group. However, they are likely to be lower those of well vascularised bone but higher than the deep zone of healthy cartilage.

1.8.4 Combination

Previous studies in chondrocytes have shown that hypoxia is able to enhance the effects of three dimensional culture in redifferentiating cells which have dedifferentiated due to monolayer passage [243-245]. Since matrix degeneration, altered tensional loading and hypoxia are all happening at once in the degenerating cuff tendon, the combined effect of disrupting the actin cytoskeleton and hypoxia will also be tested.

1.9 Aims of this Thesis

1. Test the prochondrogenic effects of lovastatin in the established ATDC5 chondrogenic model.
2. Assess the effects of cytoskeletal signalling disruptors on tenocyte phenotype.
3. Determine whether hypoxia can alter tenocyte phenotype towards that of the fibrochondrocyte.
4. Test the effect of combined hypoxia and cell shape alteration on tenocyte gene expression.

Chapter 2 - Materials and Methods

All reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

2.1 Cell Culture

2.1.1 ATDC5 Culture

2.1.1.1 Standard Monolayer Culture

The ATDC5 murine chondrocytic cell line was purchased from the European Collection of Cell Cultures and maintained in monolayer in DMEM/F12 (Lonza, Cheltenham, UK) 5% heat inactivated foetal calf serum (FCS (Biosera, Ringmer, UK)) supplemented with 3 x 10^{-8}M sodium selenite and 10μgml^{-1} apotransferrin (maintenance medium)[4]. Upon reaching ~80% confluency the cells were detached from the plastic by scraping and passaged.

2.1.1.2 Pellet Culture

In order to prevent adherence to the culture plate the bottom of each well of a 48 well plate was coated with 2% agarose (Invitrogen, Carlsbad, CA). The ATDC5s were seeded at a density of either 1x10^5 or 1x10^6 cells per well. After 24 hours pellets were observed to have formed. Three days after seeding the culture was fed by carefully removing half of the culture medium and gently replacing it with the same volume of new medium. The medium was supplemented with insulin

[4] see also Section B.3 Note Added in Corrections

($10\mu gml^{-1}$) and ascorbic acid ($20\mu gml^{-1}$)[5] (chondrogenic medium) in order to promote chondrogenesis as described by Atsumi et al. [246] and with varying concentrations of lovastatin as the experiment dictated. The cells were fed every 2-3 days. After 14 days treatment with lovastatin the pellets were harvested. Three pellet culture runs were performed per experimental design.

2.1.2 Tenocyte Culture

2.1.2.1 Tenocyte Isolation

Samples of normal hamstring tendon were obtained from 8 patients (4 males, 4 females, average age 30 (19-45y)) undergoing anterior cruciate ligament reconstruction with informed consent and in full compliance with UK Human Tissue Act and Multicentre Research Ethics Committee requirements. The protocol from Schulze-Tanzil et al. [247] was modified as follows. The tendon samples were finely chopped using a scalpel blade into sections approximately 2x2mm in length and spread evenly over the base of a 6 well tissue culture plate. These explants were cultured overnight in small drops of DMEM/F12 containing 50% FCS to encourage adherence of the explants. 2ml of DMEM/F12 50% FCS was added the following morning. A population of cells were observed to emerge from the explants during the following week. At 7 days the explants were removed and the cells cultured in DMEM/F12 10% FCS. The tenocytes were fed once every 3-4 days. Once the wells reached 70-80% confluence the tenocytes were detached from the dish using a rubber policeman, washed and replated in 10cm dishes (1 well of the 6 well plate was plated into 1 10cm dish). The 10cm

[5] see also Section B.3 Note Added in Corrections

plates reached 80% confluency approximately one month after the initial dissection of the tendon explants. The cells, at passage one, were frozen in 20% DMSO 80% FCS and placed in liquid nitrogen. Tenocytes were routinely expanded in DMEM/F12 10% FCS and maintained in DMEM/F12 5% FCS during treatment. Passage 2 tenocytes were used for RNA and passage 3 for protein assays were used as the minimum necessary to obtain sufficient numbers.

2.1.2.2 Mycoplasma Screening

All cultures were originally screened using MycoAlert (Lonza, Cheltenham, UK). However, this assay failed to reliably detect the presence of mycoplasma in primary cells (see section 2.6.1). Routine screening was subsequently carried out using the EZ-PCR Mycoplasma Test Kit (Biological Industries, Beit Haemek, Israel).

2.1.2.3 Inhibitor Treatments

The cytoskeletal inhibitor panel included: Y27632, PP2, cytochalasin D, lovastatin and mevalonate. Effective, non-toxic doses were established by dose and time course experiments. As recommended in the literature, Y27632 and PP2 were added at 24h intervals including 30min prior to harvesting [248]. Other inhibitors were added every 2-3 days. Collagen II expression was detected in tenocytes cultured on coverslips with lovastatin or DMSO for 3 weeks with twice weekly treatments.

2.1.2.4 Hypoxic Culture

Tenocytes were cultured under normoxia or continuously in 5, 3, 1 and 0.1% oxygen for 72h with 5% CO_2, balance N_2, using a miniGalaxy incubator (RS Biotech, Irvine, UK). For combination treatments, tenocytes were cultured in 1% oxygen for 72h with 2μM lovastatin. For long term chemically simulated hypoxia, tenocytes were cultured for 2 weeks with 50μM cobalt chloride.

2.1.3 Monolayer Coverslip Culture

Cells were seeded on to 13mm diameter coverslips (Thermo Scientific, Waltham, MA) and grown to 50% confluency. They were treated with varying concentrations of lovastatin for 24, 48 or 72h, fixed in 4% paraformaldehyde and stored under Dulbecco's phosphate buffered saline (PBS) (BioWhittaker, Berkshire, UK) at 4°C.

2.2 Biochemical Assays

2.2.1 Dimethylmethylene Blue (DMB) Assay

sGAG were detected using the method of Humbel [249] with the modifications proposed by Farndale et al [250]. Briefly, one pellet per condition was digested in 50μl of digest buffer (20mM sodium phosphate buffer (pH 6.8), 1mM EDTA (BDH, PA, USA) 2mM dithiothreitol and 300μg papain) for 2 hours at 65˚C and centrifuged at 13,000 rpm for 5 min to remove any undigested debris. 100μl of DMB colour reagent (40.55mM glycine, 40.55mM NaCl (Fisher, MA, USA) 0.0016% (w/v) dimethymethylene blue and 9.5mM HCl (BDH, PA, USA),

pH 3.0) was added to 4µl of digested sample and mixed briefly by pipetting. The resulting colour change was quantified using a Dynex MRX 96 well microplate reader at 520nm using Revelation 4.25 software. The portion of the digested sample not used for the DMB assay was stored at -80˚C and then subjected to the picogreen assay.

2.2.2 Picogreen Assay

The papain digested samples which had previously been subjected to the DMB assay were diluted 1 in 5 in digestion buffer and the DNA concentration determined using Picogreen (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.3 Immunostaining

2.3.1 Staining on Coverslips

2.3.1.1 Immunostaining on Coverslips

Tenocytes on coverslips were incubated with 3% hydrogen peroxide for 10min, permeabilized in 0.2% Triton X100 for 10min and blocked with normal horse serum for 20min. The coverslips were incubated with primary antibody (for dilutions and suppliers see Table 2.1) at 4°C in a humidified chamber overnight and detection carried out using the Vector Elite kit and metal-enhanced DAB (either ImmPACT DAB (Vector Laboratories Inc, Burlingame, CA) or DAB-metal substrate (Roche Diagnostics GmbH, Mannheim, Germany), with DAPI

counterstain (Vector Laboratories Inc, Burlingame, CA) before mounting in Fluorsave.

2.3.1.2 Phalloidin Staining

Coverslips containing cultured cells were incubated with 100µl per coverslip of 0.1% Triton X100 in PBS for 5min and washed. The samples were incubated with 1mgml^{-1} DAPI and 5unitsml^{-1} rhodamine labelled phalloidin (Invitrogen, Carlsbad, CA) in the dark for 30min. The coverslips were washed before being mounted in Fluorsave (Calbiochem, Darmstadt, Germany).

2.3.1.3 Live/Dead staining

A Live/Dead viability assay kit was purchased from Invitrogen (Carlsbad, CA) and the staining was performed according to the manufacturer's instructions.

2.3.2 Histological Analysis

2.3.2.1 Sample Preparation and Embedding

Processing of ATDC5 pellets was carried out as follows: one pellet per condition was washed, fixed in 1% paraformaldehyde for 20min and stored at 4˚C in PBS prior to embedding. The pellets (≤1mm diameter) were stained for 30min in 10% eosin to make them more visible.

Both pellets and tendon specimens were processed through graded alcohols and xylene (Fisher Scientific UK Ltd, Loughborough, UK), and embedded in Formula

R paraffin wax (Surgipath, Richmond, IL). 3μm sections were mounted on to either Superfrost Plus slides (Menzel GmbH and Co KG, Braunschweig, Germany) or X-tra adhesive slides (Surgipath, Richmond, IL). The slides were dried at 37°C overnight. Paraffin wax embedded sections of normal testis from 8 week old C57BL/6J mice were purchased from Abnova (Taipei City, Taiwan).

2.3.2.2 Immunohistochemistry on Paraffin Sections

Sections were dewaxed using xylene or Clearene and processed through graded alcohols. Sections were incubated with 3% hydrogen peroxide for 10min, permeabilized in 0.5% Triton X100 for 10min and blocked with normal horse serum for 20min. Primary antibody was applied overnight at 4˚C in a humidified box and detection carried out using the Vector Elite kit and metal-enhanced DAB (for 8min). Samples were counterstained with DAPI (Vector Laboratories Inc, Burlingame, CA) and mounted in Fluorsave (Calbiochem, Darmstadt, Germany) or haematoxylin (Vector Laboratories Inc, Burlingame, CA) and mounted in DePeX.

Antigen	Supplier	Product Number	Host species	Isotype	Antibody Dilution	Block	Antigen Retrieval
active caspase 3	Calbiochem	AP1027	Rabbit	P IgG	1/200	5% 1h	Sodium Citrate
Collagen II	Chemicon	MAB8887	Mouse	IgG_1 clone 6B3	1/100	5% 1h	Hyaluronidase
Scleraxis	Abcam	ab58655	Rabbit	P IgG	1/50 *	2% 20min	-

Table 2.1 Antibodies used in immunohistochemistry.

* = diluted in 2% horse serum. P = polyclonal antibody. Suppliers addresses: Abcam, Cambridge, UK; Calbiochem, Darmstadt, Germany; Chemicon, Billerica, MA.

2.3.2.2.1 Antigen Retrieval

2.3.2.2.1.1 Hyaluronidase

Hyaluronidase mediated antigen retrieval was used once the slides had been blocked using hydrogen peroxide. The Triton permeabilization step was not performed, instead the slides were incubated with 10mgml^{-1} hyaluronidase (diluted in PBS) for 10min at room temperature. The slides were washed three times in PBS and blocked with 10% horse serum for 20min at room temperature. Primary antibody was added as per the standard protocol.

2.3.2.2.1.2 Sodium Citrate

Once the slides had been dewaxed and rehydrated they were placed in boiling 10mM sodium citrate (pH 6.0) for 10min and then allowed to cool at room

temperature for 30min. The standard protocol was resumed with incubation in 3% hydrogen peroxide.

2.3.2.2.1.3 EDTA

Once the slides had been dewaxed and rehydrated they were placed in 1mM EDTA (pH 8.0) at approximately 96°C for 20min and then allowed to cool at room temperature for 30min. The standard protocol was resumed with an incubation in 3% hydrogen peroxide.

2.3.2.2.2 Proliferating Cell Nuclear Antigen (PCNA)

A PCNA staining kit was purchased from Invitrogen (Carlsbad, CA) and the staining was performed according to the manufacturer's instructions (including the optional endogenous peroxidase block) except for the following modifications. The sections were incubated with primary antibody overnight at 4°C and metal-enhanced 3,3'-diaminobenzidine tetrahydrochloride (DAB) from Roche (Basel, Switzerland) was used for development. The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in Fluorsave (Calbiochem, Damstadt, Germany).

2.3.3 Microscopy and Imaging

Coverslips and wax sections were imaged using an Olympus BX40 microscope with DP70 camera and Cell^F 2.2 software (Olympus BioSystems GmbH, Tokyo, Japan). Whole pellets were imaged using a Nikon Diaphot (Nikon, Tokyo, Japan) microscope with a Nikon Coolpix 4500 (Nikon, Tokyo, Japan) camera with a

custom adapted eyepiece. Phase contrast images were captured with a Nikon Eclipse TE300 (Nikon, Tokyo, Japan) microscope with RT Color Spot camera (Diagnostic Instruments Inc, Sterling Heights, MI) using Image Pro Plus 6.3 (Media Cybernetics Inc, Bethesda, MD).

2.4 Western Blotting

2.4.1 Sample Preparation

2.4.1.1 Pellet Sample Preparation

Three $1x10^5$ size pellets for each data point were frozen dry at -80°C for at least 2 hours followed by thawing to initiate pellet disruption. 25µl of Laemmli sample buffer (Originally described as having a final concentration of 0.0625M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol. 5% β-mercaptoethanol, 0.001% bromophenol blue [251]. For this study a x2 pre-mix (catalogue number 161-0737) was purchased from BioRad (Hercules, CA) and supplemented with 5% β-mercaptoethanol) was added to the pellets. Pellets were disrupted by vigorous pipetting followed by sonication.

2.4.1.2 Standard Sample Preparation

Sample plates were removed from the incubator and immediately placed on ice. The culture medium was removed and the samples washed twice in sample buffer (1mM EDTA, 1mM PMSF in PBS). Samples were scraped into 50µl of sample buffer, sonicated for 15s and then centrifuged at 4°C for 10min at 12,000rpm. The supernatants were stored at -80°C. The protein concentration was determined

using either the Bradford assay (Pierce Biotechnology, Rockford, IL) or the Protein 660 assay (Pierce Biotechnology, Rockford, IL)(see also Section B.5). The protein concentrations were equalised, Laemmli sample buffer (BioRad, Hercules, CA) supplemented with either β-mercaptoethanol or 100mM DTT was added and the samples were placed in 95°C for 5min.

2.4.1.3 Sample Preparation for HIF Detection

Sample plates were removed from the incubator and immediately placed on ice. The culture medium was removed and the sample scraped without washing into 50µl of ice cold HIF buffer (10% glycerol, 0.1M Tris-HCl (pH 6.8), 5µM DTT, 1% SDS, 0.5µgml^{-1} pepstatin, 1µgml^{-1} leupeptin, 2µgml^{-1} aprotinin, 200µM AEBSF, 8M urea). The samples were sonicated for 15s then centrifuged at 4°C for 10min at 12,000rpm. The supernatants were stored at -80°C. The protein concentration was determined using the BCA assay (Pierce Biotechnology, Rockford, IL). The protein concentrations were equalised, Laemmli sample buffer (BioRad, Hercules, CA) supplemented with 100mM DTT was added and the samples were placed in 95°C for 5min.

2.4.2 Western Blot Procedure

Equal volumes of each sample were run on an SDS-PAGE gel of the appropriate concentration and then transferred on to an Immobilon-P membrane (Millipore, Billerica, MA). The membrane was soaked in methanol for 30s and then allowed to dry for 20min. The dried membrane was blocked in a solution of 5% fat-free milk powder for 1h. The membrane was washed in PBS 0.1% Tween 20 (PBS-T)

and incubated with primary antibody diluted in PBS-T overnight at 4°C. The membrane was washed in PBS-T and incubated with secondary antibody diluted in PBS-T for 1h at room temperature. The membrane was washed in PBS-T and the signal developed using ECL (Pierce Biotechnology, Rockford, IL) or Supersignal (Pierce Biotechnology, Rockford, IL). The image was captured using the Chemi Doc-It Imaging System (UVP, Upland, CA) and VisionWorks LS software (VisionWorks LS v 6.7.4, UVP, Upland, CA).

Antigen	Supplier	Product Number	Host Species	Isotype	% Gel	Antibody Dilution	Detection Reagent
β-tubulin	Sigma Aldrich	T 4026	Mouse	M IgG_1 Clone TUB 2.1	N/A	1/1,000	ECL
BNIP3	Sigma Aldrich	B7931	Mouse	M IgG_{2b} Clone ANa40	12	1/1,000	SS
FOXO3a	Epitomics	2071-1	Rabbit	M IgG* Clone EP1949Y	10	1/3,000	SS
GAPDH	Abcam	ab9485	Rabbit	P IgG	N/A	1/50,000	ECL
HIF-1α	Abcam	ab8366	Mouse	M IgG_1 Clone ESEE122	10	1/1,000	SS
Rap1a	Santa Cruz Biotechnology	sc-1482	Goat	P IgG	12	1/1,000	ECL
Scleraxis	Abcam	ab58655	Rabbit	P IgG	12	1/500	SS

Table 2.2 Antibodies used in Western blotting

P = polyclonal, M = monoclonal, SS = Supersignal. Suppliers addresses: Abcam, Cambridge, UK; Epitomics, Burlingame, CA; Santa Cruz Biotechnology, Inc, Santa Cruz, CA. * = Exact isotype of this antibody unavailable see Appendix C.

2.5 Molecular Biology

Unless otherwise indicated reagents were purchased from Qiagen, Hilden, Germany.

2.5.1 RNA Extraction and Reverse Transcription

RNA extraction was performed using RNeasy kits. Concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). 100μg of RNA was reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA).

2.5.2 RT-PCR

Twenty-five microlitres of PCR mixture containing cDNA template equivalent to 15ng total RNA, as well as dNTPs and Platinum Taq polymerase (Invitrogen, Carlsbad, CA) was amplified. Quantitect primers (see Table 2.3) were diluted to give a x1 final concentration. DEC1 [104] and VEGF-A [252] primers (see Table 2.4 for sequences and annealing temperatures) were synthesised by Eurofins MWG Operon (Ebersberg, Germany) and used at 0.3μM. PCR cycle parameters were as follows, an initial melt at 95˚C for 15min followed by 35 cycles of 94˚C for 15s, Ta for 30s, and 72˚C for 30s. The PCR products were separated on a 1.2% Flashgel (Lonza, Rockland, ME). Images were taken using Gel Doc-It Imaging System (UVP, Upland, CA).

2.5.3 QPCR

QPCR was performed using a Rotorgene 3000 and Quantitect Sybr Green PCR kit with Quantitect primer assays according to the manufacturer's instructions. Non-Quantitect primers were used at 0.3μM and the reactions were performed as recommended by the manufacturers of the Quantitect Sybr Green PCR kit. Melt curve analysis confirmed a single product from each reaction. Absence of genomic DNA was confirmed using "no RT" controls for every sample. Samples were run in duplicate and analysed by comparative quantification [253] using Rotorgene software version 1.7. Expression was normalized to GAPDH or TATA box binding protein (TBP) as appropriate. Statistics were performed using GraphPad Prism version 5.0b for Mac OS X, GraphPad Software, San Diego CA.

Target Gene	Quantitect Primer Assay	Amplicon size/bp
ACAN	QT00001365	62
ACTB	QT01680476	104
BMP-2	QT00012544	148
COL1A1	QT00037793	118
COL2A1	QT00049518	94
COL2A1va1	QT00998844	95
COL3A1	QT00058233	95
GAPDH	QT01192646	119
SCX	QT01529507	91
SOX5	QT00084784	84
SOX6	QT00053697	112
SOX9	QT00001498	111
TBP	QT00000721	132

Table 2.3 Qiagen primers used for QPCR

Gene	FW sequence	RV sequence	Ta/°C
DEC-1	5'-GAAAGGATCGGCGCAATTAA-3'	5'-CATCATCCGAAAGCTGCATC-3'	55
VEGF-A	5'-CTTGCCTTGCTGCTCTACC-3'	5'-CACACAGGATGGCTTGAAG-3'	53

Table 2.4 Sequences and annealing temperature for non-Qiagen primers used for QPCR

2.6 Method Development

2.6.1 Mycoplasma Infection - Detection and Consequences

The term mycoplasma in the context of cell culture refers to a number of different bacterial species within the order Molliculites. Mycoplasma are common contaminants of cell culture, especially culture involving primary cells. Unlike other contaminating organisms, infected cultures produce no obvious immediate symptoms, although the infected eukaryotic cells may show progressively slower rates of proliferation sometimes in combination with blebbing (the shedding of small membrane bound vesicles of cytoplasm)[6]. Mycoplasma cannot be detected by routine examination of cells using phase contrast microscopy since they are extremely small and attach themselves to the outer membrane of the much larger eukaryotic cells, which they parasitize.

As is considered good practice, our laboratory carries out regular screening for the presence of mycoplasma. However, despite a recommendation from several cell banks, the Lonza Mycoalert™ kit used initially failed to detect the presence of a mycoplasma contamination in tenocytes originating from several patients. Nucleic acid staining has been used to screen for the presence of mycoplasma for many years [254]. Thus, the mycoplasma contamination was accidentally discovered during a routine DAPI counterstain as part of another experiment. In contrast to the uncontaminated tenocytes (Figure 2.1A), contaminated cells showed extranuclear nucleic acid staining (Figure 2.1B). Contamination was

[6] Both of these symptoms were observed in some but not all of the contaminated human tenocyte cultures in this study.

subsequently confirmed by PCR assay (Figure 2.1C) (Data courtesy of Dr Raewyn Poulsen).

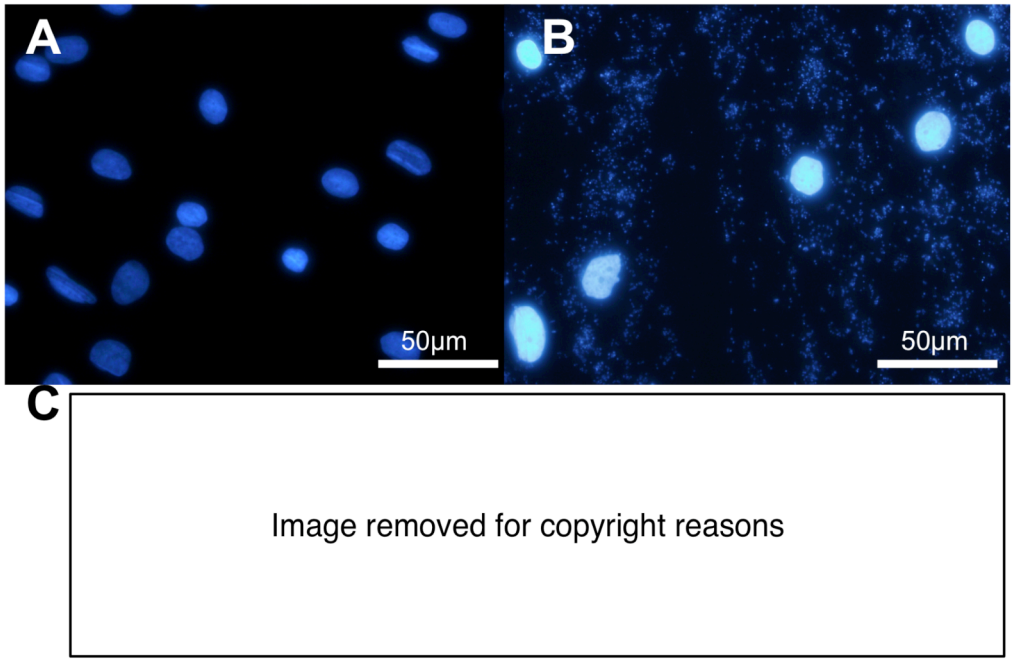


Figure 2.1 Mycoplasma contamination of human tenocytes.

Tenocytes cultured on coverslips stained with DAPI. Tenocytes were confirmed mycoplasma negative (A) or positive (B) by PCR analysis (C). Arrow indicates positive mycoplasma test band (270bp), lower bands are primer dimer. Lanes 1-10 = tenocytes from various patients, lane 11= negative control, lane 12 = mycoplasma positive control.

A full screen of all cell stocks by PCR was carried out, infected cells were disposed of and all equipment thoroughly cleaned. All experiments in which infected cells were used were repeated with cells confirmed to be uncontaminated by mycoplasma. Lovastatin was used as an inhibitor in a number of experiments subsequently found to have been performed with mycoplasma contaminated tenocytes. The rationale for the use of this particular inhibitor will be detailed in Section 3.1.2 and Section 5.1.5.4. The same response to lovastatin was seen in mycoplasma free tenocytes as was seen in tenocytes shown to be mycoplasma

infected (Figure 2.2A and B). On occasion, the mycoplasma infected tenocytes seemed to react more strongly than the uninfected tenocytes (e.g. Figure 2.2C). However, even in this instance, there is some overlap between the responses of uninfected and infected tenocytes. It is difficult to determine if the difference in responses is due to mycoplasma infection or due to natural variability in response found within the human population.

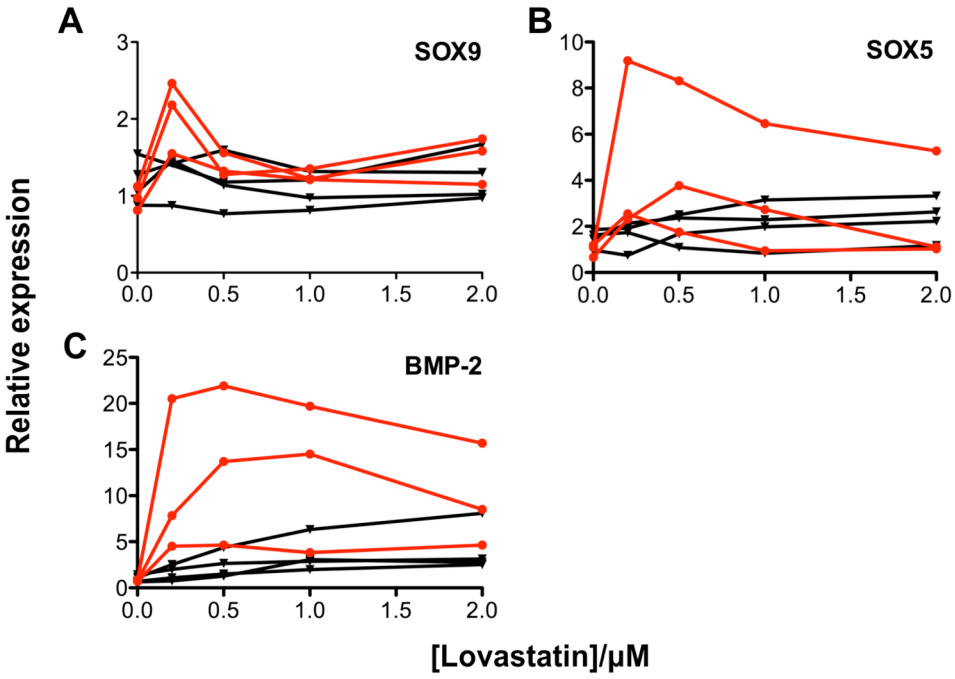


Figure 2.2 Comparison of the Response of Mycoplasma Contaminated and Uncontaminated Tenocytes to Lovastatin

Tenocytes were cultured with 0.2% DMSO vehicle control, 0.2, 0.5, 1 or 2µM lovastatin for 1 week. Sox9 (A), Sox5 (B) and BMP-2 (C) mRNA levels were assessed by QPCR and normalized to GAPDH. Tenocyte stocks confirmed mycoplasma free are shown in black. Tenocyte stocks which were mycoplasma contaminated are shown in red.

2.6.2 Housekeeper Gene Selection for QPCR

Housekeeper genes are genes whose expression is not altered by the treatment(s) to which a cell is exposed. They are used to compensate for small variations in cDNA quantities between samples that may be present when PCR reactions are set up. Genes from a wide variety of families including structural proteins (e.g. β-actin (ACTB)), metabolic pathway proteins (e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) and components of the transcriptional mechanism (e.g. TATA box binding protein (TBP)) are used as housekeepers. Importantly, there is no 'universal housekeeper' - a gene which is stable in all cells under all experimental conditions. It is therefore recommended that each selection of a housekeeping gene be validated for the particular cell type and treatment to ensure that its expression does not vary [255].

Initial selection of an appropriate housekeeping gene for experiments involving the cytoskeletal inhibitor panel was based on the available literature for the use of such reagents in chondrocytes. Since almost [219] all papers to date had used GAPDH as a housekeeping gene [78, 218, 220, 222, 256, 257] it was selected for use in this study. However, later examination of the raw Ct values revealed a subtle (<1.5 fold) dose-dependent upregulation of GAPDH in response to 7 days of lovastatin treatment (Figure 2.3).

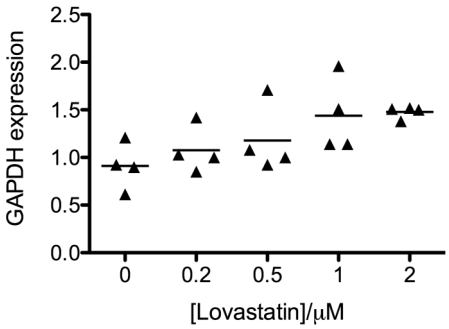


Figure 2.3 GAPDH Expression in Response to Lovastatin Treatment

Tenocytes were cultured with 0.2% DMSO vehicle control, 0.2, 0.5, 1 or 2μM lovastatin for 1 week. GAPDH mRNA levels were assessed by QPCR. Four independent experiments are shown.

Additionally, GAPDH has been reported to be an unsuitable housekeeping gene for the hypoxia paradigm [258]. Indeed, a preliminary study examining the effects of hypoxia on tenocytes using the hypoxia mimic cobalt chloride (the mechanism of action of this molecule is detailed in Section 6.2.3), revealed a significant upregulation of GAPDH expression upon exposure to the mimic (Figure 2.4). Since studies combining cytoskeletal inhibitors and hypoxia were planned, a housekeeping gene whose expression was stable under both conditions was desirable.

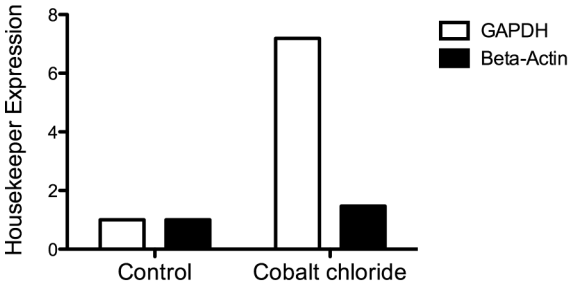


Figure 2.4 Comparison of GAPDH and β-actin as Housekeeper Genes for Cobalt Chloride Treatment

Tenocytes were cultured with or without 50μM cobalt chloride for 1 week. GAPDH and beta-actin mRNA levels were assessed by QPCR. One experiment is shown.

Fink et al. investigated several genes as potential housekeepers for use in chondrogenic differentiation assays using adipose-tissue derived stem cells [259]. Conditions assayed included hypoxia (1% oxygen). One of the least variable genes under these conditions was TATA box binding protein (TBP). The response of TBP to lovastatin and hypoxia was therefore determined by QPCR. TBP was found to be less responsive than GAPDH to lovastatin treatment (Figure 2.5A) and low oxygen concentrations (Figure 2.5B). It also proved to have stable expression under a combination of hypoxia and lovastatin treatment (Figure 2.6). It was therefore used as a housekeeper for all studies involving lovastatin alone, hypoxia or lovastatin in combination with hypoxia. Interestingly, a recent publication [260] has found that TBP has high stability of expression in chondrocytes in monolayer, 3D culture and varying oxygen tensions.

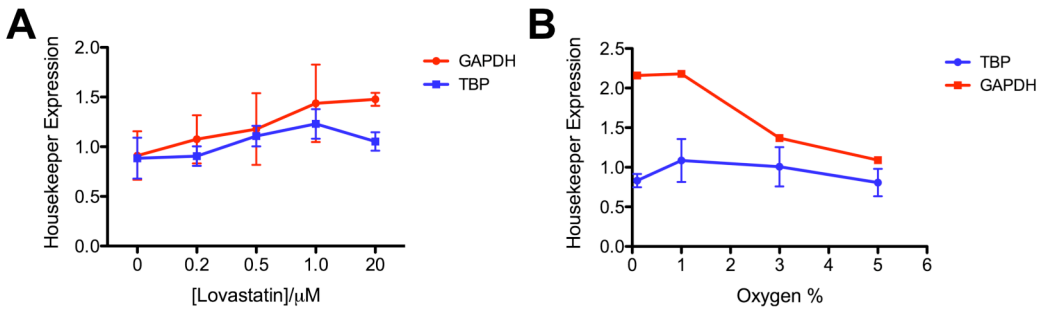


Figure 2.5 Comparison of GAPDH and TBP as Housekeeper Genes for Lovastatin Treatment and Hypoxia

A Tenocytes were cultured with 0.2% DMSO vehicle control, 0.2, 0.5, 1 or 2μM lovastatin for 1 week. GAPDH and TBP mRNA levels were assessed by QPCR. Mean and SD from 4 independent experiments are shown. B Tenocytes were cultured for 72h in 5%, 3%, 1% or 0.1% oxygen. GAPDH and TBP mRNA levels were assessed by QPCR. Results are relative to tenocytes cultured simultaneously in 20% oxygen. Mean and SD from 3 independent experiments with different donors are shown for TBP. GAPDH results are from a single experiment.

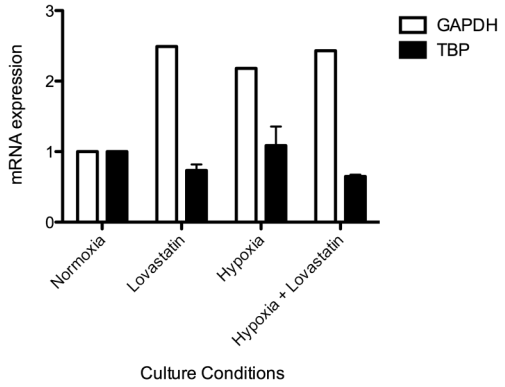


Figure 2.6 Comparison of GAPDH and TBP as Housekeeper Genes for Combination Treatments

Tenocytes were cultured in 1% (hypoxia) or 20% (normoxia) oxygen in the presence or absence of 2μM lovastatin for 72h (the rationale for the precise experimental set-up can be found in Section 6.2.4). GAPDH and TBP mRNA levels were assessed by QPCR. Mean and SD from 3 independent experiments are shown for TBP. GAPDH values relate to one experiment only.

Chapter 3 - Effects of Lovastatin Treatment on the ATDC5 Model

3.1 Introduction

This thesis aims to determine if alterations in cell shape and/or oxygen tension can push the phenotype of adult human tenocytes towards that of chondrocytes. In order to determine the effect of these conditions it is necessary to have both a proven model of chondrogenesis and sensitive and appropriate assays to measure chondrocyte markers.

This chapter describes initial preparatory studies performed using a well characterised chondrogenic cell line in a three-dimensional (3D) pellet culture system. The effects of lovastatin, a modifier of small GTPase signalling, on cell shape and chondrocyte marker expression are determined using this model. Disadvantages and a possible alternative to this culture system are discussed. This chapter additionally describes the development of appropriate assays to measure cell cycle status and matrix molecule output (both of which are of importance to the generation of healthy cartilage).

3.1.1 Three Dimensional Culture and Chondrogenesis

As has been previously noted (Section 1.8.2), the degree of roundedness of an individual chondrocyte appears to correspond to its ability to obtain and maintain a chondrocytic phenotype. In rotator cuff degeneration it is not clear how much degeneration and chondroplasia takes place before the tear and how much after.

However, it is likely that the altered matrix and/or decreased tensional load seen in torn rotator cuff tendons favours or even induces an alteration in tenocyte cell shape from elongated fibroblast to round. Such a change in cell shape may be necessary for the transdifferentiation of tenocytes to chondrocyte-like cells. Indeed, the cytoskeletal inhibitors previously reported to upregulate chondrocyte phenotypic markers may act as enhancers to a fundamental alteration in cell shape which is responsible for changes in cell phenotype.

The majority of cartilage differentiation studies cite the need for 3D culture to induce or maintain normal levels of cartilage markers [261]. Therefore in order to firstly assess the effects of proposed prochondrogenic agent, lovastatin, in ATDC5 chondrocytes and secondly to set up the methodology of 3D culture for studies using tenocytes a model which incorporates 3D cell culture was set up. Various culture systems are available for the production and maintenance of such an environment. The pellet culture system was chosen because it is inexpensive (unlike limb bud micromass culture), is capable of producing tissue-like structure (unlike hydrogels such as agarose or alginate) and is a well-accepted method in the field.

To confirm that the 3D culture system developed was capable of sustaining and even inducing chondrogenesis, and that the assays developed were capable of detecting chondrocyte markers, it was necessary to test the system using cells known to be capable of undergoing chondrogenesis. The murine cell line ATDC5 recapitulates chondrogenesis in monolayer when treated with insulin [246, 262]. It has been well characterized and used extensively in studies of chondrocyte

development. 3D pellet culture has been demonstrated to enhance the expression of chondrocyte matrix molecule genes in ATDC5 cells [263]. The form of pellet culture used in this instance involved seeding cells on to a surface to which they are unable to adhere, namely agarose. The cells form one central clump or pellet overnight.

3.1.2 Statins as Pro-chondrogenic Agents

Once the 3D pellet culture system was established, it was used to determine the effect of the cytoskeletal inhibitor lovastatin on the chondrogenic process. Statins inhibit HMG Co A reductase, an enzyme which catalyses the conversion of HMG Co A to mevalonate, an early step in the cholesterol synthesis pathway (Figure 3.1)[264]. The downstream metabolites farnesyl diphosphate and geranylgeranyl diphosphate are involved in the post-translational modification of small GTPases known as prenylation [264]. Inhibition of prenylation alters the subcellular location of small GTPases and disrupts small GTPase signalling.

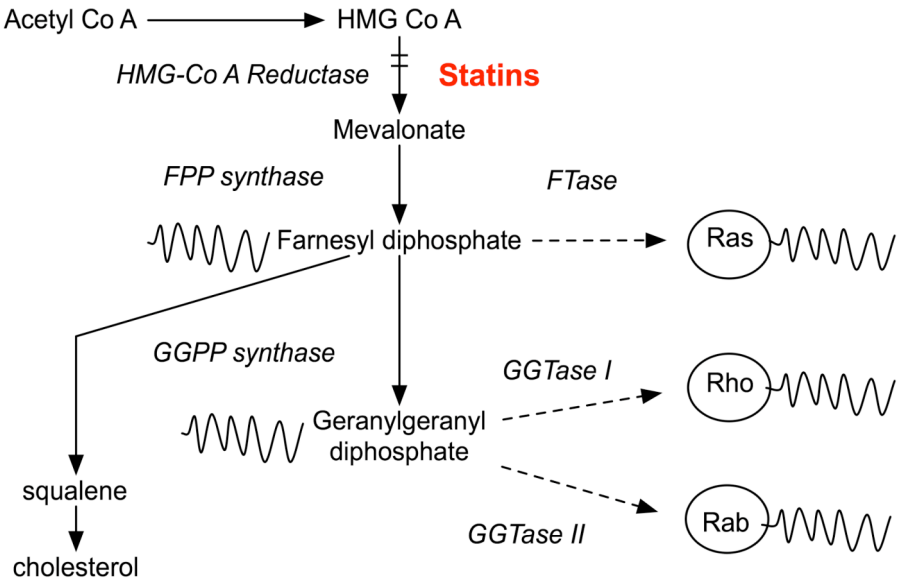


Figure 3.1 Cholesterol synthesis pathway

Work which had been published when beginning this series of experiments suggested a pro-chondrogenic role for statins, with exposure increasing mRNA expression of collagen II and aggrecan core protein in alginate encapsulated foetal rat chondrocytes [222]. Additionally, our group found elevated collagen II protein and sGAG levels in adult bovine articular chondrocytes grown in 3D pellet culture and treated with lovastatin [221]. However, both of these models examine the maintenance or enhancing effects of statins on cells which are already running a chondrocyte programme. No one has attempted to ascertain what response, if any, statins would elicit from cells in a state of pre-differentiation. Additionally, the structure of tissue produced in the presence of statins has never been examined. The effect of a broad spectrum inhibition of small GTPases on the process of chondrogenesis in a well defined chondrocyte cell line is of interest to the overall thesis because an ability to promote or enhance chondrogenesis from a less differentiated phenotype suggests a more powerful driver of differentiation.

3.2 Results

3.2.1 Lovastatin Disrupts ATDC5 Actin Cytoskeleton Arrangement

To confirm that lovastatin treatment disrupts the actin cytoskeleton, ATDC5 cells were grown on coverslips for two days in the presence of varying concentrations of lovastatin, fixed and stained with rhodamine-phalloidin. Initially doses ranging from 1-10μM were chosen since these were effective in promoting chondrogenesis of adult bovine chondrocytes in 3D culture in our laboratory over a 1-3 week period [221]. Prominent stress fibres were present in the control cells (Figure 3.2). Progressive dose-dependent disruption of actin cytoskeleton morphology was seen with increasing doses of lovastatin, with many rounded cells apparent at higher doses. However cell detachment was severe at the higher doses used in these ATDC5 cells after only 2 days of culture, therefore the dose range was lowered. The range of doses used for the rest of this study (0.2-2μM lovastatin) is similar to that used by Hatano et al. in their study using foetal rat chondrocytes [222] (0.1-2μM mevastatin). It should be noted, however, that lovastatin (K_i = 0.64nM) is a slightly more effective inhibitor of HMG Co A reductase than mevastatin (K_i = 1.4nM) [265].

3.2.2 Lovastatin Inhibits Prenylation in ATDC5 Pellets

Rap1a is a member of the Ras subfamily of small GTPases which are post translationally modified by the addition of an isoprenoid lipid that serves to anchor them to the plasma membrane [266]. An antibody which recognises only the unprenylated form of this protein was used to confirm that the doses of

lovastatin used for this study were capable of altering small GTPase prenylation status in ATDC5 pellet culture. Pellets cultured for five days in the presence of 0.01-1μM of lovastatin were lysed in Laemmli sample buffer and the proteins separated on a polyacrylamide gel (Figure 3.3). Unprenylated Rap1a was present when the concentration of lovastatin equalled or exceeded 0.1μM. The concentrations of lovastatin used for the remainder of the study were 0.2μM, 0.5μM and 1μM.

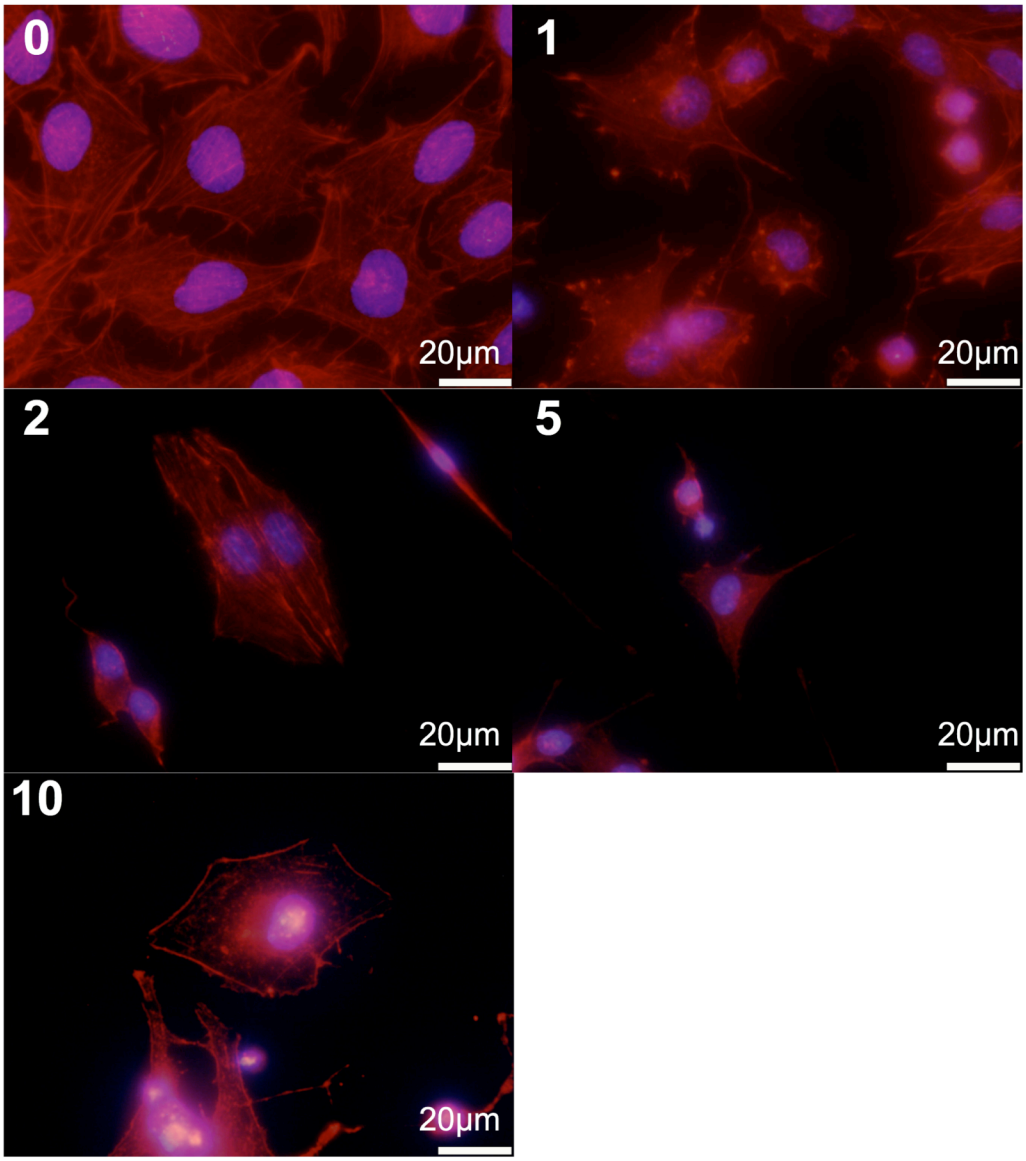


Figure 3.2 Lovastatin Treatment Causes Actin Cytoskeleton Rearrangement

ATDC5 cells were grown on coverslips for two days in the presence of varying concentrations of lovastatin (1-10μM). They were then fixed and stained with rhodamine-phalloidin and counter-stained with DAPI. Note the alterations in stress fibres seen at all doses and the cell rounding at high doses.

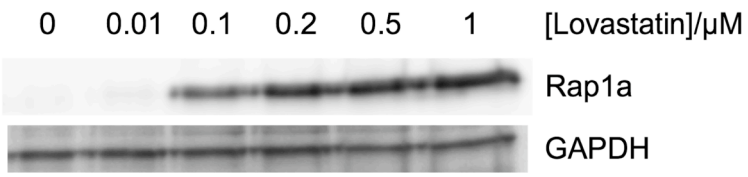


Figure 3.3 Lovastatin Treatment Inhibits Small GTPase Prenylation

Western blot of pellets seeded at a concentration of $1x10^5$ cells per well cultured in the presence of varying concentrations of lovastatin for five days. The blots were probed with antibodies to the unprenylated form of the small GTPase Rap1a and the house keeping protein GAPDH. Results are representative of two independent experiments.

3.2.3 Effect of Lovastatin on Pellet Volume

During the course of experiments to determine the effect of lovastatin on sGAG production (see Section 3.2.7) variations in pellet size were noted. Twenty-four hours after seeding the newly formed ATDC5 pellets were uniform in size (data not shown), however, as the culture progressed a difference in size became apparent. Pellets cultured in chondrogenic medium were noticeably larger than the control pellets (Figure 3.4). The addition of lovastatin to the pellets grown in maintenance medium caused a dose-dependent increase in pellet size, with the pellets grown in 1μM lovastatin almost reaching the size of those grown in chondrogenic medium alone. These changes in pellet size could either be due to alterations in matrix production or in cell number or a combination of the two. Lovastatin did not induce an increase in overall pellet size in the larger chondrogenic medium-treated pellets. We noted that in these pellet cultures the medium became very yellow during rapid pellet growth and attribute the frayed edges and poor morphology of some pellets to poor nutrient exchange. This was confirmed upon histological examination which demonstrated necrotic centres in all treatment groups.

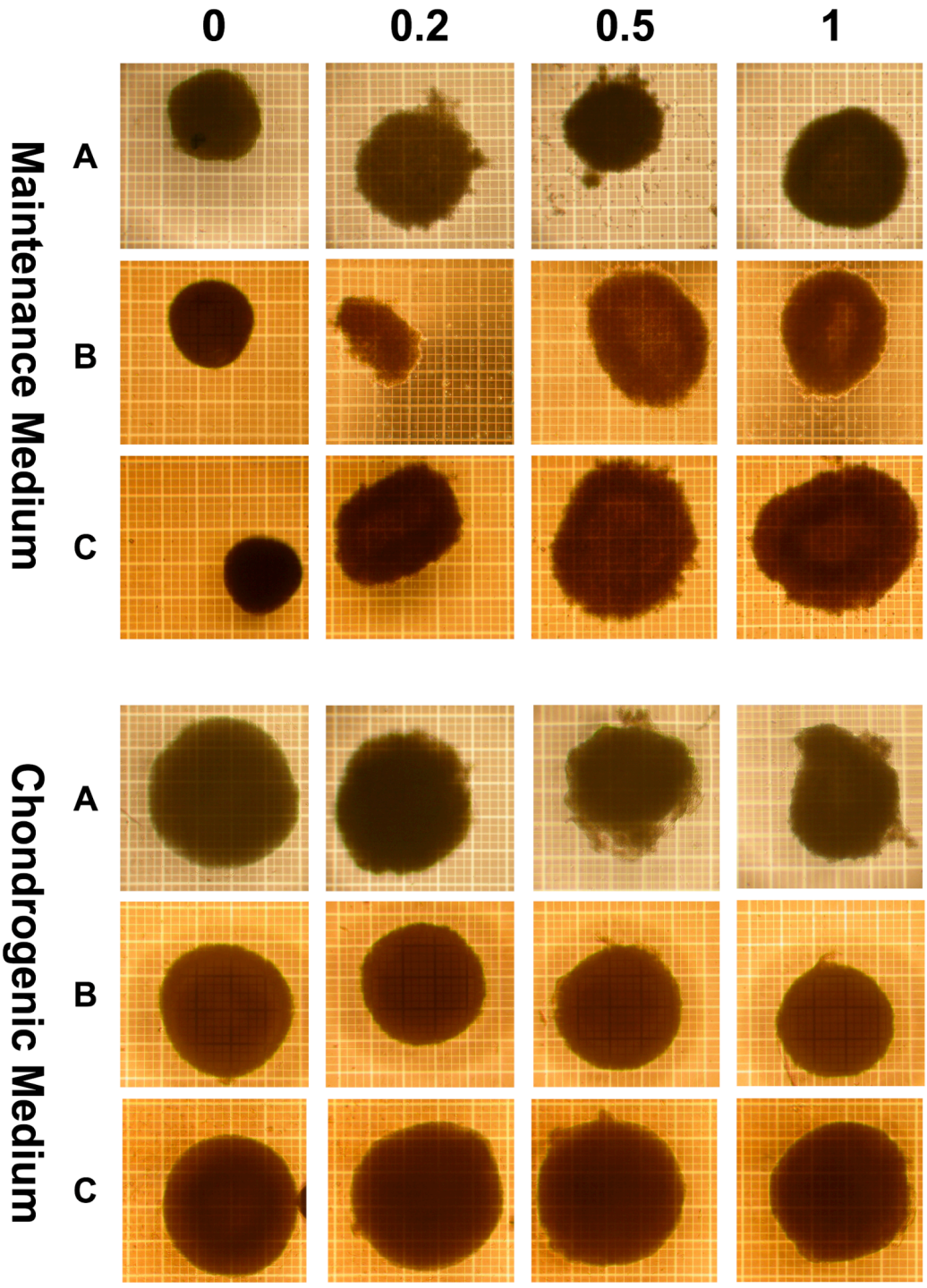


Figure 3.4 Effect of Lovastatin on Pellet Volume

Pellets seeded at a concentration of $1x10^5$ cells per well were cultured in the presence of varying concentrations of lovastatin with or without chondrogenic medium for two weeks. The pellets were photographed at harvest on a Neubauer improved haemocytometer. Three independent experiments are shown.

3.2.4 Lovastatin Decreases Pellet DNA Content

To determine the effect of lovastatin on cell number, pellets were assayed using Picogreen assay. The tiny maintenance medium pellets were at the lower limit of detection for the assay, but larger pellets in chondrogenic medium were more readily quantifiable. Culture in chondrogenic medium resulted in an increase in DNA concentration compared to control pellets (Figure 3.5), suggestive of an increased cell number. The increase in pellet size seen with the addition of chondrogenic medium may therefore be due to an increase in cell number, in addition to possible increases in matrix.

Combination treatment with lovastatin and chondrogenic medium resulted in statistically significant decreases in DNA concentration ($p<0.05$) (Figure 3.5). This could be due to cell cycle arrest, apoptosis or necrosis.

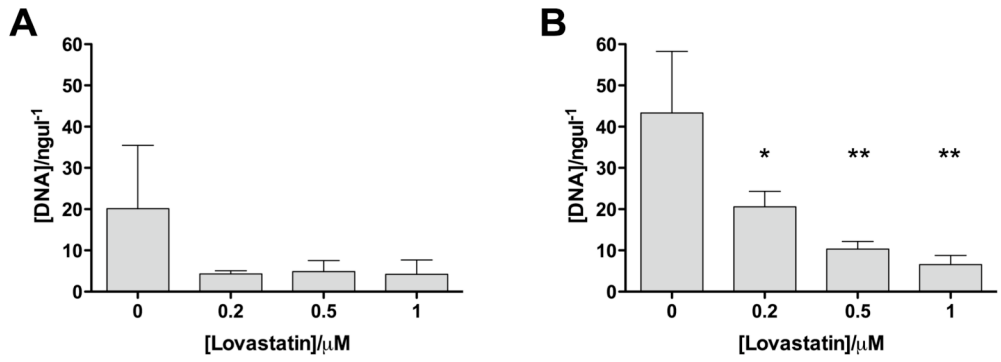


Figure 3.5 Lovastatin Treatment causes Statistically Significant Decreases in DNA Concentration

ATDC5 pellets seeded at a concentration of $1x10^6$ cells per well were cultured with varying concentrations of lovastatin with or without chondrogenic medium. Samples were digested with papain and the dsDNA concentration measured by picogreen assay. **A** Pellets fed with control medium. **B** Pellets fed with chondrogenic medium. Data shown are mean results of three independent experiments ±SD. Significance was determined by one way analysis of variance with Dunnett's post-hoc test. (* $p<0.05$, ** $p<0.01$)

3.2.5 Lovastatin Decreases Cell Proliferation

Since the decrease in DNA content observed in lovastatin treated samples could be the result of cell cycle arrest it was necessary to determine if the pellets were still proliferating. Proliferating cell nuclear antigen (PCNA) is a DNA clamp protein which is only expressed in the nuclei of actively proliferating cells [267]. Diffuse positive staining was seen throughout the control pellet (Figure 3.6A), while the pellet grown in chondrogenic medium (Figure 3.6B) showed dense positive staining on its outer surface with no staining towards the core. Given the variance in size between the two pellets this could be caused by a poor diffusion of nutrients into the core of the larger chondrogenic pellet with associated cell death or by the central cells having ceased proliferation and having begun to differentiate.

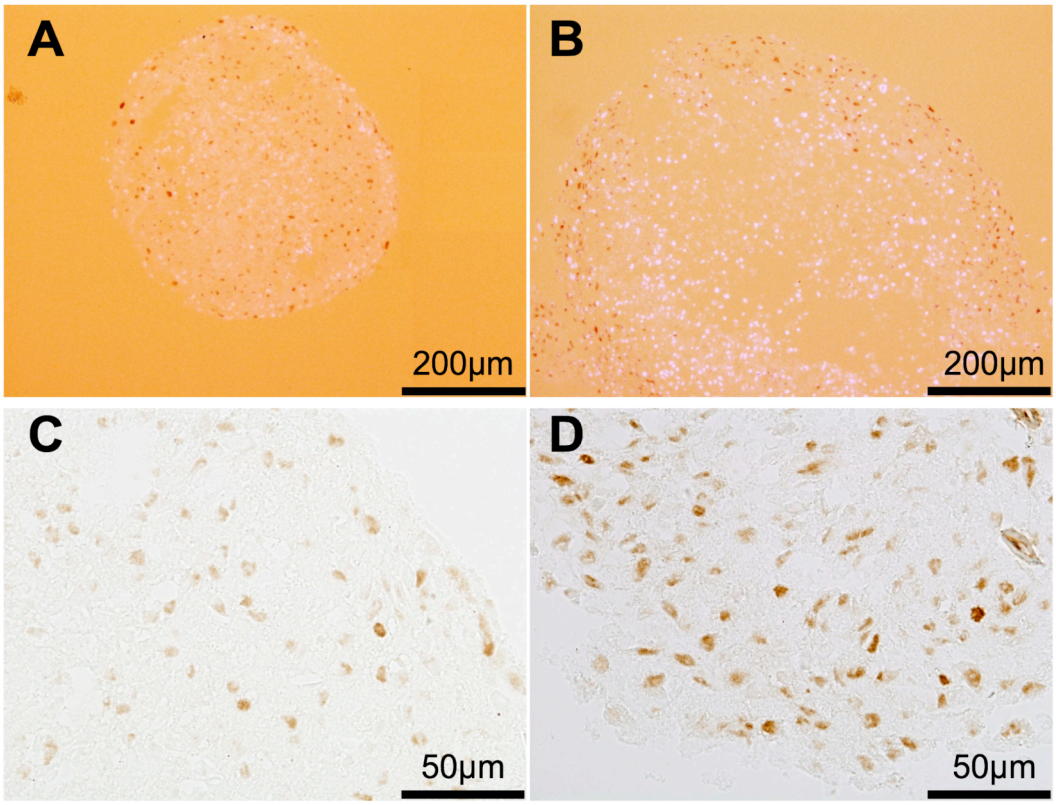


Figure 3.6 Chondrogenic Medium Increases Cell Proliferation

Pellets were seeded at a concentration of $1x10^6$ cells per well and cultured in maintenance medium (A and C) or chondrogenic medium (B and D) for two weeks. 3µm wax embedded sections were stained with anti-PCNA and counterstained with DAPI. Results are representative of three independent experiments.

A dose dependent decrease in PCNA staining was seen in the pellets treated with lovastatin alone, with very little positive staining observed in 0.5µM or 1µM treated pellets (Figure 3.7). However, the presence of some positive staining at these doses suggests that some proliferation continues even with high dose lovastatin.

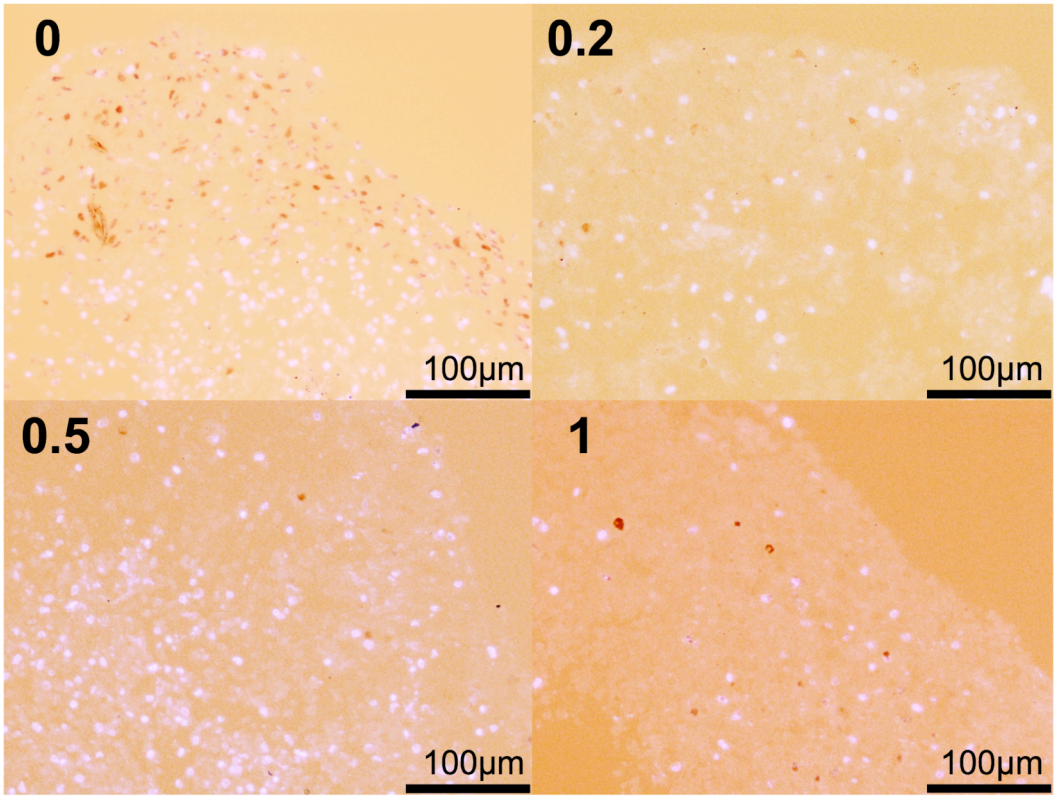


Figure 3.7 Lovastatin Treatment Decreases Cell Proliferation

Pellets were seeded at a concentration of $1x10^6$ cells per well and cultured for two weeks with 0, 0.2, 0.5 and 1µM lovastatin with chondrogenic medium. 3µm wax embedded sections were stained with anti-proliferating cell nuclear antigen and counterstained with DAPI. Results are from a single experiment.

3.2.6 Effect of Lovastatin on Apoptosis

Since lovastatin has been documented to be toxic at high doses [268] it was necessary to determine if apoptosis contributed to the observed decrease in DNA concentrations. Initial studies using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) to detect DNA fragmentation, a feature of end-stage apoptotic cells, were unsuccessful due to high levels of non-specific background staining. It was therefore decided to use an alternative marker of apoptosis, active caspase-3.

Caspase-3 is a cysteine protease with aspartic specificity involved in the final stages of apoptotic cell death. In non-apoptotic cells it exists in an inactive form (pro-caspase-3). In cells undergoing apoptosis it is cleaved to produce active caspase-3 which is then able to activate other caspases and to participate in the process of apoptosis. Sections of statin treated pellets were stained for active caspase-3. The results were inconclusive. No obvious macro-distribution or large scale changes in active caspase-3 were observed. The exact numbers were difficult to quantify further since the pellets had poor internal structure and gained an accurate overall cell count was not possible. It was not possible to identify cell boundaries, nor to be certain whether an area of positive staining belonged to one or two cells.

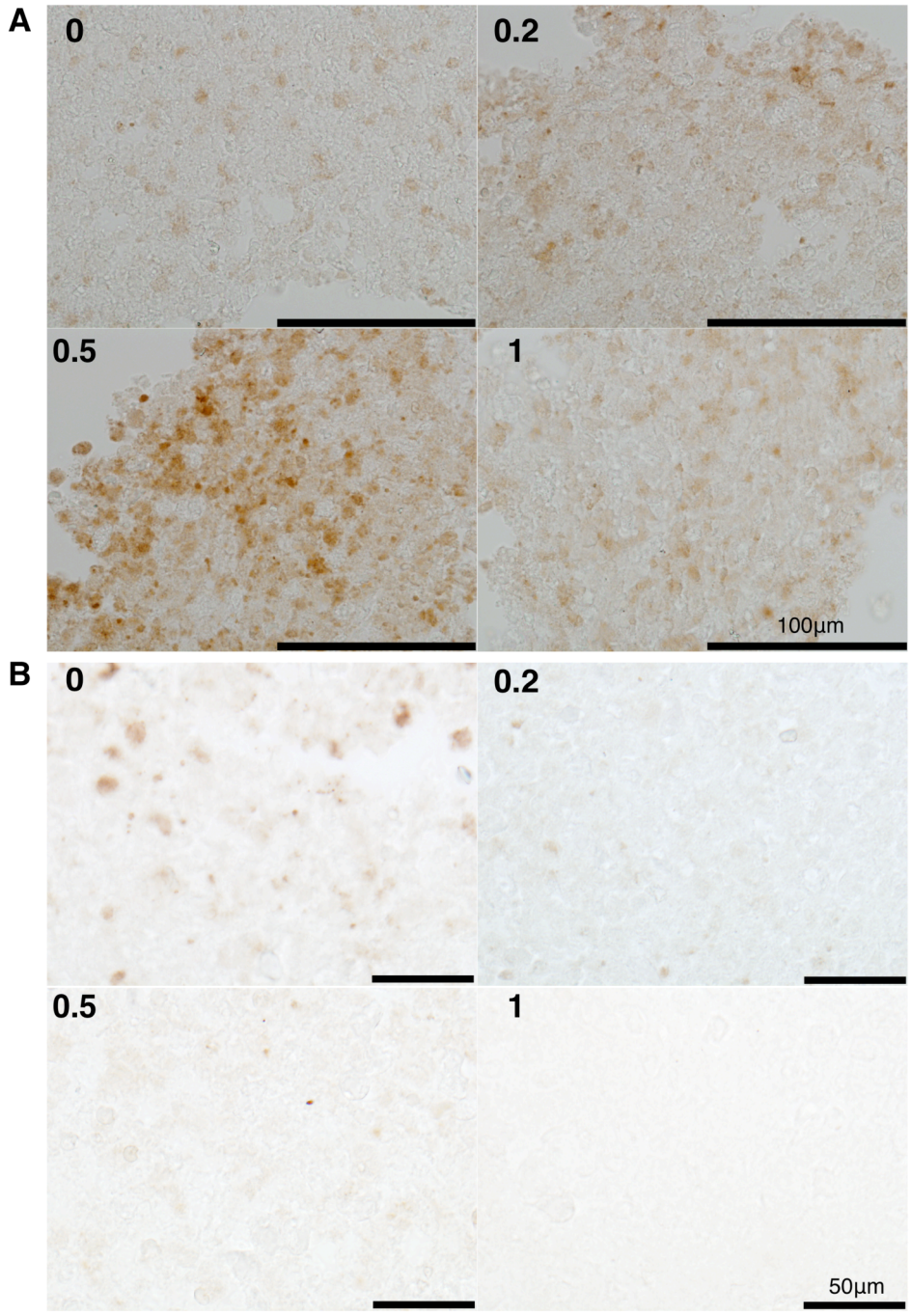


Figure 3.8 Effect of Lovastatin on Caspase 3

Pellets were seeded at a concentration of $1x10^6$ cells per well and cultured for two weeks with 0, 0.2, 0.5 and 1µM lovastatin with chondrogenic medium. 3µm wax embedded sections were stained with anti-active caspase 3. Results are shown from two experiments A and B. n = 3 in total.

3.2.7 Lovastatin Increases Sulphated Glycosaminoglycan Concentration

Sulphated glycosaminoglycan accumulation is a marker of chondrogenesis. In order to determine the effects of lovastatin on sGAG concentration, ATDC5 pellets were cultured in varying concentrations of lovastatin with and without chondrogenic medium. Sulphated glycosaminoglycan concentrations were measured using the DMB assay, which was effective on the larger pellets but smaller basal medium pellets fell under the accurate limits of detection of the assay (Figure 3.9A). Lovastatin caused a dose dependent increase in sGAG production per nanogram of DNA in pellets treated with chondrogenic medium (Figure 3.9B). This reached significance ($p<0.01$) at 1μM dose.

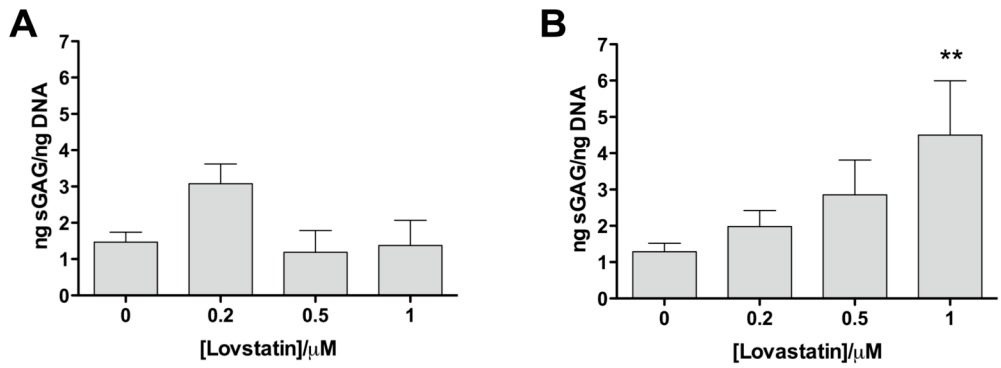


Figure 3.9 Effects of Lovastatin Treatment on Pellet sGAG Concentration

ATDC5 pellets seeded at a concentration of $1x10^6$ cells per well (of a 96 well plate) were cultured with varying concentrations of lovastatin with or without chondrogenic medium. Samples were digested with papain and the sGAG concentration measured by DMB assay. **A** Pellets fed with control medium. **B** Pellets fed with chondrogenic medium. Data shown are mean results of three independent experiments ±SD. Significance was determined by one-way analysis of variance with Dunnett's post-hoc test. ** $p<0.01$

3.2.8 Lovastatin Increases Collagen II Production

Collagen II is an extracellular matrix protein uniquely present in cartilage. Pellets grown in varying concentrations of lovastatin in the presence of chondrogenic medium were sectioned and stained for collagen II[7]. Pellets treated with chondrogenic medium alone for 14 days showed negligible staining for collagen II (Figure 3.10A). Pellets treated with lovastatin in addition to chondrogenic medium showed dose-dependent increase in staining for collagen II (Figure 3.10). This staining was located around the periphery of the pellets closest to the medium. Failure of the core of the pellets to stain could be attributed either to cell death or to poor diffusion of the statin causing these cells to remain less differentiated. The tissue which stains positively for collagen II displays the lacunar structure typical of cartilage.

[7] The verification of this antibody by Western blot to ensure that it does not cross-react with other collagen molecules is the gold standard, although technically challenging both to undertake (since mature collagen is not readily soluble) and to interpret (since the solubilisation process often generates many fragments of varying sizes, each of which may cross react with the antibody). Although the antibody used in these studies has not been verified by Western blot, it has been used on sections of tibial plateau where staining was observed only in the cartilaginous area and on sections of skin where no staining was observed.

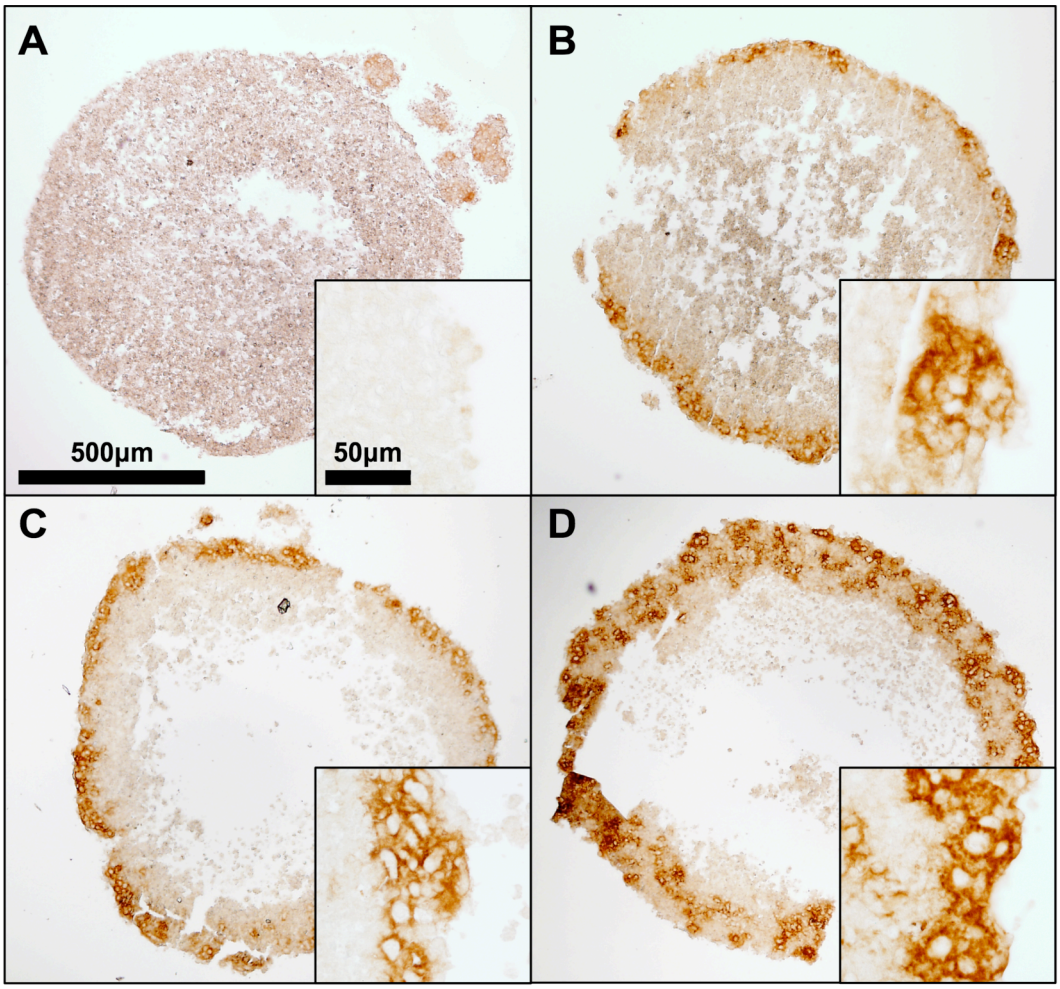


Figure 3.10 Lovastatin increases collagen II production

ATDC5 pellets seeded at a concentration of $1x10^6$ cells per well were cultured for 14 days in chondrogenic medium with 0 (A), 0.2 (B), 0.5 (C) or 1 (D) µM lovastatin, fixed and wax embedded. 3µm sections were probed with anti-collagen II. Results are representative of three independent experiments.

3.3 Discussion

This section of work had 3 aims:

1. Develop a 3D culture system which could be used with the tenocytes.
2. Develop assays for detection and quantification (if appropriate) of chondrocytic markers.

3. Assess the effects of lovastatin on ATDC5 differentiation in a 3D pellet culture system.

3.3.1 Effectiveness of Pellet Culture as a Three Dimensional Culture System

The chosen method of 3D culturing, that of pellet culture, whilst in favour at the start of the study, created a number of difficulties. The size of the pellets produced proved problematic. Initial experiments were undertaken using pellets seeded at $1x10^5$ cells (as recommended by Tare et al. [263]). These proved to be too small to handle easily. Several pellets had to be combined for Western blotting purposes. Additionally, even when several pellets were combined, they frequently contained too little sGAG to be detected by the DMB assay. This problem was especially acute in pellets cultured in maintenance medium treated with lovastatin. The small size additionally made these pellets extremely difficult to examine histologically.

To overcome these issues, pellets were seeded at $1x10^6$ cells. However, these larger pellets also suffer from size-related issues. The centre of many of the larger pellets is uncohesive and, in many cases, totally absent. Where it is present, the tissue does not look healthy. This is a recognised phenomenon, with some authors attributing the problem to oxygen diffusion problems. Although it is not possible to say for certain the cause of the difficulty in this instance, it is more likely to be caused by poor nutrient diffusion. The culture medium volumes used are probably insufficient to maintain the number of cells present in the pellets. Additionally, the size of the pellets makes the penetration of nutrients from the

medium into the core of the pellet difficult. To determine if these conditions are limiting, a bioreactor could be used to ensure better medium delivery.

Other groups have drawn attention to the drawbacks of the pellet culture system in generating healthy cartilage [269]. As work was drawing to a close on this section of work, a paper was published which proposed an alternative method of three dimensional cell culture for use in chondrocyte studies, utilizing trans-well culture on a permeable filter base [269]. This system delivers increased sGAG levels when compared to the pellet culture system. It also does not appear to suffer from the necrotic centre, present in many pellets. Although not stained for in this chapter, pellets frequently exhibit positive staining for collagen I in their outer layer [269]. This does not occur in the transwell culture system, presumably because of improved mechanics. If Murdoch et al. [269] are correct in their hypothesis that the expression of collagen II was inhibited by the presence of tensional forces within the periphery of the growing pellet this could have implications for the progression of chondroplasia within the degenerate rotator cuff. It is conceivable that the tensional force exerted upon the tendon by movement of the muscles of the rotator cuff acts to suppress the chondrocytic phenotype, particularly the expression of the cartilage matrix molecule collagen II. Upon tearing, this tensional force is removed which removes a brake on the chondroplastic process.

3.3.2 Possible Alterations and Improvements to the Methods Undertaken in this Chapter

The following section details some possible adaptations to and, in some cases replacements for, the methods and assays used in this chapter which might have improved the data presented herein.

3.3.2.1 Alternative Methods for Examining Cytoskeletal Structure

Alternative methods for examining the effect of lovastatin on cytoskeletal structure include replacing phalloidin with an anti-actin antibody. Although this would enable the use of DAB as a substrate in place of fluorescence, a measure that enhances sensitivity somewhat, poor signal quality was not an issue in this particular assay. Additionally, using an antibody would involve using many more steps and reagents than phalloidin alone.

Another alternative to staining cells grown on coverslips with phalloidin would have been to treat additional pellets with statins and to assay them by transmission electron microscopy. This has the advantage of revealing the fate of other components of the cell. If necessary, to confirm the fate of actin as distinct from other structures of the cytoskeleton, antibodies conjugated to gold could be used. This technique would also allow the visualisation of various features of apoptosis. Although features such as pyknotic nuclei are identifiable by epifluorescence, electron microscopy would have enabled the effects of statins on cells inside the pellets to have been determined (something not possible using coverslips). The disadvantage with electron microscopy, however, is that it would have involved

taking a two dimensional slice through a three dimensional object. To ensure that the data is truly representative careful selection of such a section is necessary.

3.3.2.2 DMB Assay - Alternative Method of Normalisation

An alternative method of normalising the sGAG concentration as determined by the DMB assay to dsDNA concentration would have been to normalise it to pellet dry weight. Although normalising sGAG concentration to dry weight provides a more accurate measure, particularly when dealing with samples of degenerate tendon where the matrix may well have been laid down by cells which subsequently die, it is questionable as to whether it would have been an appropriate normaliser for this system. Since chondrogenesis involves an increase in per cell output of sulphated glycosaminoglycans it is vital to ascertain sGAG output per cell, something that can not be done if sGAG concentration is normalised to pellet dry weight alone. This is all the more important when one considers that the cells treated with chondrogenic medium alone continued to proliferate, whereas the cells treated with statin did not. Normalising to dry weight alone would not necessarily allow a distinction to be made between many cells producing a small quantity of sGAG (such as might be produced by the pellets treated with chondrogenic medium alone) and a small number of cells producing a large quantity of sGAG (such as might be produced by the statin treated cells).

Additionally, this method would have been technically difficult to undertake. The small pellets on which the DMB assay was performed measured approximately 1mm in length when wet. Once dried these samples would have been extremely

tiny and we do not have a balance that would accurately record such a weight. Even the larger pellets used for histology would have been difficult to weigh. These also, as previously mentioned, suffered from problems of probable necrosis and where difficult to handle.

3.3.2.3 Viability, Apoptosis and Necrosis

The issue of pellet viability, especially in the presence of lovastatin, is an important one. The MTT assay (corrected to a measure of cell number such as DNA concentration to compensate for lovastatin's inhibitory effect on cell proliferation) could be used as one potential measure of pellet viability. The lactate dehydrogenase assay which measures the presence in the medium of a key enzyme in the glycolytic pathway is another alternative, measuring cytotoxicity rather than viability. If, as seems likely, pellet viability was found to decrease with the addition of lovastatin then the cells could be dying by apoptosis or necrosis. Currently no specific markers are available for necrosis, which is often diagnosed as cell death which in the absence of markers of apoptosis.

3.3.2.4 Active Caspase 3

Inconsistent active caspase 3 staining was observed across the experimental repeats (Figure 3.8). Some antibodies do not perform well on paraffin embedded sections (as used in this study). It is possible that this particular antibody would perform better on cryosections.

3.3.2.5 Collagen II Detection and Quantification

The enhanced collagen II expression could have additionally been confirmed by RT-PCR. The use of QPCR would also enable a quantitation of the increase in collagen II expression. Since hydroxyproline is only found in biological systems in collagen, determining the hydroxyproline content of each pellet would enable quantification of the increased collagen deposition.

3.3.3 Effect of Statins on Cell Cycle

Control of cell cycle is an important regulator of chondrogenesis within the growth plate. Cell cycle exit is required for hypertrophic differentiation [270]. The increase in DNA content in the pellets cultured in chondrogenic medium alone when compared with those cultured in maintenance medium alone indicates an increase in cell number in the pellets cultured in chondrogenic medium. Whilst this might seem counterintuitive in a differentiation model it appears to be a feature of this particular cell line, indeed it is commonly reported in the ATDC5 literature. Shukunami et al. report that monolayer ATDC5 cultures treated with chondrogenic medium contact inhibit at confluency but re-enter the growth phase 3 days later [262]. Chen et al. [271] report an increased number of cells in plates treated with chondrogenic medium versus control medium at 14 days in monolayer ATDC5 culture. These cells show upregulation of Collagen II and Collagen X mRNA as well as increased secretion of sGAG indicating that despite an overall increase in cell number overall the colony differentiates. Likewise, Tare et al. [263] reported the presence of PCNA in ATDC5 pellets cultured in chondrogenic medium for 21 days, a longer time period than used in this current

study. Indeed, in this study, pellets cultured in chondrogenic medium alone for 14 days were found to contain a large number of PCNA positive cells. Thus, it appears that the insulin used to induce chondrogenesis in ATDC5s additionally promotes cell growth.

Statins have been reported to cause cell cycle arrest at G_1 in primary cells and a large number of cell lines [272, 273]. They are reported to inhibit DNA replication [274, 275], induce the expression of cyclin dependent kinase inhibitors [276-278] and decrease transcription of cyclin D1 [279]. Used at clinical doses they are regarded as relatively safe and non-toxic drugs. However at high doses in vitro they also induce apoptosis in many tumour cell lines [280]. It seems likely that in chondrocyte cultures both cell cycle exit and apoptosis are possible outcomes of statin treatment and this may be dose-dependent, with lower doses affecting cell cycle only and higher doses inducing cell death. The doses used in this study are lower than those used to synchronise the cell cycle in cultured cell lines [273].

To date no published study has examined the effect of statins on cell cycle in chondrocytes. However, two previous studies have examined the effect of statins on chondrocytes using the MTT assay. This assay relies on the conversion of tetrazolium salts to formazan by dehydrogenases present within living cells [281]. The MTT assay has been recommended by some authors for the measurement of cell proliferation but is better used, with caution, as a measure of cell viability [282].

Hatano et al. examined the effects of mevastatin on rat foetal chondrocytes using the MTT assay. The authors interpreted the data as indicative of cell number and drew conclusions concerning the effect of mevastatin on chondrocyte proliferation, finding that mevastatin did not enhance chondrocyte proliferation [222]. The study reported a decrease in formazan levels in monolayer primary rat foetal chondrocyte cultures exposed to 2μM mevastatin when compared to control cultures over a 12 day period. The authors note the absence of a positive effect on formazan levels by mevastatin but do not comment on the possible reasons for the comparative decrease in formazan levels seen in the cultures treated with 2μM mevastatin. Assuming that statins do not affect either dehydrogenase enzyme levels or activity (i.e. that formazan levels correspond to cell number), the relative decrease in formazan levels in cultures treated with mevastatin would be consistent with cell cycle exit. It should further be noted that at no point does the reported value for 2μM mevastatin cultures dip below that observed on day 0 suggesting that cell death is unlikely to be responsible for the relative decrease in formazan. However, these conjectures need to be formally verified by assaying for cell proliferation and death.

Likewise, Zhang et al. reported a decrease in formazan levels in rat intervertebral disc cells cultured in alginate beads and exposed to 1 and 3μM simvastatin [256]. In this instance the authors interpreted the MTT assay in terms of cell viability, concluding that simvastatin decreases cell viability in a dose-and time-dependent manner. Unfortunately, unlike the data presented by Hatano et al.[222], the data in this paper is normalised to the vehicle control. Consequently it is impossible to determine if the observed decrease in formazan levels was a true decrease in cell

viability (and therefore potentially cell number) caused by toxic effects of the statin or a relative decrease caused by formazan production levels remaining constant in the statin treated samples (possibly due to cell cycle exit), while formazan production levels (and potentially cell number) increased in the control.

Although neither of the two published studies prove a reduced proliferation of chondrocytes with statin treatment the data could be interpreted as supporting such a hypothesis. Indeed, our group has previously observed just such a reduced rate of increase in cell number in adult bovine articular chondrocytes cultured in 3D pellets in the presence of lovastatin [221].

In the current study lovastatin caused a dose-dependent decrease in DNA concentration with lovastatin treated pellets containing fewer PCNA positive cells. These data are consistent with lovastatin promoting cell cycle exit. Indeed, positive PCNA staining is restricted to the S phase of cell cycle [267]. The decrease in PCNA positive staining with lovastatin treatment is therefore consistent with cell cycle arrest at the G_1/S transition. However, these hypotheses would need to be confirmed with analysis of cyclin/cdk content.

Wang et al. [219] report that transcription from the cyclin D1 promoter is under the control of RhoA in primary mouse chondrocytes. Overexpression of wild type RhoA or expression of the constitutively active form of RhoA, Q63L, upregulates expression of cyclin D1, while expression of the dominant negative form of RhoA, T19N, down regulates cyclin D1 promoter activity. Lack of cytotoxicity was confirmed by MTT assay. Wang et al. further report that inhibition of the

effectors of the small GTPase RhoA ROCK1 and 2 by 10μM of the specific inhibitor Y27632 reduces the proliferation of chondrocyte cell lines, including monolayer ATDC5s [219], while upregulating markers of hypertrophy. This study did not check for cytotoxicity with Y27632, however the dose used is that recommended for the inhibition of ROCK and it does not cause apoptosis in the other cell types in which it has been used. It therefore seems likely that the statin induced decrease in cell number seen in the current study could be attributable, at least in part, to inhibition of RhoA and ROCK signalling.

3.3.4 Effect of Statins on Matrix Production

In the current study lovastatin treatment caused a dose dependent increase in sGAG concentration. This corresponds well to the published data, with both mevastatin and simvastatin reported to dose dependently increase sGAG production and secretion in foetal chondrocytes [222] and intervertebral disc cells [256]. Statins correspondingly increase not only ACAN (aggrecan core protein) mRNA levels but also COL2A1 (collagen II) mRNA in a dose dependent manner [222, 256]. In the latter study, the effects of statin on ECM molecule mRNA levels were overcome by the addition of the downstream metabolite mevalonate confirming the specificity of the effect. Although no published work has investigated the effect of statins on collagen II protein, previous work by our group has demonstrated a dose dependent increase in response to lovastatin treatment in adult bovine chondrocytes [221]. I have confirmed that lovastatin not only significantly enhances the production of cartilage matrix molecules but that it does so without disrupting the specialised structure of the tissue.

Recent work implicates the small GTPase RhoA and its downstream effectors ROCK1/2 in the control of cartilage matrix production. Woods et al. report that over expression of RhoA decreases collagen II and aggrecan core protein mRNA levels in monolayer cultured ATDC5s [257]. Likewise, transfection of micromass cultured chick limb bud cells with a dominant negative form of RhoA or treatment of the same with C3 transferase (which selectively inhibits the Rho subfamily) increases collagen II and aggrecan core protein mRNA [283]. Increased staining with Alcian blue is also seen.

Inhibition of ROCK1/2 by Y27632 increases sGAG levels [218]. It additionally increases collagen II and aggrecan core protein mRNA levels in monolayer cultured ATDC5s [257]. Lovastatin is known to disrupt small GTPase signalling therefore it is possible that lovastatin may exert its prochondrogenic effects via disruption to the RhoA and ROCK signalling pathways.

3.3.5 Summary

Lovastatin promotes cell cycle exit and the expression of a cartilaginous matrix. This suggests enhanced chondrocyte differentiation. This could be of further use in applications such as autologous chondrocyte implantation. However, the timing of the application of statins would need to be carefully controlled. One could envisage the application of growth factors designed to multiply cell numbers, followed by the application of statins, which would then cause differentiation, yielding good quality cartilaginous matrix. This would quite nicely model the process seen in vivo.

Chapter 4 - Tenocyte Phenotyping

4.1 Introduction

In order to establish an in vitro system for determining if cell shape change or hypoxia are pro-chondrogenic it is necessary to use a population of well characterised healthy tenocytes. This chapter is concerned with the isolation and characterisation of normal human tenocytes. As such it considers both the current knowledge (and limits thereof) of tenocyte phenotype and characterises the populations of tenocytes used during preparation of this thesis.

This chapter discusses three principal questions:

1. What are the markers that distinguish tenocytes from other mesenchymal cells?
2. How long do cultured tenocytes maintain expression of these markers (i.e. how long do they maintain their differentiated phenotype)?
3. Which markers do tenocytes share with chondrocytes and which distinguish them from each other?

4.1.1 Phenotypic Marker Selection

A good marker of phenotype ought to be unique, that is, expressed in no other cell type, and changes in its levels ought to indicate an alteration in the function of the cell. Little work has been done on defining specific markers of tenocytes. Unlike cartilage and bone there is no consensus panel of markers, although some molecules are better accepted than others. Table 4.1 contains tenocyte phenotype

markers as they existed at the start of this thesis. The markers broadly split into two categories, ECM molecules and non-ECM molecules arising from developmental studies. Each category has both strengths and weaknesses when considered for phenotyping the tenocytes used in this thesis.

ECM molecules, such as decorin [123] and cartilage oligomeric matrix protein (COMP) [153], are definitely expressed in adult tendon tissue. They are also highly likely to be functionally significant, given the structural role tendon plays within the body. However, as previously discussed (Section 1.5.1) many ECM molecules are not specific to tendon, being shared with other fibroblasts, even other cells from the mesenchymal lineage. A good example is collagen I, which is expressed by many connective tissue cells, including dermal fibroblasts [284] and osteoblasts [285].

Developmental markers, in contrast, are identified precisely because they are not expressed in other joint tissues. However, the relevance of these markers to adult tendon phenotype has not been investigated in many incidences. The markers in question may either not be expressed, not be specific or may have no functional role within the adult tissue (some authors [67] are sceptical of a role for scleraxis beyond the embryonic stage, for example).

This field badly needs a better description of adult tenocyte phenotype. Some of these problems have begun to be addressed with recent publications. Since these were not available when the phenotyping for this thesis was undertaken, however, their findings will be detailed in the Discussion section for this chapter.

Marker	Type	Description	Usage
Aggrecan	Lectican	Tendon ECM component	-
Collagen I	Fibrillar collagen	Major ECM component in tendons.	[35, 53, 247, 286, 287]
COMP	Glycoprotein	Significant ECM component in tendons.	[287]
Decorin	class I SLRP	Significant ECM component in tendons.	[53, 286]
EphA4	Receptor tyrosine kinase	Expression in the developing limb bud localises at sites associated with tendon formation [288].	[35, 287]
Eya1 and Eya2	Transcription factors	Appear to participate in the patterning of distal limb tendons during development [289].	[35]
Scleraxis	bHLH transcription factor	Putative master transcription factor for tendon phenotype (see Section 1.3.2.2.4.1).	[35, 53, 247, 287]
Six1 and Six2	Homeobox transcription factors	Originally identified as a specific marker(s) for limb tendons during development [290]. A recent report suggests that Six1 doesn't mark tendon primordia or tendons and that limb tendon formation is independent of Six1 [291].	[35, 287]
Tenascin-C	Glycoprotein	Originally described as strongly expressed at the MTJ [292]. Posited as pivotal for the correct alignment and orientation of the collagen fibres [293]. Known to be strongly expressed in tissues other than tendon including cartilage [292].	-
Tenomodulin	Type II transmembrane glycoprotein	Positively regulated by scleraxis [74]. Suggested as a promising phenotype marker for the later events of tendon development [74].	-

Table 4.1 Tenocyte Phenotype Markers as Posited in Summer 2007.

4.1.2 The Panel of Tenocyte Markers used in this study

The panel of markers selected for initial tenocyte phenotyping for this study were collagen I, aggrecan and scleraxis.

Collagen I is the main matrix molecule produced by tenocytes. It was therefore important to confirm that it is expressed by the explanted fibroblasts and that this expression is stable in long-term culture. It has been used frequently in past tenocyte phenotyping studies and has shown decreased levels on extended passage, suggesting a sensitivity to differentiation status [286].

Aggrecan is a normal non-collagenous component of tendon ECM. It is used as a marker of differentiation status in chondrocytes and, as such, may be more sensitive to monolayer culture than collagen I.

Scleraxis is the putative master transcription factor for tendon and, as such, its expression has been used in many studies as an indicator of tendon phenotype. No study published at the start of this thesis had examined the effect of monolayer culture on scleraxis although Schulze-Tanzil et al. did use scleraxis expression as a marker of tenocyte differentiation status for cells in high-density culture [247]. It is likely that expression of this molecule is sensitive to differentiation status.

4.2 Results

4.2.1 Tenocyte Isolation

Reconstruction of the anterior cruciate ligament involves the replacement of the ruptured ligament with a section of hamstring tendon. Pieces of tendon excised from the patient but not used in repair are routinely discarded. This constitutes a good source of healthy tendon tissue for culture.

Samples of normal human hamstring tendon were obtained from patients undergoing anterior cruciate ligament reconstruction. Explants from these were cultured overnight in small drops of DMEM/F12 containing 50% FCS to promote explant adhesion and consequent tenocyte migration. 2ml of DMEM/F12 50% FCS was added within 72h. A population of cells were observed to migrate out from the explants typically after 7-14 days (Figure 4.1A)(Image courtesy of Dr Philippa Hulley). Once robust sheets of cells had emerged from most of the explant pieces, the tissue was removed and the cells cultured in DMEM/F12 10% FCS. Typical morphology is fibroblastic (Figure 4.1B)(Image courtesy of Dr Philippa Hulley). Cells display a strong tendency to align in parallel with their neighbours, producing a "basket-weave" appearance of multiply aligned clusters in confluent culture.



Figure 4.1 Explanted Tenocyte Population

A Cells emerging from tendon explants. B Phase contrast image of explanted cells. Images courtesy of Dr Philippa Hulley.

4.2.2 Tenocyte Phenotyping

In order to confirm that the emigrant fibroblast population were tenocytes it was necessary to characterize their phenotype. Selection of the phenotype marker panel has been previously described (Section 4.1.2).

4.2.2.1 Tenocytes Express Scleraxis Protein

Specificity of a scleraxis antibody was confirmed by Western blot (Figure 4.2A). A band of 22kDa (the molecular weight of scleraxis) was detected in the ATDC5 lysate (a). In contrast no band was seen in the Jurkat (a T-cell line, b) lysate. Scleraxis is reported to be expressed in both differentiating [66] and mature cartilage [67]. It is therefore not unexpected to find a 22kDa band in the ATDC5 lysate. A band of the same molecular weight was observed in monolayer cultured human tenocytes from all passages tested (passage 1 - passage 5, c-f) indicating that the cultured cells do, indeed, express the tenocyte marker scleraxis. The

presence of this band in the passage 5 tenocyte lysates suggests that the cells retain their differentiated phenotype until at least passage 5.

To further confirm the specificity of the scleraxis antibody sections of mouse testis were stained. In the testis, scleraxis expression is restricted to the Sertoli cells [69, 294]. The scleraxis antibody specifically stained Sertoli cell nuclei in wax embedded adult mouse testis sections (Figure 4.2B). Healthy human tenocytes cultured on coverslips were therefore probed with the same antibody. A specific nuclear stain was seen in a small number of tenocytes (Figure 4.2C). The expected proportion of positive nuclei in adult tendon tissue is unknown.

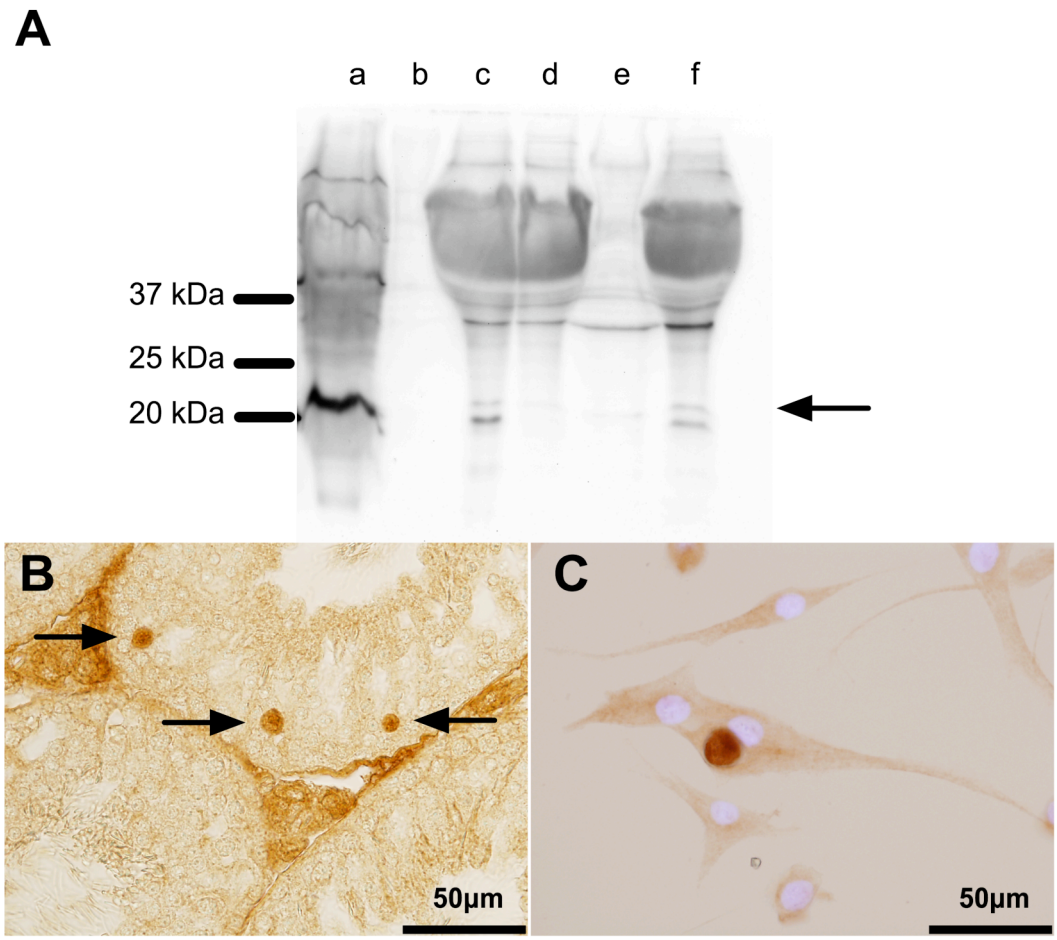


Figure 4.2 Scleraxis is Expressed in Sertoli Cells and Explanted Tenocytes

A Western blot of cell lysates probed with anti-scleraxis antibody. a = ATDC5, b = Jurkat, c-f monolayer cultured tenocytes c = passage 1, d = passage 3, e = passage 5 (extracted in HIF buffer), f = passage 5. Arrow indicates band of approximately 22kDa, which is the molecular weight of scleraxis. B Wax embedded testis sections stained with anti-scleraxis (DAB). Arrows indicate the nuclei of Sertoli cells, which stained positively for scleraxis. C Tenocytes (passage 2) cultured on coverslips were stained by immunocytochemistry for scleraxis (DAB) and counterstained with DAPI.

4.2.3 Maintenance of Tenocyte Phenotype in Monolayer Culture

Dedifferentiation of tenocytes in monolayer culture has been reported in the literature [53, 286]. To confirm the stability of the phenotype, tenocytes were grown for eight passages and the mRNA expression of three tenocyte markers was assessed by QPCR. The tenocytes stably express mRNA for the tendon matrix

molecules collagen I (COL1A1) and aggrecan (ACAN) (Figure 4.3). They also express mRNA for the putative master transcription factor for tendon, scleraxis (SCX). Expression of this mRNA is stable until about passage 5. After this mRNA levels drop, suggesting a possible dedifferentiation (Figure 4.3). The dedifferentiation of cultured cells after passage 5 is broadly consistent with the previous protein data (see Section 4.2.2.1) and with previous findings. Juvenile rabbit tenocytes [286] and human Achilles tenocytes [53] isolated by collagenase treatment are reported to begin dedifferentiation after passage 2 and passage 3 respectively. To ensure a stable tenocytic phenotype for this study, only cells at or below passage 3 were used for further experiments.

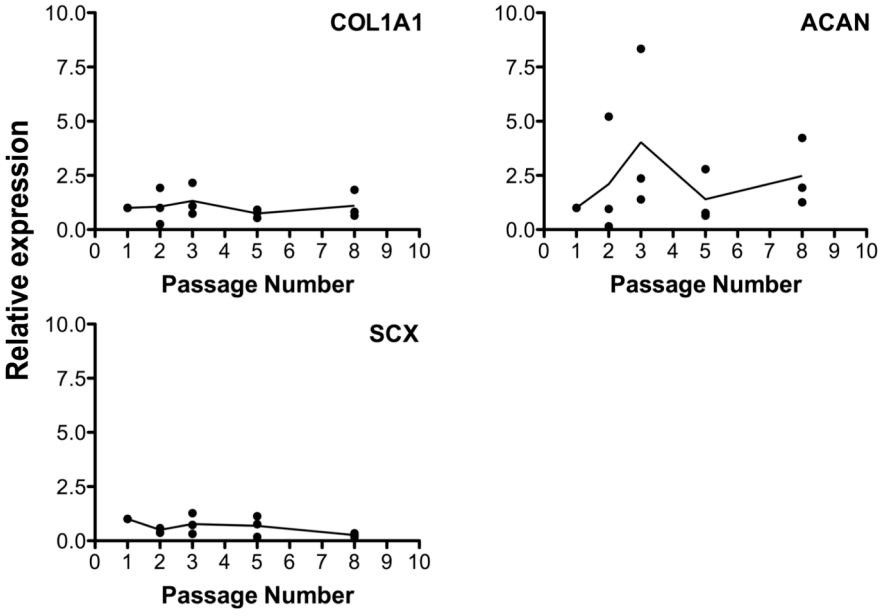


Figure 4.3 Stability of Tenocyte Phenotype

Human hamstring tenocytes were cultured in monolayer for eight passages. COL1A1, ACAN and SCX mRNA levels were assessed by QPCR and normalized to GAPDH. (Cell culture and RNA extraction performed by Dr Min Liang. Reverse transcription and QPCR performed by the author).

4.2.4 Further Characterisation of the Tenocytes with regard to Chondrocytic Markers

Normal tenocyte phenotype is poorly characterised, especially with regard to the expression of molecules usually associated with cartilage. A comparison was therefore made of the expression of various markers normally associated with cartilage in healthy human tenocytes and human cartilage by RT-PCR. Tenocytes were found to express mRNA for the chondrocytic markers Sox5, Sox6 and Sox9. In contrast the matrix protein collagen II was only expressed in cartilage and not at all in tendon.

Although high levels of aggrecan expression are associated with chondrocytes, tenocytes are also reported to express mRNA for this proteoglycan [194]. In line with this report mRNA for aggrecan core protein was detected in the cultured hamstring tenocytes. The expression of low levels of Sox9 protein by human tenocytes has been described by Tempfer et al. [295]. Sox9 was also found to be expressed at the mRNA level by the cultured tenocytes. To date no group has examined the expression of Sox5 or Sox6 by tenocytes. The data presented in Figure 4.4 demonstrate for the first time the expression of mRNA for both molecules in cultured normal human hamstring tenocytes. In contrast the matrix protein collagen II was only expressed in cartilage and not at all in tendon. This was the case for both the 'pre-chondrocyte' long form of COL2A1va1 (encoding type IIA collagen) and the 'chondrocyte' short form of COL2A1 (encoding type IIB collagen) [296, 297].

Relative levels of expression cannot be judged from these figures since the concentration of cDNA in the cartilage extract was not measured and therefore it can not be stated with certainty that the same amount of cDNA was loaded in to each reaction. Additionally, none of the reactions were optimised to reveal differences in relative expression level but were run for 40 cycles in order to determine if any transcript were present in each cell type. It should also be noted that attempting a comparison using this figure would have little meaning since it compares cartilage tissue with cultured tendon cells. A more meaningful experiment for these purposes would compare expression levels in cartilage and tendon tissue and between cultured chondrocytes and tenocytes. Indeed, such studies have been published recently [67, 298].

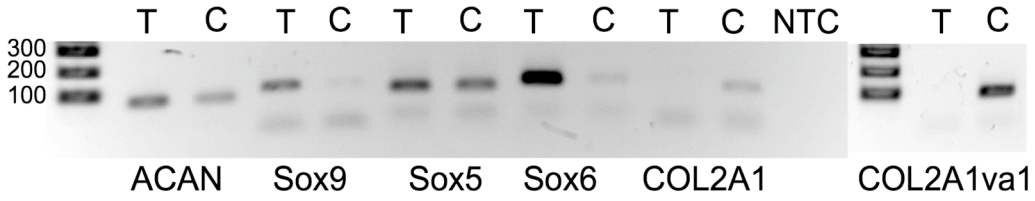


Figure 4.4 Cultured Tenocytes Express some Chondrocyte Markers but not Collagen II

The expression of ACAN, Sox9, Sox5, Sox6, COL2A1 and COL2A1va1 mRNA from cultured healthy human tenocytes (T) and human cartilage (C) assayed by RT-PCR (40 cycle reaction). NTC = no template control. Results are from a single experiment.

4.3 Discussion

That tenocytes have not been well defined can be illustrated both by the lack of consensus on a panel of comprehensive markers and by the publication of several papers during the time period of preparation for this thesis which impact on this issue. The papers fall into two broad categories, those that attempt to refine the

panel of known potential markers of tenocyte phenotype and those that, by design or by accident, suggest potential new phenotypic markers.

4.3.1 Significance of Recent Studies

Taylor et al. [67] assembled a panel of commonly used phenotypic markers and attempted to determine which of them distinguished tendon from other mesenchymal tissues with a particular interest in those markers which could be used to investigate tenogenesis. In contrast Jelinsky et al. [76] attempted to identify novel tendon markers by comparing gene expression in both human and rat mesenchymal tissues by microarray. These studies are significant to the field of tendon phenotyping since they represent the first concerted effort to identify tendon phenotype markers in adult tissue.

4.3.2 Recommended Established Markers

4.3.2.1 Distinguishing Tenocytes from Other Mesenchymal Cell Types

Taylor et al. [67] attempted to refine the definition of tenocyte as it pertains to mature tissues. The authors identified low expression of tenascin-C with high expression of scleraxis and COL1A2 as being characteristic of tensional tendon. While both tenascin C and scleraxis expression are significantly different in tendon and bone, there is no significant difference between expression levels in tendon and cartilage. From a perspective of positively identifying tendon from cartilage in this study better markers for tendon would be COL1A2, decorin and

tenomodulin[8]. The findings of the study by Taylor et al. were substantially supported and found to be relevant to human cells in monolayer culture by another recent study [298].

Jelinsky et al. [76] adopted a more hypothesis free approach and searched for novel markers of adult tendon tissue (rat Achilles, patellar and supraspinatus tendons and unspecified adult human tendon) by microarray. The study identifies approximately 18 genes, which appear to have specific expression in tendons, some of which have been previously suggested as markers of tenocyte phenotype. Of these, the authors identified tenomodulin as showing particularly enhanced mRNA expression in human tendon. Interestingly, decorin was also identified as showing enhanced expression in human tendon.

4.3.2.2 Determining Maintenance of Phenotype In Vitro

Recent studies have also impacted on the selection of markers, which enable detection of dedifferentiation in monolayer culture. Stoll et al. compared the expression of various genes from freshly isolated human hamstring tendon with tenocytes subject to extensive monolayer passage [299]. They found significant decreases in the mRNA levels of the tendon matrix genes decorin, COMP and ACAN in monolayer cultured tenocytes compared to the levels in native tissue. They also found a significant increase in COL3A1 and an increase in COL1A1 mRNA in monolayer cultured tenocytes. The transcript levels of elastin and β_1

[8] This chapter concerns itself with the characterisation of tenocytes. The question of suitable markers for chondrocytic transformation of tenocytes will be addressed in Section 5.1.3, however it is interesting to note in passing that Taylor et al. [67] also identify significantly greater expression of SOX9 and COL2A1 mRNA in cartilage compared to tendon.

integrin were unchanged. The change in levels of the selected transcripts between the isolated tissue and the late passage monolayer tenocytes suggests that these transcripts could be useful as potential markers of dedifferentiation in culture and supports the use of COL1A1 and ACAN as tenocyte differentiation status markers in this study.

Pauly et al. compared the mRNA expression levels of various putative tenocyte markers in monolayer cultured human tenocytes, chondrocytes and osteoblasts [298]. The data strongly paralleled the findings of Taylor et al. [67], who compared tendon, cartilage and bone tissue. Interestingly, however, Pauly et al. [298] failed to find the differences in scleraxis and tenomodulin levels between tenocytes, chondrocytes and osteoblasts that Taylor et al. [67] observed between tendon, cartilage and bone. Taylor et al. [67] noted a decrease in scleraxis expression in monolayer horse superficial digital flexor tenocyte culture at both passage 1 and passage 5 compared to the levels found in tissue, a finding confirmed in much later passage human hamstring tenocytes by a subsequent study [299]. Taylor et al. also failed to find tenomodulin expression in monolayer cultured tenocytes, while Jelinsky et al noted that this molecule (in common with thrombospondin 4) was downregulated in monolayer cultured cells compared to fresh tissue [76]. The lack of differences between scleraxis and tenomodulin expression in monolayer cultured cells in the work of Pauly et al. [298] may be attributable to a sensitivity to monolayer culture in scleraxis and tenomodulin expression. This suggests that these genes may be particularly good markers of tenocyte differentiation status and supports the use of scleraxis as a phenotype marker in this study.

4.3.3 Criticism of Some Established Tenocyte Markers

As well as identifying potential markers of adult tendon tissue, both Taylor et al. [67] and Jelinsky et al. [76] make additional recommendations concerning the unsuitability of certain markers for this task.

4.3.3.1 Scleraxis

Jelinsky et al. [76] are critical of the use of scleraxis for the identification of adult tendon tissue. This is because, although scleraxis did pass the filter for rat tendon-specific expression, it was additionally expressed in many tissues including muscle.

Taylor et al. [67] fail to find a significant difference in scleraxis expression between cartilage and tendon. They attribute this to the inclusion of perichondrium in the cartilage sample, noting that this is derived from scleraxis expressing cells. They are additionally somewhat sceptical of the relevance of scleraxis to adult tenocyte phenotype.

4.3.3.2 The Developmental Markers - Six1, Six2, Eya1 and Eya2

In the light of this statement it is important to note that Jelinsky et al. [76] specifically state that the proposed tendon markers Six1, Six2, Eya1 and Eya2, which were first identified through developmental studies, did not emerge as tendon specific genes. This suggests that, while these markers may be appropriate for studies of tenogenesis, they are inappropriate for phenotyping mature adult cells.

4.3.3.3 Novel Potential Markers of Tenocyte Phenotype

4.3.3.3.1 Mohawk

Recent work [300] has implicated mohawk, a member of the Three Amino acid Loop Extension superclass of atypical homeobox genes [301], as an important transcription factor in the maturation of the tenocyte phenotype, particularly with respect to expression of the major tendon ECM molecule, collagen I. Mohawk knockout mice show impaired expression of collagen I despite the presence of scleraxis [300]. There also appears to be an additional effect on the major tendon SLRP decorin, which showed decreased levels [300]. Although the focus of this paper was the role of mohawk in tenocyte maturation the authors speculate briefly that mohawk may have a role in tendon homeostasis in adult tissue. Additionally mohawk was identified as a specific marker of adult human tendon in a recent microarray paper [76]. It would therefore be interesting to investigate the expression of mohawk in our cultured tenocytes, especially with respect to expression over extended monolayer passage.

4.3.3.3.2 Thrombospondin 4

Thrombospondin 4 is a large pentameric glycoprotein capable of binding collagen and COMP. Jelinsky et al. identified thrombospondin 4 as having strongly enhanced mRNA expression in adult human tendon compared to other adult tissues of mesenchymal origin [76].

4.3.4 Tenocyte Phenotype and Three Dimensional Culture

Recent studies have suggested that tenocyte phenotype may be better maintained in three-dimensional culture. As has already been noted, scleraxis and tenomodulin appear to have a particular sensitivity to monolayer culture. Two recent studies confirm this hypotheses (for scleraxis) and demonstrate upregulation of this transcript in 3D culture [299, 302]. Other tenocytic molecules including aggrecan, COMP and decorin which show downregulation in monolayer culture compared to their levels in freshly isolated tissue are also upregulated by 3D culture [299]. Future work in this field should investigate the potential of various 3D culture systems for maintaining tenocyte phenotype.

Summary

The emigrant fibroblast population therefore stably expresses major markers of tenocytes but does not express the major cartilage matrix molecule, collagen II. Given the highly specific nature of the tissue from which the fibroblasts originated it seems justified to identify these cells as tenocytes. The tenocytes were found to additionally express mRNA for the 'chondrocytic' transcription factors Sox5, Sox6 and Sox9. The expression of Sox5 and Sox6 by tenocytes is, to our knowledge, a unique finding and suggests a high degree of relatedness between tenocytes and chondrocytes. The field of tenocyte phenotyping is still emerging. As in the cartilage field, time and ongoing studies will undoubtedly reveal the tenocyte phenotype more exactly. Full phenotypic expression of tenocytes in culture may require use of 3D, mechanically loaded constructs under reduced oxygen tension.

Chapter 5 - Cell Shape Change and Tenocyte Phenotype

5.1 Introduction

5.1.1 Chondroplasia and Cell Shape Change

This thesis is concerned with investigating potential drivers of chondroplasia in degenerate rotator cuff tendons. An examination of the published literature and of the features of the degenerate cuff lead to the identification of several possible drivers of chondroplasia. One of these drivers is cell shape change which is particularly linked with the structure of the actin cytoskeleton. This chapter is concerned with examining the effects of cell shape change, particularly that caused by alterations to actin polymer structure, on the expression of chondrocytic markers by tenocytes.

5.1.2 Culture System Selection

In order to determine if cell shape and the structure of the actin cytoskeleton has an effect on tenocyte phenotype, it is necessary to use an in vitro culture system. This in vitro culture system requires both well characterised tenocytes and an established method of culture which should, ideally, mimic the natural environment of the tendon. Chapter 4 has detailed the establishment of well characterised populations of normal human tenocytes and has determined the limits of phenotype stability for these cells in monolayer culture.

It was initially believed that to maintain the tenocytic phenotype of the cells culture in three dimensions would be necessary. The preparatory work detailed in chapter 3 therefore partly focused on establishing the 3D pellet culture system, which previous studies have recommended for the maintenance of chondrocyte phenotype. However this work revealed problems with the pellet culture system, including high levels of cell death at the centre of the pellets. It was therefore decided to perform some initial studies in monolayer with an optional shift into an alternative 3D culture system (a modified version of that used by Murdoch et al. [269]) if the results in monolayer proved unpromising.

5.1.3 Phenotypic Marker Selection

In order to assess chondrocytic transformation it is necessary to have a panel of markers of cell phenotype (see Table 5.1). Collagen II was chosen as a marker of cartilage phenotype because it is only produced by chondrocyte-like cells and because it has been reported as a feature of chondroplastic rotator cuff tendon matrix. The tendon matrix molecules COL1A1 and COL3A1 were also selected because in order to be confident a true transdifferentiation is occurring it is important to see not only increases in chondrocyte markers but a decrease in tenocyte markers.

Work done for this thesis has unexpectedly shown that Sox5 and Sox9 are expressed in both chondrocytes and normal human tenocytes (see Section 4.2.4). These transcription factors were included because both Sox5 and Sox9 are necessary for the expression of collagen II, a key component of cartilage matrix. Since collagen II is not expressed in tensional tendon and is not expressed in the

monolayer cultured tenocytes, it is reasonable to assume that Sox gene levels may increase with transdifferentiation. Indeed work published subsequent to the completion of practical work on this thesis found that Sox9 levels were significantly higher in cartilage compared to tendon [67].

Marker	Type	Phenotype
COL1A1	Main ECM component in tendon	Tenocyte
Collagen II	Main ECM component in cartilage	Chondrocyte
COL3A1	Tendon ECM component	Tenocyte
SOX5	Transcription factor	Shared (but chondrocyte associated)
SOX9	Transcription factor	Shared (but chondrocyte associated)

Table 5.1 Cell phenotype markers.

5.1.4 Proposed signalling link

As previously described in Section 1.8.2.3, there is a common signalling pathway for the transmission of both mechanical signals and signals from the ECM to the cell nucleus which involves integrins, RhoA, ROCK and the actin cytoskeleton. The proposed signalling link between the ECM, through integrins to RhoA and Src and on to actin structure and a potential repression of Sox9 signalling is detailed in Figure 5.1A. Figure 5.1B shows the point of action of each of the panel of cytoskeletal inhibitors on this scheme including a hypothetical upregulation of Sox9 gene transcription.

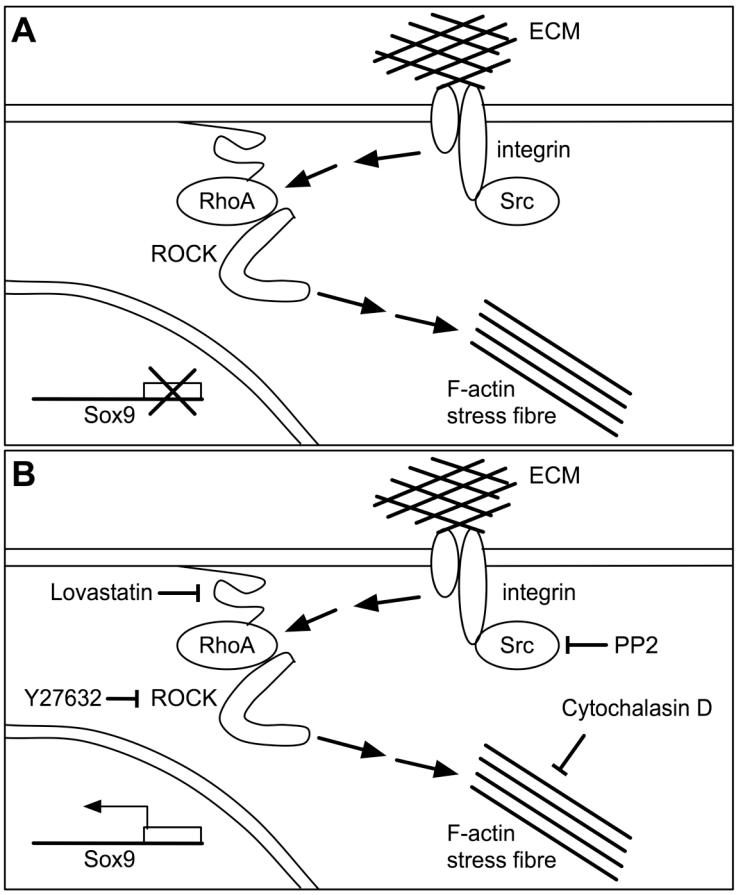


Figure 5.1 Cytoskeletal Signalling Disruptors

A. Proposed signalling mechanism in healthy tenocytes. B. Points of action of cytoskeletal inhibitor panel.

5.1.5 The Panel of Inhibitors of Cytoskeletal Signalling

Given the interdependence of cell shape as mediated by the actin cytoskeleton and chondrocyte phenotype and the dramatic changes in cell shape observed in the degenerate rotator cuff it was hypothesised that disruption to signalling pathways affecting the actin cytoskeleton might induce expression of chondrocytic markers in healthy tenocytes. A panel of reagents was therefore assembled which is capable of affecting either the actin cytoskeleton directly or of various actin-regulating signal transduction pathways. Each reagent selected has reported pro-chondrogenic action in at least one model of chondrogenesis.

5.1.5.1 Cytochalasin D

Cytochalasin D has been used for decades to disrupt the actin cytoskeleton and has been reported to induce chondrogenic marker expression in several cell systems [216, 220, 303, 304]. The concentration of cytochalasin D used in chondrocyte experiments varies between about 1μM [220, 257] and about 4μM [216, 303], although one study uses up to 20μM [304]. 3μM was chosen for this study because this concentration upregulated Sox9 transcript levels in limb bud micromass cultures [218].

5.1.5.2 PP2

4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyramidine (PP2) inhibits the Src family of non-receptor tyrosine kinases. It prevents dedifferentiation of chondrocytes in monolayer culture including the downregulation of Sox9 expression. It induces cell rounding, a cortical arrangement of actin and upregulates Sox5 and collagen II expression [78]. The PP2 concentration that produces these effects in chondrocytes (10μM) was chosen for use with tenocytes.

5.1.5.3 Y27632

Recent work suggests a role for small GTPases in the control of chondrocyte phenotype. RhoA and its downstream effectors ROCK1, ROCK2 and mDia1 have been implicated as negative regulators of chondrogenesis [212, 283]. Treatment of the chondrogenic cell line ATDC5 with ROCK1/2 inhibitor Y27632 causes a re-organisation of the actin cytoskeleton. Concurrently Sox9 and sGAG

production is upregulated [218]. ROCK inhibition also accelerates hypertrophy [219]. The concentration of ROCK used in this thesis (10μM) was chosen because is sufficient to abolish stress fibres in a number of cell types [248] including ATDC5s and chondrocytes [218] and because it concurrently upregulates Sox9 transcript levels and sGAG accumulation in chondrocytes [218].

5.1.5.4 Lovastatin

Statins are widely used small molecule drugs that affect signalling via multiple GTPases. They inhibit HMG-CoA reductase, an enzyme which catalyses the conversion of HMG-CoA to mevalonate, an early step in the cholesterol synthetic pathway. Downstream metabolites of this enzyme are involved in prenylation, a posttranslational modification of small GTPases. Inhibition of prenylation alters the subcellular location of small GTPases and disrupts signalling. Within the musculoskeletal field statins were originally reported as osteogenic agents that induced expression of bone morphogenic protein-2 (BMP-2) in osteoblasts [305]. Recent work has suggested a pro-chondrogenic role with statin treatment increasing collagen II and aggrecan mRNA expression in chondrocytes [222] and intervertebral disc cells [256]. In the latter case, the effect seems to be due, in part, to the upregulation of BMP-2.

In order to determine the dose of lovastatin to use with the tenocytes it was necessary to perform a dose response curve. It was decided to begin with the lovastatin dose range that enhanced chondrogenesis in the ATDC5 pellet culture model. An initial dose response curve examining the effects of lovastatin on the expression of Sox9, Sox5 and Sox6 mRNA levels at the end of seven days of

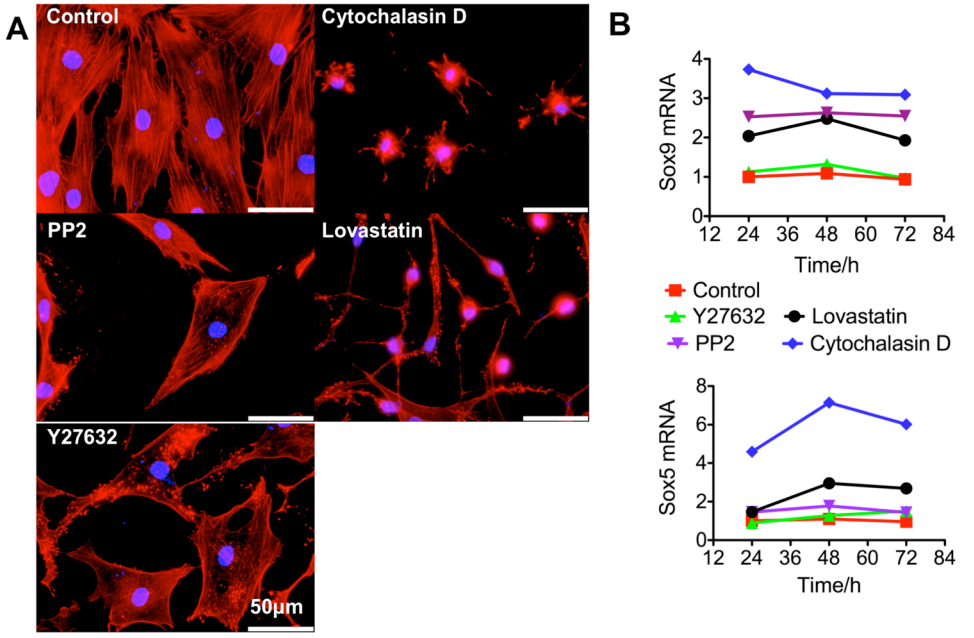
culture was performed (data not shown). These data suggested that 0.5μM was optimal for increasing levels of transcript for the chondrogenic Sox trio. However, it was subsequently discovered that the tenocytes used for this dose response curve were infected with mycoplasma. At this point, however, a large number of experiments had been performed using the doses calculated from this experiment. Were I to repeat these experiments, I would begin with a new dose response curve including 2-10μM since I suspect that many of the experiments detailed use too low a concentration of lovastatin.

5.2 Results

5.2.1 Cytoskeletal Inhibitors cause Tenocyte Actin Reorganization

In initial experiments to confirm that the panel of inhibitors would alter F-actin structure, normal human hamstring tenocytes at passage 2 were cultured for 72h in the presence of DMSO vehicle control, 3μM cytochalasin D, 0.5μM lovastatin, 10μM PP2 or 10μM Y27632. Control tenocytes (Figure 5.2A) show prominent F-actin stress fibres. As seen in vivo these are often arranged along a single long axis within individual cells. The stress fibres within tenocytes in a given culture area are often aligned with each other. Treatment with cytochalasin D caused cell rounding within 30mins (data not shown) and profound re-organisation of the actin cytoskeleton. Y27632 and PP2 treatment caused some loss of F-actin. The morphology remained fibroblastic, although the cells appeared to "shrink" with PP2 treatment. The response to lovastatin was mixed. Some cells rounded up rapidly while others retained an essentially fibroblastic morphology, with severely

reduced or absent F-actin. High doses caused complete cell detachment. Cells may be vulnerable to lower doses at times of reduced adherence e.g. during mitosis or when attached to plastic directly rather than to secreted matrix.



C

Sox9

Time/h	Control	Y27632	PP2	Lovastatin	Cytochalasin D
24	1	1.13	2.53	2.04	3.73
48	1.09	1.32	2.63	2.48	3.12
72	0.94	0.97	2.55	1.93	3.09

Sox5

Time/h	Control	Y27632	PP2	Lovastatin	Cytochalasin D
24	1	0.89	1.46	1.46	4.59
48	1.1	1.28	1.78	2.96	7.15
72	0.96	1.51	1.44	2.69	6.02

Figure 5.2 Actin Arrangement in Tenocytes treated with Cytoskeletal Signalling Disruptors

A Tenocytes were cultured with 10µM PP2, 3µM cytochalasin D, 0.5µM lovastatin or 10µM Y27632 or 0.1% DMSO vehicle control for 48h. Staining for F-Actin (red) and nuclei (DAPI-blue) was performed. **B** and **C** Sox9 and Sox5 mRNA levels were assessed by QPCR at 24, 48 and 72h and normalized to GAPDH. n=1.

5.2.2 Short term treatment with Cytoskeletal Inhibitors Increases Sox9 and Sox5 mRNA Expression

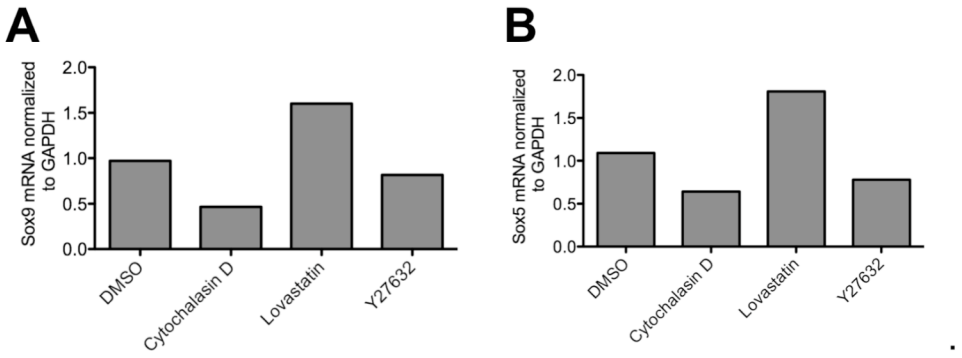
To determine the effects on chondrocytic gene expression, tenocytes at passage 2 were cultured with the inhibitors for 24, 48 and 72h. Messenger RNA levels for Sox9 and Sox5 were determined by RT-QPCR. Initial experiments showed that lovastatin, cytochalasin D and PP2 increased Sox9 mRNA levels between 2 and 3 fold at 24 hours (Figure 5.2B). This increase was steadily maintained over 3 days. Only the ROCK inhibitor Y27632 failed to increase Sox9 mRNA levels.

Cytochalasin D increased Sox5 mRNA levels at 24 hours. Cytochalasin D and lovastatin increased Sox5 mRNA levels at 48 hours (Figure 5.2B), and it remained high for 2 days. Both PP2 and Y27632 failed to increase Sox5 mRNA levels.

5.2.3 Only Lovastatin Maintains the Upregulation of the Sox Genes Long Term and Long Term Lovastatin Treatment Induces Collagen II Expression

To determine if the elevated Sox gene mRNA levels were maintained over an extended treatment period tenocytes at passage 2 were cultured with the panel of inhibitors for 3 weeks. Y27632 was included to determine if it would generate an elevation in chondrogenic gene expression over a longer time frame. Only lovastatin treatment maintained the increase in Sox9 mRNA levels (Figure 5.3A). Additionally, an increase in Sox5 mRNA was found in the lovastatin treated samples (Figure 5.3B). Cytochalasin D was unsurprisingly rather toxic over this extended treatment period.

To determine if the increased levels of Sox9 and Sox5 mRNA would result in cartilage matrix production, tenocytes at passage 3 were cultured in the presence of lovastatin for 3 weeks. Positive collagen II staining was seen in the rounded cells and not in those maintaining a fibroblastic morphology (Figure 5.3E). This was the only treatment paradigm that generated detectable collagen II expression in tenocytes.



C

Target	Control	DMSO	Cytchalasin D	Lovastatin	Y27632
Sox9	1	0.97	0.47	1.60	0.82
Sox5	1	1.09	0.64	1.81	0.78

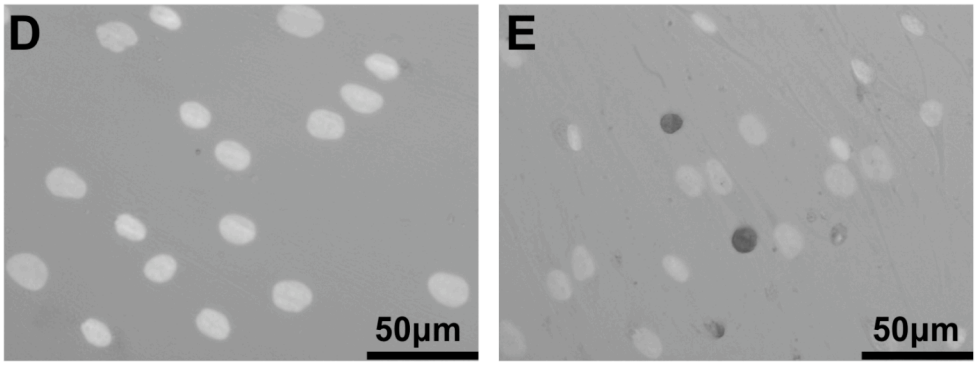


Figure 5.3 Long Term Treatment of Tenocytes with Cytoskeletal Signalling Disruptors

Tenocytes were cultured with 0.4µM cytochalasin D, 0.5 µM lovastatin, 20µM Y27632 or 0.2% DMSO for 3 weeks. Sox9 (A) and Sox5 (B) mRNA levels were assessed by QPCR and normalized to GAPDH. Also presented in table C. Tenocytes were additionally cultured with 0.05% DMSO (D) or 0.5 µM lovastatin (E) for 3 weeks. Coverslips were stained by immunocytochemistry for collagen II (DAB; arrows) and counterstained with DAPI. n=1

5.2.4 Lovastatin Dose-Dependently Regulates Expression of Chondrocytic and Tenocytic Markers

To determine if the effect of lovastatin on tenocytes was dose-dependent, cells at passage 2 were treated with 0.2-2µM lovastatin. Live/Dead staining confirmed

that, even at the highest concentration, lovastatin did not cause cell death (Figure 5.4, note that the rounded cells stain positively with calcein AM (Live stain) and actively exclude ethidium bromide (Dead stain)). As previously noted, GAPDH mRNA expression displayed a subtle, dose-dependent increase with lovastatin treatment (Figure 2.3). The levels of TBP were stable and it was therefore used in preference as a housekeeper in all further lovastatin experiments. Lovastatin increased Sox9 and Sox5 mRNA levels (Figure 5.5). Lovastatin additionally dose-dependently increased BMP-2 mRNA levels, reaching statistical significance at 1μM. Messenger RNA levels for tendon matrix components collagen I (COL1A1) and collagen III (COL3A1) were dose-dependently decreased by lovastatin treatment, reaching significance at 0.5μM.

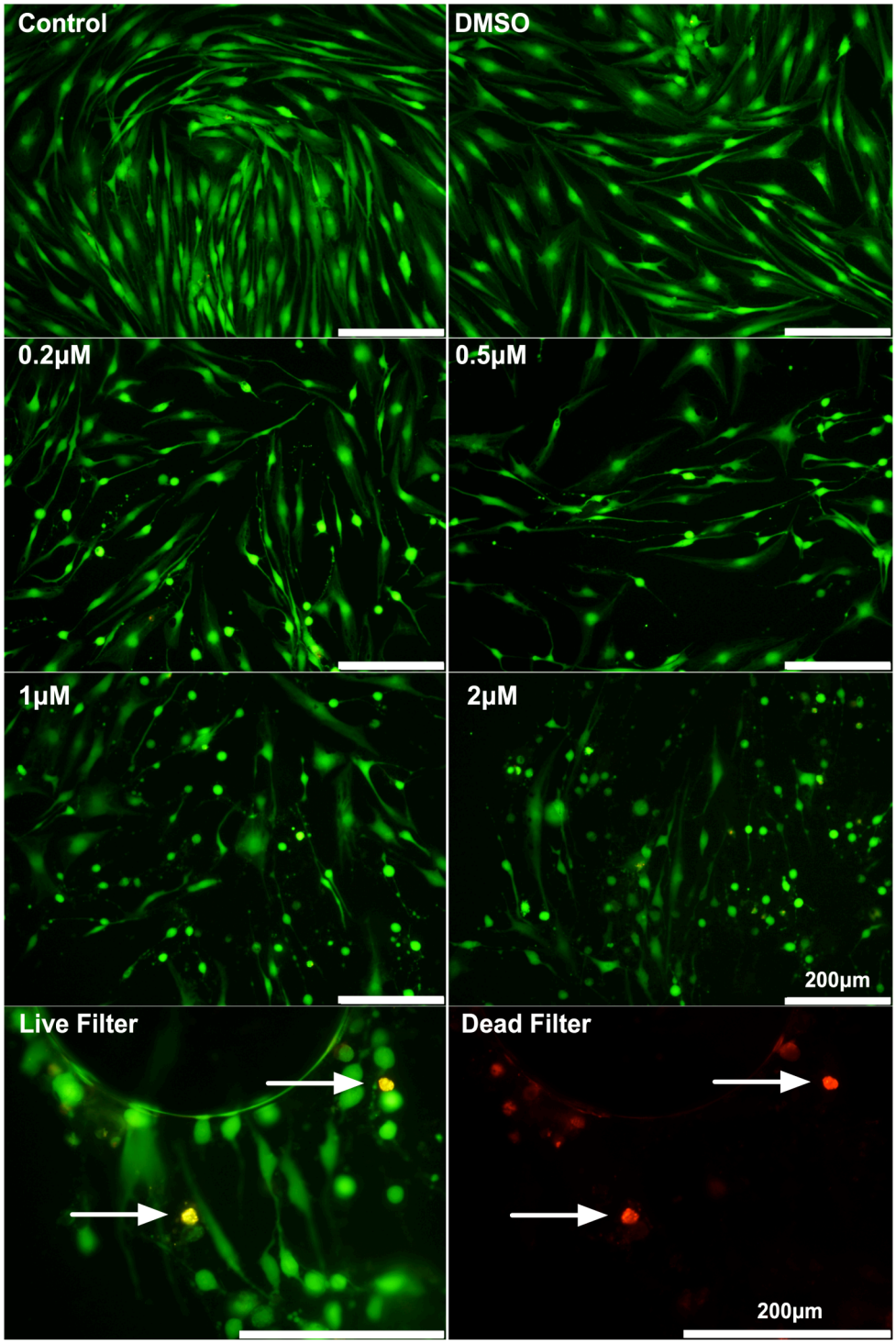


Figure 5.4 Effect of Lovastatin on Tenocyte Viability

Tenocytes were cultured in plain medium (control) or with 0.2% DMSO vehicle control, 0.2, 0.5, 1 or 2µM lovastatin for 1 week then stained with Live/Dead. Green = live cells, Red/Yellow = dead cells. Arrows indicate cells staining positively for ethidium bromide (Dead stain) using the live filter and dead filters as labelled. Our live filter is broad spectrum. The Dead stained cells are visible as yellow objects.

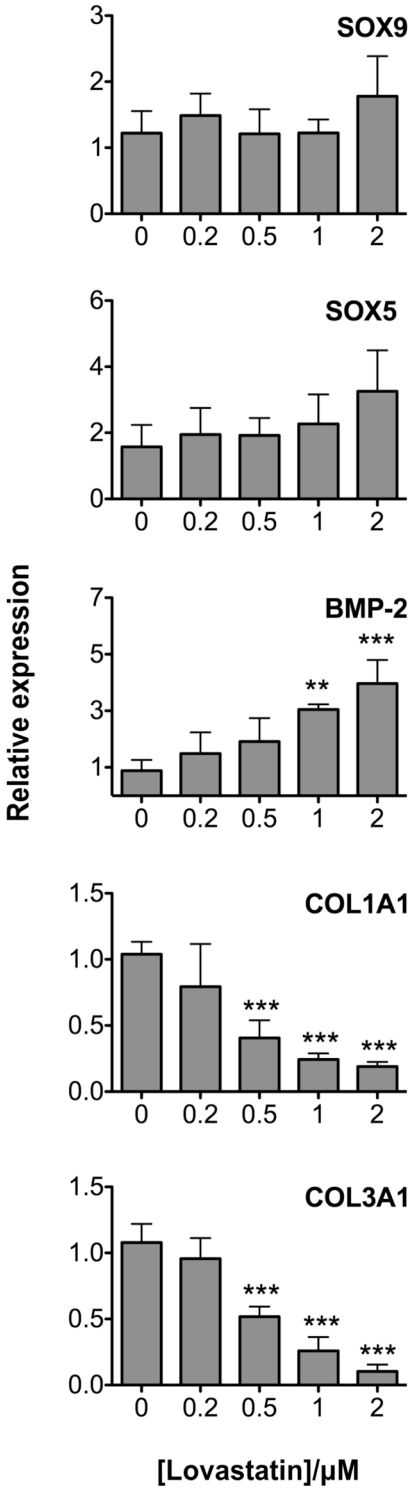


Figure 5.5 Effect of Lovastatin on Phenotypic Marker mRNA Levels

Tenocytes were cultured with 0.2% DMSO vehicle control, 0.2, 0.5, 1 or 2μM lovastatin for 1 week. Sox9, Sox5, BMP-2, COL1A1 and COL3A1 mRNA levels were assessed by QPCR and normalized to TBP. Mean and SD from 4 independent experiments are shown. One-way ANOVA with Dunnett post-hoc was performed. ** $p<0.01$, *** $p<0.001$

5.2.5 Lovastatin-mediated Regulation of Chondrocytic and Tenocytic Markers Correlates with Alterations in Prenylation Status

The effects of lovastatin on F-actin and cell signalling are believed to result from its inhibition of HMG-CoA reductase, an enzyme which catalyses an early step in the cholesterol synthesis pathway. Downstream metabolites of this enzyme are involved in a post-translational modification of small GTPases, known as prenylation. Inhibition of prenylation disrupts the intracellular location of these proteins and consequently their downstream signalling. To confirm that alterations in Sox, BMP-2 and collagen gene expression levels were due to inhibition of HMG-CoA reductase, add-back experiments were performed using an excess of the downstream metabolite mevalonate. Tenocytes that showed a strong response to lovastatin were used in these experiments.

Lovastatin treatment caused cell rounding (Figure 5.6A). Mevalonate prevented this, maintaining the fibroblastic morphology of the tenocytes. Western blotting for the unprenylated form of the small GTPase Rap1a confirmed that lovastatin inhibited protein prenylation at these doses. Mevalonate restored protein prenylation (Figure 5.6B). It prevented the lovastatin-induced increase in Sox5 ($p<0.01$) and BMP-2 mRNA ($p<0.01$) (Figure 5.6C). Additionally, mevalonate prevented the decrease in COL1A1 ($p<0.01$) and COL3A1 mRNA levels (Figure 5.6C).

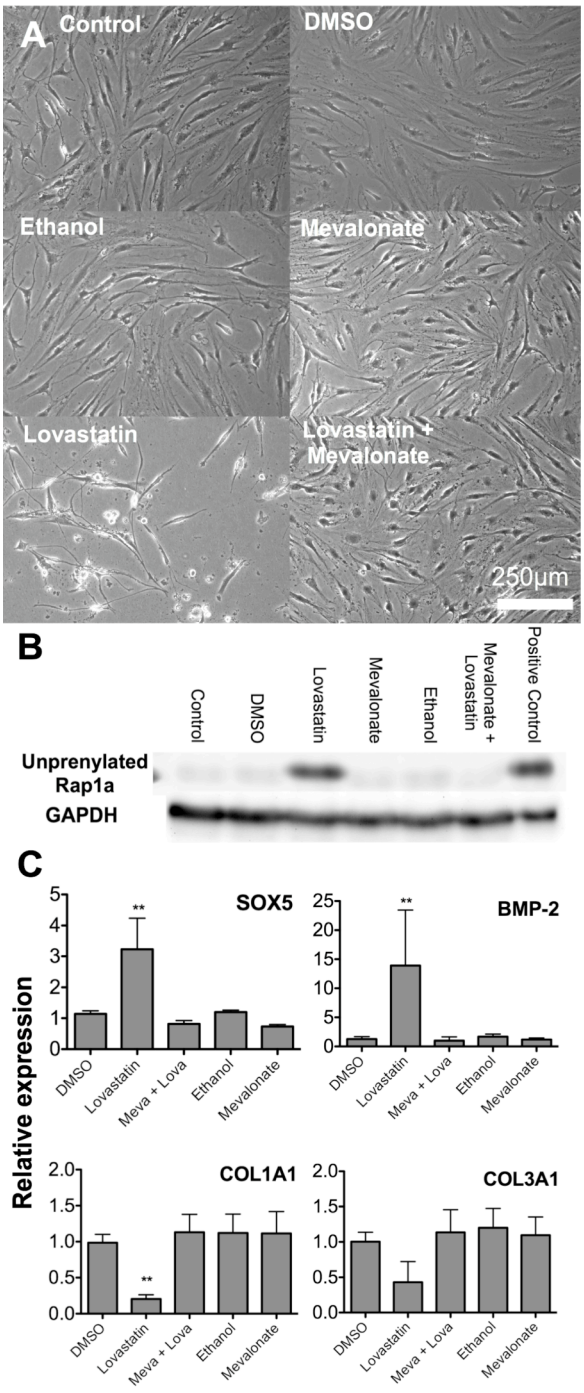


Figure 5.6 Reversibility of Lovastatin Effects with Mevalonate

Tenocytes at passage 2 were cultured with medium alone, vehicle control (DMSO or ethanol), 100µM mevalonate, 0.5µM lovastatin or lovastatin and mevalonate (Meva + Lova) for 1 week. Bright field images demonstrating cell morphology are presented (A). Unprenylated Rap1a and GAPDH were detected by Western blotting (B). The "Positive control" is a sample known to contain unprenylated Rap1a and was used to confirm that the Western blot itself was correctly performed. Sox5, BMP-2, COL1A1 and COL3A1 mRNA levels were assessed by QPCR and normalized to TBP (C). Mean and SD from 4 independent experiments are shown. One-way ANOVA with Tukey post-hoc was performed. ** $p<0.01$

5.3 Discussion

In order to determine the effects of disruption to signalling linked to the actin cytoskeleton a panel of reagents was assembled capable of affecting either the actin cytoskeleton directly or of various pathways, which can signal, to the actin cytoskeleton. The reagents were confirmed to alter actin distribution and, in some cases, cell shape. Cytochalasin D, PP2 and lovastatin were found to increase levels of Sox9 while cytochalasin D and lovastatin also increased Sox5 mRNA after 48h of treatment. However, this increase was only maintained long term in the lovastatin treated cells. It is interesting to note that both lovastatin and cytochalasin D had quite dramatic effects on cell shape and produced the strongest gene expression changes.

While both cytochalasin D and lovastatin alter actin distribution, statins have the additional ability to inhibit prenylation of proteins and in some cell types upregulate expression of BMP-2. The data in the current study show that lovastatin strongly impaired prenylation of small GTPases in tenocytes, demonstrated with the example of Rap1a and that this correlated with altered cell shape and the dose dependent upregulation of Sox9 and Sox5 mRNA. Sox9 is the master regulator of chondrogenesis [213]. Sox5 binds to a chondrocyte-specific enhancer region within intron 1 of COL2A1 and increases transcription [213]. Increased expression of Sox9 and Sox5 suggests a switch towards a more chondrocytic programme and would favour the expression of cartilage matrix molecules including collagen II. Indeed, prolonged exposure to lovastatin induced expression of collagen II protein in the tenocytes. However, it is notable

that this expression was not universal but confined to those cells displaying a rounded morphology.

Chondrocytes grown in culture systems that favour a rounded cell morphology frequently show decreased collagen I and III mRNA levels compared to those grown in monolayer [306, 307]. In this study it is shown that treatment with lovastatin decreases COL1A1 and COL3A1 mRNA levels whilst inducing a rounded cell morphology. Both collagen I and collagen III are tendon matrix molecules whose expression is not associated with healthy chondrocytes. The downregulation of these genes may be indicative of a phenotypic switch.

Statin treatment has previously been found to decrease collagen I expression in other cell types including dermal fibroblasts [308]. Similarly collagen III mRNA levels are decreased by fluvastatin treatment in a kidney fibroblast line [309]. GGTI-298, a specific inhibitor of geranylgeranyl transferase I (GGTase I), decreases COL1A1 and COL3A1 mRNA levels in dermal fibroblasts [310]. GGTase I only prenylates members of the Rho and Rac families implicating these small GTPases in the control of collagen transcription. C3 transferase treatment, which inactivates the Rho subfamily, downregulates COL1A1 mRNA levels in chondrocytes. Transient transfection with the constitutively active RhoA mutant RhoQ63L increases COL1A1 mRNA levels [311]. It is further notable that fluvastatin treatment reduced the levels of activated Rho and COL3A1 mRNA in kidney fibroblasts [309]. These data strongly implicate the Rho family, particularly RhoA in the control of tenocytic collagen matrix production.

The importance of RhoA signalling to chondrocyte phenotype is well documented. High levels of RhoA correlate with fibroblastic morphology and the presence of stress fibres in de-differentiated chondrocytes. Upon re-differentiation by alginate bead culture RhoA protein levels drop and a loss of stress fibres is observed [283]. Inhibition of the Rho subfamily by C3 transferase restores chondrocytic gene expression in de-differentiated chondrocytes [283].

It is therefore conceivable that signalling involving RhoA may play a key role in the determination of phenotype. In this model (see Figure 5.7), active RhoA promotes a tenocyte phenotype i.e. the expression of collagen I, the formation of stress fibres and a fibroblastic morphology. Inactive RhoA promotes a chondrocytic phenotype i.e. a rounded morphology, a cortical arrangement of actin and the expression of Sox5, Sox6, Sox9 and collagen II. Indeed, Novakofski et al. show that C3 transferase inhibition of chondrocytes decreases COL1A1 expression whilst simultaneously increasing Sox9 levels [311].

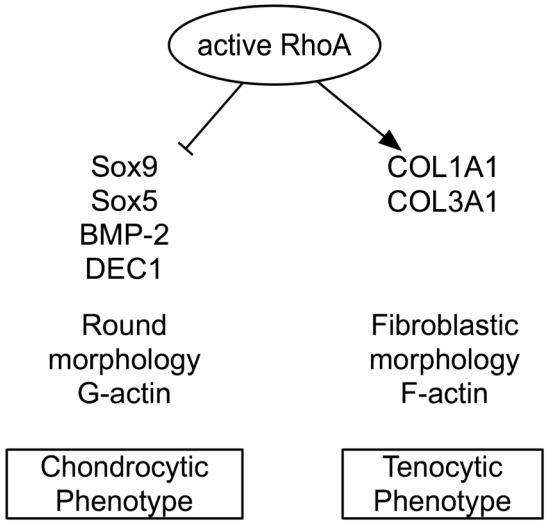


Figure 5.7 Proposed model for cell phenotype in which activation status of RhoA functions as a switch between tenocytic and chondrocytic phenotype.

To more closely assess the mechanism by which lovastatin induces phenotypic changes in tenocytes we restored an excess of mevalonate, the downstream metabolite of statin-targeted HMG-CoA-reductase. Mevalonate completely reversed all lovastatin-induced changes in gene expression as well as restoring cell shape. This indicates that the changes seen in cells treated with lovastatin are due to inhibition of HMG CoA reductase by the drug and not due to other cytotoxic effects.

Given the literature showing impaired small GTPase signalling in statin treated cells and the very strong implicating impaired GTPase signalling in chondrogenesis, this is likely to be a causal mechanism in tenocytes. However, to be sure, it would be necessary to observe the same gene expression changes when using FTI or GGTI inhibitors which directly mediate prenylation. Similarly, in this study it is shown that statin treatment increases BMP-2 mRNA levels in tenocytes as previously reported in osteoblasts [305] and chondrocytes [222]. In order to determine whether this contributes to the upregulation of pro-chondrogenic markers, BMP-2 would need to be neutralised with either antibodies or the secreted antagonist noggin. These experiments were planned but several months of work had to be repeated due to the mycoplasma infections and we decided to prioritise work on the hypoxia paradigm instead (See Chapter 6)

When these experiments were originally conceived they were planned to take place in 3D culture, rather than in monolayer. However, given both the problems with the widely published pellet culture system and the upregulation of chondrocytic markers in monolayer culture by the cytoskeletal inhibitors it was

decided that monolayer culture would be sufficient. Indeed, the upregulation of markers that are normally downregulated by monolayer culture suggests that lovastatin is a strong pro-chondrocytic agent.

5.3.1 Potential Future Work

Since the literature strongly implicates RhoA as a critical signalling molecule in chondrogenesis and the data presented in this thesis would be consistent with such a hypothesis it would be interesting to determine if RhoA plays an important role in tenocyte transdifferentiation. Initial experiments could utilise specific inhibitors of FTase, GGTase I and GGTase II to determine i) if inhibition of protein prenylation is crucial to increased BMP-2, DEC-1 and Sox gene transcription and ii) to help narrow which family of small GTPases could be responsible. Use of C3 transferase as a specific inhibitor of the Rho subfamily would further narrow the possible proteins. Transfection of primary tenocytes with the dominant negative form of RhoA (RhoAQ63L) would finally determine which effects, if any, are due to inhibition of RhoA signalling.

Chapter 6 - Hypoxia and Combination and Tenocyte Phenotype

6.1 Introduction

As has already been discussed in Section 1.8.3.2, hypoxic conditions favour the expression of chondrocytic markers in chondrocytes. Studies of samples of torn rotator cuff tendons suggest that the tenocytes are subject to low oxygen concentration. The response of tenocytes to hypoxic conditions is extremely poorly characterised. It is therefore appropriate to determine if the hypoxic conditions promote a chondrocytic phenotype either alone or in combination with lovastatin, which this thesis has previously shown to alter cell shape and actin arrangement and to upregulate chondrocytic markers.

6.1.1 Aims of this chapter

The aims of this chapter therefore are:

1. To further characterise the tenocyte response to hypoxia, with special focus on the response of chondrocytic markers.
2. To determine if hypoxia and lovastatin act synergistically to upregulate chondrogenic markers.

6.2 Results

6.2.1 Tenocytes show Increased Levels of HIF-1α, VEGF-A and FOXO3a in Hypoxic Conditions

The in vitro response of tenocytes to low oxygen tension has been very poorly characterised. Work done by our group has shown that normal human tenocytes rapidly accumulate HIF-1α when exposed to 0.1% oxygen or the hypoxia mimic cobalt chloride (Figure 6.1A and C). Additionally, 0.1% oxygen increased expression of at least three HIF target proteins. Pro-apoptotic BNIP3 and Nix were upregulated while both expression and secretion of the pro-angiogenic growth factor VEGF-A was increased [312]. In addition, assays performed for this thesis show that tenocytes cultured in 10% serum for 16 hours[9] in 0.1% oxygen (virtual anoxia) upregulated expression of the stress-responsive transcription factor forkhead box O3a (FOXO3a) (Figure 6.1B).

[9] FOXO3a induction under hypoxic conditions has previously been shown to be dependent on HIF-1 [313]. Expression of FOXO3a at the protein level is transient, peaking at 8h in mouse embryonic fibroblasts and 24h in NIH 3T3 cells [313]. Since peak protein level can vary between cell types it was decided to pick 16h as the time point for tenocytes since 16h lies between 8h and 24h increasing the possibility of observing an obvious difference in FOXO3a levels.

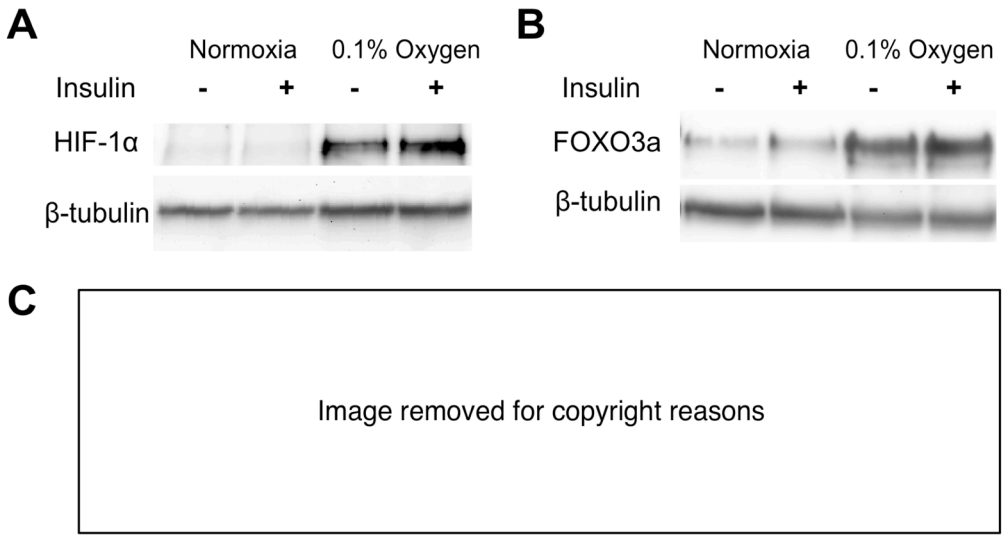


Figure 6.1 Tenocyte Response to Hypoxia – Non-Phenotypic Markers

A Tenocytes were cultured for 6h[10] in medium containing 10% FCS in 0.1% oxygen with and without insulin (3 μgml^{-1}). HIF-1α protein levels were determined by Western blotting. β-tubulin was used as a loading control. Results are representative of two experiments. B Tenocytes were cultured for 16h in medium containing 10% FCS in 0.1% oxygen with and without insulin (3 μgml^{-1}) (as part of an unrelated study). FOXO3a protein levels were determined by Western blotting. β-tubulin was used as a loading control. Results are representative of two experiments. C Tenocytes were cultured in 0.1% oxygen for 1, 4, 8, 16, 24 and 48h. HIF-1α protein levels were determined by Western blotting. β-tubulin was used as a loading control. HIF-1α time course data shown in C courtesy of Dr Min Liang [312].

6.2.2 Low Oxygen Concentration Stimulated VEGF and DEC1 Expression but did not alter other Chondrocytic and Tenocytic Marker Gene Expression over 3 days

Chondrocytes have been described to respond to reduced oxygen concentration by upregulating chondrogenic marker genes. Since statins induce clear changes in chondrogenic markers in tenocytes within 3 days the effects of 3 days of culture in

[10] A 6h time point was selected for this experiment because it was believed that substantial levels of HIF-1α would remain in the cells (indeed, the levels could, conceivably, be higher than those observed at 4h) and because it enabled other experiments to be performed in parallel with this one.

varying oxygen concentrations were assessed. Tenocytes upregulated expression of the classical hypoxia response gene VEGF-A (Figure 6.2) in response to low oxygen concentration. This reached significance ($p<0.001$) at 1% oxygen. In contrast to lovastatin, hypoxia did not alter Sox9, COL1A1 or COL3A1 mRNA levels. Sox5 mRNA levels remained similarly unaltered.

The response of the tenocytes to 0.1% oxygen (virtual anoxia) was varied with some samples showing upregulation of the transcription factors and VEGF-A, while others showed downregulation of the same genes. This might, perhaps, correlate with increasing cell death. However, the expression levels of the collagen genes, particularly COL1A1, remained steady and tightly clustered even at 0.1% oxygen, which suggests that the cell population remains alive.

The basic helix-loop-helix protein Differentially Expressed in Chondrocytes 1 (DEC1) is a recently described regulator of chondrogenesis [100]. Overexpression of DEC1 in mesenchymal stem cells promotes chondrogenic differentiation [100] while inhibiting adipogenesis and myogenesis [101, 102]. DEC1 contains a hypoxia response element in its promoter [104] and, like Sox9, is upregulated in response to low oxygen concentration. It is not routinely included in chondrogenic marker panels but as a transcription factor it provides a potential convergence point for multiple regulatory pathways and is therefore an interesting target. We report for the first time that DEC1 is present in tenocytes and is upregulated by hypoxia ($p<0.001$)(Figure 6.2).

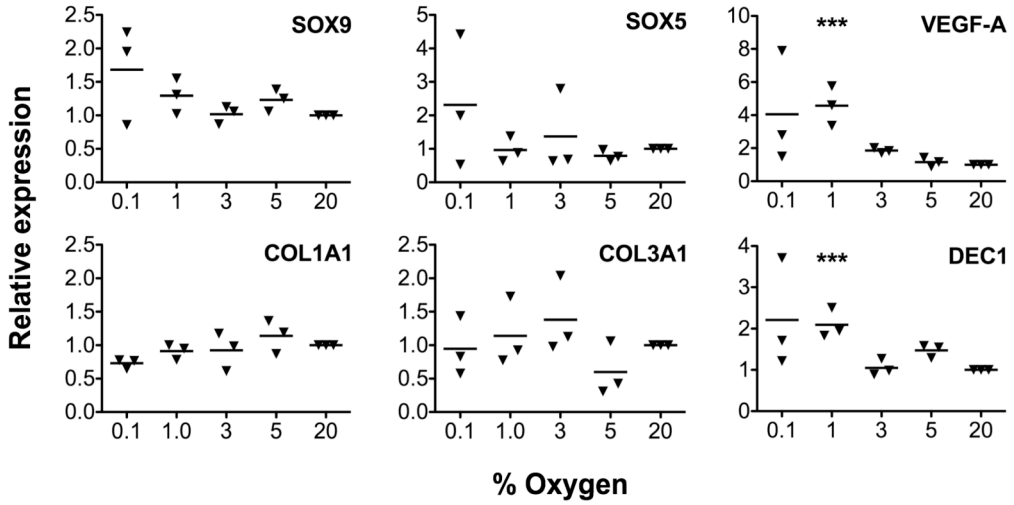


Figure 6.2 Tenocyte Response to Graded Hypoxia – Phenotypic Markers

Tenocytes were cultured for 72h in 5%, 3%, 1% or 0.1% oxygen. Sox9, Sox5, COL1A1, COL3A1, DEC1 and VEGF-A mRNA levels were assessed by QPCR and normalized to TBP. Results are relative to tenocytes cultured simultaneously in 20% oxygen. Mean and SD from 3 independent experiments with different donors are shown. One-way ANOVA with Dunnett post-hoc was performed. *** $p<0.001$

6.2.3 Long Term Cobalt Chloride Treatment Upregulates Sox9 Expression

In the torn rotator cuff the disruption to the local blood supply and hence the potential for hypoxia appears to persist for some time. In preliminary experiments to determine the potential long-term effects of exposure to low oxygen tension, tenocytes were treated with the hypoxia mimic cobalt chloride for two weeks. This acts by occupying the iron-binding site of the enzymes prolyl-4-hydroxylase and Factor inhibiting HIF-1 inactivating them, thus preventing the degradation of HIF-α subunits under normoxic conditions [314]. It also occupies the binding site for von Hippel–Lindau protein preventing the degradation of HIF-α subunits [315]. The HIF-α subunits translocate to the nucleus where they bind to the HIF-β subunits and CBP/p300. This complex binds to hypoxia response elements in

the promoters of hypoxia responsive genes and upregulates transcription. VEGF-A has at least one such HRE in its promoter [316]. A HRE has been located in the mouse Sox9 promoter [234] which is known to bind HIF-1α [317]. The human equivalent has proved more elusive with at least one study which examined the Sox9 promoter region reporting a failure to locate a HRE [318]. Recent work by Duval et al., however, shows EMSA data of HIF-1α binding to a portion of Sox9 promoter which, according to the authors, contains "multiple hypoxia-responsive elements" [245][11].

As shown in (Figure 2.5) the expression of β-actin is stable in tenocytes treated with cobalt chloride and is therefore suitable for use as a housekeeping gene. Cobalt chloride strongly increased the expression of VEGF-A by approximately 8 fold (Figure 6.3A). It additionally increased the expression of Sox9 by 4.5 fold. However, no expression of COL2A1 was seen (Figure 6.3B).

[11] Since Aprelikova et al. [318] do not state the region of DNA they regard as comprising the Sox9 promoter it is difficult to compare the findings of these two papers. The difference in findings could be attributable to one paper analysing a region of DNA that the other did not. This seems a slightly unlikely scenario, however, since the region used in the EMSA binding study was less than 500bp from the transcription start site (-236 to -212) [245].

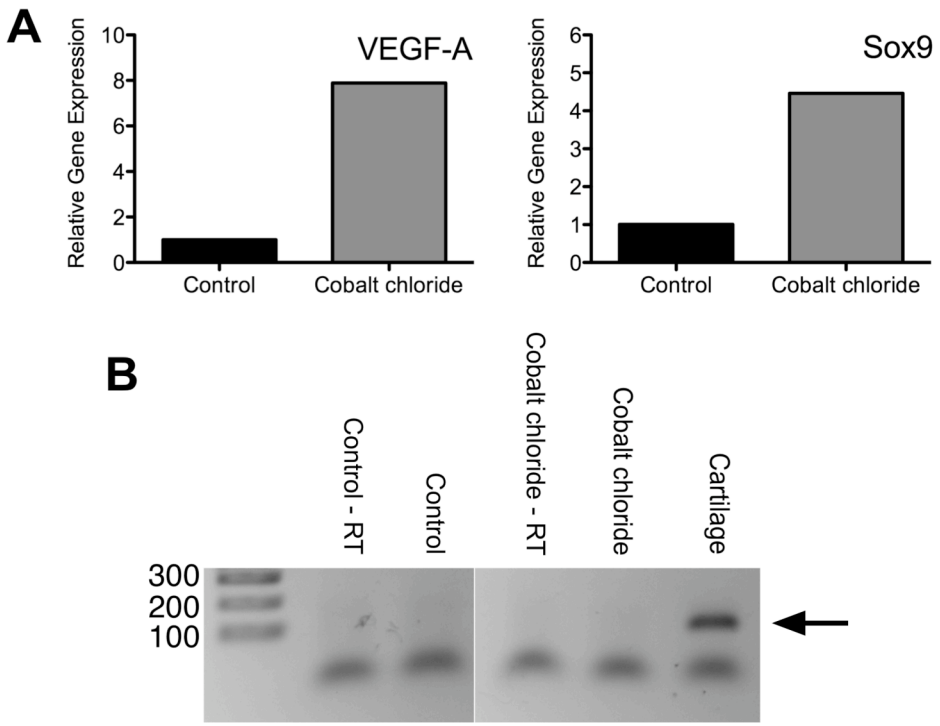


Figure 6.3 Response of Tenocytes to Long Term Chemically Simulated Hypoxia

Tenocytes were cultured with and without 50μM cobalt chloride for two weeks. **A** VEGF-A and Sox9 mRNA levels were assessed by QPCR and normalized to β-actin. Results from a single experiment. **B** COL2A1 mRNA expression was assessed by RT-PCR. Arrow indicates expected position of PCR product from COL2A1 assay. Results from a single experiment.

6.2.4 Hypoxia and Lovastatin do not Interact and Affect Chondrocytic and Tenocytic Marker Gene Expression over 3 Days of Co-culture

The hypoxia and actin cytoskeleton regulatory pathways are not known to interact directly. However, chondrocytes upregulate cartilage markers in response to both cell shape change and hypoxia and both paradigms are relevant to the torn rotator cuff. The effect of combining both treatments on chondrocytic marker gene expression in tenocytes was therefore tested.

Lovastatin significantly increased Sox9 ($p=0.0034$), Sox5 ($p=0.0105$) and BMP-2 ($p=0.0210$) mRNA levels (Figure 6.4). It significantly decreased COL1A1 ($p<0.0001$) and COL3A1 ($p=0.0011$) mRNA levels. Hypoxia significantly increased VEGF-A ($p=0.0002$) mRNA levels. The chondrogenic transcription factor DEC1 was induced by both hypoxia ($p=0.0440$) and interestingly also by statin alone ($p=0.0006$). However in combination there was no additive or synergistic effect on DEC1 expression. Hypoxia and lovastatin did not exert a combined effect on the levels of mRNA of any other selected genes within this 3-day culture period. While not significant, there is a trend towards increased Sox 9 with hypoxia alone and also towards an additive effect in combination (Figure 6.2 and Figure 6.4). This suggests longer or sequential treatments might be more effective. Treatment paradigms that are effective in chondrocytes might take longer in tenocytes that are being transformed into chondrocytes. However, lovastatin works very quickly, within 3 days, and we were therefore interested to assess the combination effect at this stage.

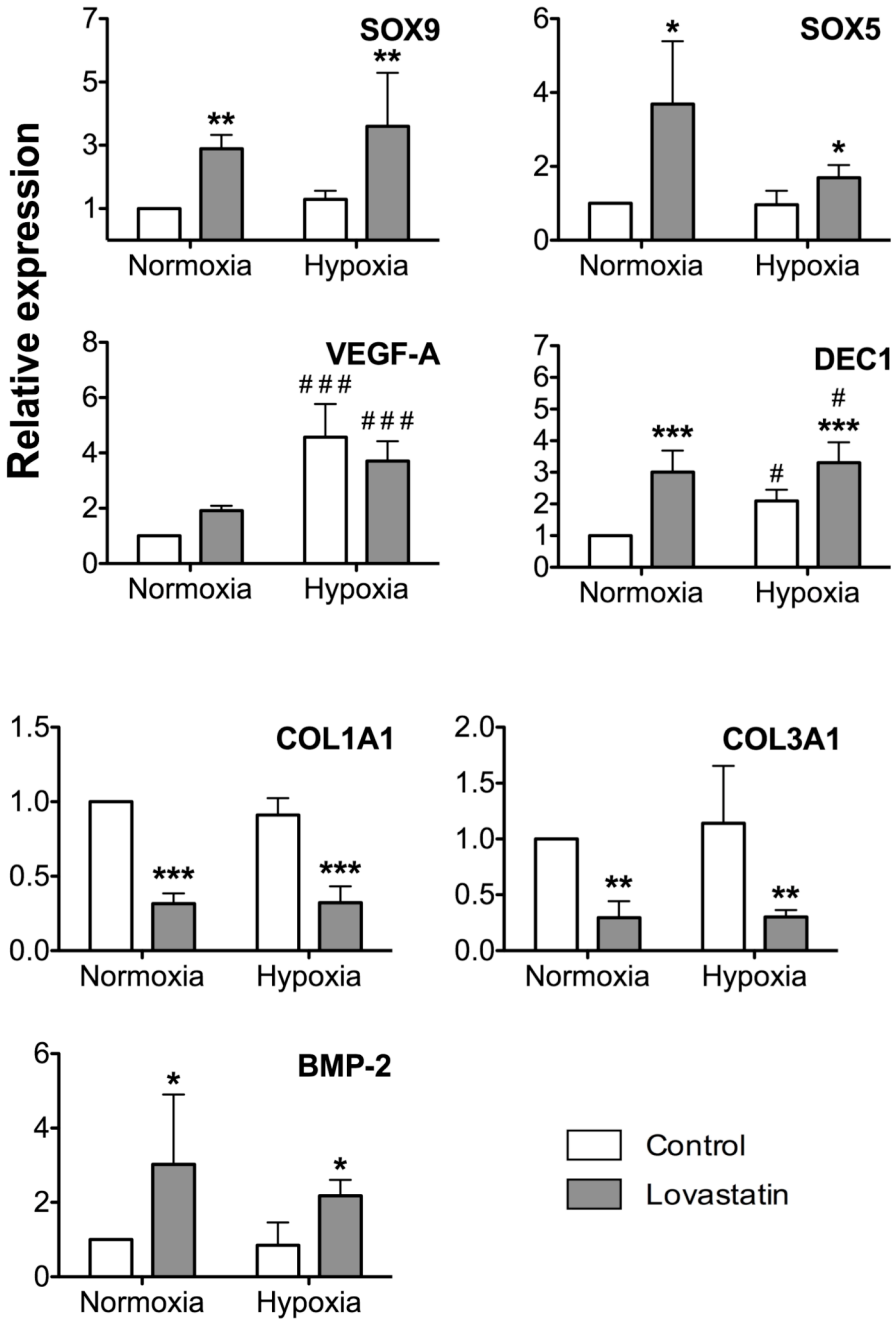


Figure 6.4 Response of Tenocytes to a Combination of Hypoxia and Lovastatin Treatment

Tenocytes were cultured in 1% (hypoxia) or 20% (normoxia) oxygen in the presence (filled bars) or absence (clear bars) of 2μM lovastatin for 72h. Sox9, Sox5, VEGF-A, DEC1, COL1A1, COL3A1 and BMP-2 mRNA levels were assessed by QPCR and normalized to TBP. Results are relative to tenocytes cultured simultaneously in 20% oxygen without lovastatin. Mean and SD from 3 independent experiments are shown. Two-way ANOVA was performed. Variation attributable to lovastatin: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Variation attributable to hypoxia: # $p<0.05$. ### $p<0.001$

6.3 Discussion

This section of work had 2 aims:

1. To further characterise the tenocyte response to hypoxia, with special focus on the response of chondrocytic markers.
2. To determine if hypoxia and lovastatin act synergistically to upregulate chondrogenic markers.

6.3.1 Tenocyte Response to Hypoxia

6.3.1.1 Classical Markers of Hypoxia

The in vitro response of tenocytes to low oxygen tension has been very poorly characterised. Of the studies published by other groups, none were performed using normal human tenocytes. Work done by our group has established that tenocytes exposed to 0.1% oxygen rapidly accumulate stabilised HIF-1α [312]. Rat tenocytes have been shown to respond to hypoxia (5% oxygen) by increasing VEGF-A secretion [319, 320]. We have confirmed that normal human tenocytes respond to 0.1% oxygen by secreting VEGF-A and by increasing expression of BNIP3 and Nix [312]. Thus tenocytes can respond to hypoxia by upregulating classical HIF driven pathways.

6.3.1.2 FOXO3a - A Hypoxia Responsive Protein

Upregulation of FOXO3a at the protein and the mRNA level in response to low oxygen concentration was first reported in mouse embryonic fibroblasts and NIH 3T3 cells, where it was described as HIF-1 dependent [313]. In this study, normal

human tenocytes upregulated FOXO3a protein levels in response to 0.1% oxygen. FOXO3a is reported to have a pro-survival role in cells adapting to hypoxic stress [313].

6.3.2 Phenotypic Genes

6.3.2.1 Tenocytic Gene Expression

The stability of expression of COL1A1 and COL3A1 mRNA in tenocytes exposed to 72h of hypoxia observed in this study is consistent with the majority of published literature on the behaviour of collagen gene expression in monolayer chondrocyte culture under hypoxic conditions. Murphy and Sambanis [307] reported that exposure of dedifferentiated chondrocytes in monolayer culture to 5% oxygen failed to alter collagen I mRNA levels even after extended culture.

One study in this area, the work of Duval et al. [245], is difficult to interpret, however. The authors fail to find an additive effect of hypoxia and alginate bead culture on the expression of COL1A1, COL1A2 and COL3A1 in dedifferentiated chondrocytes (the effects of a combination of 3D culture and hypoxia on tenocytic collagen gene expression will be discussed in Section 6.3.3.1.2). They therefore hypothesise that hypoxia is able to inhibit these genes but that the effect is not additive with the strong effect produced by 3D culture. It is worth noting that Duval et al. do not present evidence that monolayer culture of dedifferentiated chondrocytes in hypoxia downregulates tenocytic collagen gene expression. They do, however, report a statistically significant downregulation of all three genes in monolayer culture with the hypoxia mimic cobalt chloride. They also present

further data including the use of dominant negatives and siRNA which, for reasons that will be outlined in Section 6.3.5.2, I interpret as indicating that HIF may be involved in the downregulation of these genes. Given that it is not possible to identify a comparative oxygen concentration for cobalt chloride treated cells and that 5% oxygen is not a particularly stringent form of hypoxia, it is possible that the monolayer results reported by Duval et al. could represent the response of dedifferentiated cells to the equivalent of a lower oxygen concentration.

If this hypothesis is correct, it may represent a fundamental difference in the hypoxic response of tenocytes and chondrocytes with respect to tenocytic collagen mRNA levels. Regardless, the data in this thesis is supported by a recent study which found that monolayer culture of porcine tenocytes in 2% oxygen did not alter the expression of collagen I or collagen III mRNA [321].

6.3.2.2 Chondrocytic gene expression

6.3.2.2.1 Sox Genes

Studies of dedifferentiated human chondrocytes have shown that exposure of monolayer cultures to hypoxia (1% oxygen) upregulates Sox9 expression [232, 322]. The response of Sox5 (and, indeed of Sox6) to hypoxia in monolayer cultures is unknown. It is however it is reasonable to hypothesise that it may be upregulated by hypoxia since the expression of collagen II (a gene which is not believed to contain a HRE) is upregulated by hypoxia. Sox5 regulates collagen II expression by binding to an enhancer element present within intron 1 [93].

In this thesis, hypoxia had no significant effect on the mRNA levels of either Sox5 or Sox9 in monolayer cultured tenocytes. Possible reasons for this will be discussed in Section 6.3.4.

6.3.2.2.2 DEC1

The response of DEC1 to hypoxia in adult chondrocytes is unknown, although its promoter region contains a HRE and it is upregulated by hypoxia in differentiating cells [104]. Overexpression of DEC1 in mesenchymal stem cells promotes chondrogenic differentiation [100] while inhibiting adipogenesis [101]. The upregulation of DEC1 by hypoxia in this study may indicate a shift in tenocyte phenotype, although it should be noted that the role of DEC1 in tenocytes is not known, indeed we believe this to be the first report of the expression of DEC1 by tenocytes.

DEC1 has been shown, in other cell types, to be a transcriptional repressor [65]. The exact mechanism of this repression seems to vary but it may include the direct binding of an E-box in the promoter of a target gene followed by the recruitment of corepressor components including histone deacetylase 1 or the formation of heterodimers with a non-class I bHLH preventing the non-class I bHLH from binding the E-box and transactivating its target genes [103]. The mechanism of action of DEC1 during chondrogenesis has therefore been hypothesised to be that of repression of differentiation towards alternative lineages. Scleraxis is a bHLH transcription factor and several classical tendon genes are known to contain E-boxes in their promoter regions. The upregulation of DEC1 observed in this study might lead to repression of scleraxis activated

genes either by heterodimerisation of scleraxis with DEC1 or by competition for E-box binding. Such a mechanism appears to occur in the DEC2 mediated repression of myogenesis [102]. However, this hypothetical mechanism of action in tenocytes depends, in part, upon scleraxis playing a central role in the maintenance of phenotype in adult tenocytes.

6.3.3 The effect of Combination Treatments

Several authors have examined the effects of a combination of culture in 3D systems (including alginate beads and pellet culture) and hypoxic culture on the expression of chondrocytic and fibroblastic markers.

6.3.3.1.1 Combination treatment and Chondrocytic genes

In both the re-differentiation of monolayer expanded chondrocytes and chondrogenesis from cells possessing stem-like qualities, culture at oxygen tensions at or below 5% enhances the expression of Sox5, Sox6 and Sox9 induced by 3D culture [243, 244, 323]. In this study no additive effect was seen with hypoxic culture and lovastatin treatment. This could be for the reason detailed in Section 6.3.4.

6.3.3.1.2 Combination treatment and Tenocytic genes

Murphy and Polak [243] report that a combination of culture in 5% oxygen and alginate bead encapsulation does not significantly alter COL1A1 mRNA levels beyond that of alginate bead encapsulation alone. Duval et al. [245] confirmed

this finding, additionally reporting that a combination of alginate bead encapsulation and culture in 5% oxygen did not significantly alter COL1A2 or COL3A1 mRNA levels beyond that of alginate bead encapsulation alone. This lack of synergistic effect has also been noted in studies involving chondrocytic differentiation from stem cells [244, 323]. The findings of the current study that there was no synergistic effect of lovastatin treatment and hypoxic culture on either COL1A1 or COL3A1 expression in tenocytes is in agreement with the published literature pertaining to the combination of three dimensional culture and hypoxia in chondrocytes.

Marker	3D Culture	Hypoxia	Combination
COL1A1	↓ [245, 306]	*	↓ [243-245]
COL1A2	↓ [245, 306, 307]	↔ * [307]	↓ [245]
COL3A1	↓ [245, 306]	*	↓ [245]
SOX5	↑ [244]	?	↑↑ [243, 244]
SOX9	↑ [243, 244]	↑ [232, 322]	↑↑ [243, 244]
DEC1	?	↑ [104]	?

Table 6.1 Table showing published effects in chondrocytes of hypoxia, 3D culture and combination treatment on the markers used in this study (and COL1A2).

* = work by Duval et al. [245] (see main text). ↑ = increase in expression. ↓ = decrease in expression ↑↑ = additive increase in expression.

6.3.3.2 Issues Arising from Combination Treatment Results

6.3.3.2.1 The Effect of Lovastatin on VEGF-A mRNA Expression

In this experiment lovastatin did not upregulate the expression of VEGF-A mRNA by tenocytes at 72h. Although the primary purpose in assaying for VEGF-A mRNA was as a positive control for hypoxia statins have previously been reported to upregulate the expression of VEGF-A in various cell types [324-326] including the osteoblastic cell line MC3T3-E1 [327]. However, in all instances a shorter time point was measured. Indeed in MC3T3-E1 cells the increase in VEGF-A mRNA induced by a single treatment of 1μM simvastatin peaked at 12h with levels returning to baseline by 24h. The increase in VEGF-A mRNA caused by a single dose of statin treatment appears to be a transient response. It is therefore possible that the tenocytes responded to lovastatin treatment by transiently increasing VEGF-A mRNA levels but that the time point measured in this experiment was not correct to detect this. The relevance of such a transient increase in VEGF-A mRNA is hard to interpret without substantiating ELSIA data.

6.3.3.2.2 Lovastatin and DEC1 Regulation

This is the first account of the upregulation of DEC1 by statin treatment. It should be noted, however, that the time point assessed does not allow us to determine if the DEC1 promoter is under direct control of signalling pathways including small GTPases. In this study statins also upregulated BMP-2. Shen et al. have shown that treatment of ATDC5s with BMP-2 upregulates the expression of DEC1 after 48h [100]. Thus, it is possible that the increased DEC1 mRNA seen in this study

could either result from direct signalling involving small GTPases or as a result of statin induced BMP-2 signalling.

Irrespective of the precise signalling pathway(s) involved, the upregulation of DEC1 gene expression by lovastatin is of importance to the tissue engineering of cartilage. From the data presented in this thesis statins have the potential to promote chondrogenesis by upregulating the expression of chondrogenic Sox factors (as demonstrated in this chapter) resulting in increased production of sGAG (Chapter 3) and collagen II (Chapter 3 and this chapter) while simultaneously preventing differentiation to alternative phenotypes (via the upregulation of DEC1).

6.3.4 The Possible Impact of Time upon Hypoxia as a Chondrogenic Driver

While lovastatin upregulated multiple markers of chondrogenesis within 72h, hypoxia only upregulated expression of DEC1. The timeframe, oxygen concentration and age of the donors used are comparable to previous chondrocyte studies. This could reflect differences between tenocyte and chondrocyte response to hypoxia i.e. that hypoxia is capable of promoting a chondrocytic phenotype in chondrocytes but tenocytes never respond in such a manner. Or it could simply mean that tenocytes take longer to upregulate chondrocytic genes in response to hypoxia than do chondrocytes. In favour of the latter, long term exposure to the hypoxia mimic cobalt chloride increased the expression of Sox9. This result needs to be confirmed and the study extended using a hypoxic workstation and a defined oxygen concentration. Nevertheless, it raises the

interesting prospect that long term exposure to hypoxia may play a part in the formation of chondrocyte-like cells in tendon.

If this is the case then recent work attempting to dissect out the precise signalling pathways involved in the upregulation of chondrocytic genes in chondrocytes by hypoxia are of interest since they could inform future research directions. As has been previously stated an important component of the oxygen response mechanism of many cells is hypoxia inducible factor (HIF). Recent years have seen much interest in the role of HIF in hypoxia-induced chondrocytic gene expression. The next section considers the current state of the literature in this area.

6.3.5 The Role of HIF-1α and HIF-2α

That hypoxia promotes a chondrocytic phenotype in cells which are already undergoing chondrogenesis is well supported in the literature. There is strong evidence that hypoxia upregulates the expression of collagen II by a HIF mediated process. The HIF dimer does not directly regulate collagen II but acts through intermediates such as Sox9. What is less clear, however, is whether a specific HIF-α subunit is involved in the upregulation of Sox9 and, if so, which HIF-α subunit this is. Work by Schipani et al. shows that HIF-1α is necessary for chondrocyte survival [328]. There is some disagreement, however, concerning whether HIF-1α has a role beyond simple pro-survival, particularly whether it directly regulates pro-chondrogenic genes.

6.3.5.1 Summary of the Arguments in Favour of a Central Role for HIF-1α or HIF-2α as they are Presented in the Literature

Many studies have been published in which the authors implicate HIF-1α as playing an important role in the production and deposition of cartilaginous matrix [234, 245, 317, 329, 330]. Recent work by Lafont et al [232], however, suggests that it is not HIF-1α but HIF-2α which is critical for the expression of cartilage matrix molecules. This section summarises the arguments as they are presented by the various authors in the literature.

Grimer et al. reported that culture of osteoarthritic chondrocytes with the HIF inhibitor 2-methoxyestradiol abolished the nuclear localisation of HIF-1α and prevented the upregulation of collagen II by hypoxia [329]. Mouse micromass cultures depleted of HIF-1α expression using the Cre-lox system show decreased nodule formation under hypoxia [317]. The hypoxia induced increase in collagen II and aggrecan expression is abolished. Expression of the chondrocytic Sox trio is decreased in normoxia and the hypoxia induced increase is abolished [317]. Kanichai et al. report that the transfection of differentiating rat MSC with HIF-1α siRNA abolished the upregulation of safranin O, collagen II and Sox9 staining induced by hypoxia [330]. Likewise, Duval et al. report that HIF-1α siRNA decreased the expression of collagen II in dedifferentiated human chondrocytes while upregulating the expression of collagen I [245]. They further report that transfection of the cells with a dominant negative form of HIF-1α inhibited the hypoxia induced expression of collagen II, aggrecan and Sox9, while upregulating

the expression of collagen I and collagen III [245]. The over-expression of HIF-1α resulted in the opposite effects [245]. Work by Amarilio et al. suggests that HIF-1α directly regulates Sox9 in the developing cartilage anlagen in mice [317]. Both this study and a previous study identified HREs in the mouse Sox9 promoter region [234, 317]. Recent work by Duval et al. reports the binding of HIF-1α to putative HREs in the human Sox9 and aggrecan promoter regions [245].

In contrast, work by Lafont et al. suggests that HIF-2α and not HIF-1α upregulates the chondrogenic genes in human chondrocytes dedifferentiated by repeated passage and redifferentiated by exposure to hypoxia [232]. Additionally, Khan et al. report that hypoxia increased transcription of HIF-2α in infrapatellar fat pad stem cells stimulated to undergo chondrogenesis while the levels of HIF-1α transcript remained constant [244].

6.3.5.2 A Critical Review of the Literature

In the light of the recently published work by Lafont et al. it is important to reassess the work of other groups in the field in order to try to place this important work within its correct context.

6.3.5.2.1 A Critical Review of the Findings of Khan et al.

Khan et al. found a selective upregulation of HIF-2α mRNA in pellets of infrapatellar fat pad cells (IPFP) cultured in chondrogenic medium in 5% oxygen, compared to those cultured in normoxia. Since this correlated with an upregulation of chondrogenic Sox trio the authors suggest that in IPFP cells the hypoxia driven pro-chondrogenic response may be driven by HIF-2α [244].

While the relative increase in HIF-2α transcript is an interesting observation the suggestion that the hypoxia driven pro-chondrogenic response is mediated by HIF-2α should be treated with caution since the authors observed changes in the mRNA levels only and did not assay for HIF-α subunit protein levels. Previous studies have shown a poor correlation between HIF transcript levels and hypoxic response, at least in short term extreme hypoxia and there appears to be no reason from the current understanding of the mechanism of oxygen sensing that this should alter with longer term treatment.

6.3.5.2.2 A Critical Review of the Findings of Grimmer et al.

Grimmer et al report that osteoarthritic articular chondrocytes cultured in 1% oxygen upregulated the expression of collagen II mRNA [329]. They note a localisation of HIF-1α to the nucleus of these cells. Addition of 2-methoxyestradiol abolished this nuclear localisation and prevented the upregulation of collagen II. The authors interpret these data as strongly suggesting that HIF-1α is involved in the hypoxia induced upregulation of collagen II synthesis. However, 2-methoxyestradiol has been shown to inhibit both HIF-1α and HIF-2α protein expression [331]. The figure is probably better interpreted to suggest that HIF-α subunit(s) are involved in the hypoxia induced upregulation of collagen II.

6.3.5.2.3 A Critical Review of the Findings of Duval et al (Including a Critical Review of the Findings of Kanichai et al.)

Duval et al. begin their case for specific regulation of chondrocytic markers by HIF-1α by describing the upregulation of chondrocytic genes during a transient transfection of dedifferentiated chondrocytes with pc-DNA3-HIF-1α, a HIF-1α overexpression vector (In [245] Figure 3). It should be noted, however, that Warnecke et al. provide evidence of a loss of target gene specificity during transient overexpression of both HIF-1α and HIF-2α [332]. It would therefore seem appropriate to be cautious when interpreting these data.

Both Kanichai et al [330] and Duval et al. [245] present data which they claim shows that inhibition of HIF-1α impairs the expression of chondrogenic genes, particularly collagen II. However, the failure to consider the possibility of a role for HIF-2α leads the authors to make some unfortunate omissions. Duval et al. use cadmium chloride to counteract the effects of hypoxia on HIF-1α (In [245] Figures 4A and 4B). It should be noted that the effect of cadmium chloride on HIF-2α is not known. However, in the paper describing the effect of cadmium ions on HIF-1α degradation, hypoxia induced transcription of EPO in Hep3B cells is shown to be prevented by cadmium treatment [333]. RNA interference studies have indicated that the hypoxic induction of this gene is largely dependent on HIF-2α in this cell line [332]. This suggests that cadmium treatment may not be specific for HIF-1α, thus the downregulation of chondrocytic genes observed by Duval et al. in the presence of cadmium chloride can not be solely attributed to the removal of HIF-1α.

Duval et al. report that transfection of a dominant negative form of HIF-1α reduced hypoxia induced expression of chondrocytic genes (In [245] Figure 4C). The dominant negative form of HIF-1α lacks the C-terminal transactivation domain and is proposed to act via binding to HIF-1β resulting in the production of a non-functional dimer [334]. However, HIF-1β is also the binding partner for HIF-2α [335]. Although the effects of the dominant negative HIF-1α on HIF-2α signalling have not been reported, the dominant negative form of HIF-2α, which similarly lacks the C-terminal transactivation domain, is known to inhibit HIF-1α signalling by sequestering HIF-1β [336]. The figure may therefore show, not the effects of removing HIF-1α, but the effects of removing the common dimerisation partner HIF-1β. These data are probably better interpreted as demonstrating that HIF-α subunit(s) are involved in the hypoxia induced upregulation of collagen II. Both Kanichai et al. and Duval et al. present HIF-1α siRNA data which shows the prevention of hypoxia-induced upregulation of chondrocytic genes (In [330] Figure 5, in [245] Figure 4D). However, both papers use siRNA purchased from commercial suppliers to inhibit expression of HIF-1α with the consequence that they are not able to state the precise sequences used. Unfortunately, in both cases, the authors fail to show any PCR and/or protein data indicating that the siRNA in question silences only HIF-1α and not HIF-2α. This makes their data difficult to interpret conclusively.

6.3.5.2.4 A Summary of the Difficulties of the Current Literature

A summary of the evidence presented by each paper reviewed, the authors' interpretation and my preferred interpretation is presented in Table 6.2. The

difficulty in interpreting the data presented by the various groups is largely created by the failure of the papers implicating HIF-1α to investigate and definitively rule out HIF-2α as a causal factor. This failure to consider HIF-2α is understandable in the light of the original report of HIF-2α expression being limited to endothelial cells [335]. However, it is to be hoped that the papers that will be published in the coming years will rectify these early problems by including a consideration for the potential role of HIF-2α in the hypoxic promotion of chondrogenesis.

Paper	Author Interpretation	Criticism	Figs	Preferred conclusion
Khan [244]	Enhancement of chondrogenesis by hypoxia may be driven by HIF-2α	mRNA only not protein	3	Possible, but caution
Grimmer [329]	Strongly suggest that HIF1α is involved in hypoxia induced upregulation of collagen synthesis	Non-specific inhibitor	4	HIF (without specified a subunit) is involved in the hypoxia induced upregulation of collagen synthesis.
Kanichai [330]	Inhibition of HIF-1α impairs the expression of chondrogenic genes especially COL2A1	Unknown specificity of siRNA	5	?
Duval [245]	HIF-1α plays a bifunctional role in the redifferentiation of chondrocytes through the upregulation of cartilage matrix genes and the inhibition of dedifferentiation genes.	Questionable reliability of over-expression method Non-specific inhibitor Non-specific dominant negative Unknown specificity of siRNA	3 4A, 4B 4C 4D	HIF (without specified a subunit) is involved in the hypoxia induced upregulation of prochondrogenic genes. This upregulation may be HIF-1α dependent.

Table 6.2 Summary of Critical Review of Papers Concerned with Determining HIF-α Subunit Role in the Response of Chondrocytes to Hypoxia

6.3.5.2.5 Is Sox9 a HIF-α Subunit Selective Gene?

One consequence of considering, particularly, the data of Duval et al. and assuming that the siRNA used is, indeed, specific to HIF-1α is that the findings directly contradict those of Murphy et al. Given that the findings of Duval et al. are supported by the data from the mouse models this is not an unlikely scenario. It also begins to beg the question, is Sox9 a HIF-α subunit selective gene at all? Many genes that are hypoxia responsive do not appear to be regulated exclusively by HIF-1α or HIF-2α [337].

Two independent studies with a virtually identical design published simultaneously identified Sox9 as a gene upregulated by hypoxia in the breast cancer cell line MCF-7 [318, 338]. Notably, however, one study identifies Sox9 as regulated by HIF-1α [338] while the other identifies it as regulated by HIF-2α [318]. One obvious difference in the culture protocols used was that one study cultured the cells in 1% oxygen for 16h [338] while the other cultured the same cells in 0.5% for 8h [318]. While there are other additional procedural differences (such as the use of transfection reagents from different companies) that could account for the differences observed that two almost identical studies could reach opposite conclusions is striking. Indeed, the same studies additionally identified DEC1 as upregulated by hypoxia. However, both agreed that this gene is regulated by HIF-1α (see supplementary information for both papers). It is perhaps indicative that the results obtained for some hypoxia regulated genes may be peculiarly sensitive to experimental conditions, with different groups claiming dependence on one α-subunit or the other dependent on the cell source, timing and oxygen concentration used. Whatever the case, it would be ill advised for a

study finding the presence of HIF-2α in torn rotator cuff tendon to take this as an early step in chondroplasia.

6.3.6 Potential New Chondrocytic Markers Induced by Hypoxia

Recent work has a suggested a number of interesting new markers of the chondrocytic phenotype that may be hypoxia regulated. Lafont et al. investigated gene expression by microarray, selecting for genes that were downregulated by monolayer passage and upregulated by redifferentiation of the chondrocytes with hypoxia [322]. In addition to finding upregulation of many classical chondrocyte matrix genes (including COL2A1, ACAN, collagen IX and PRELP) they identified the transcription factors Sox9 and DEC2 as downregulated by monolayer passage but upregulated by hypoxia. This is suggestive of DEC2 playing an important role in creating a chondrocytic phenotype.

Lafont et al. additionally identify the anti-angiogenic tenomodulin homolog chondromodulin and growth and differentiation factor 10 (GDF10) as downregulated by monolayer passage and subsequently upregulated by hypoxia. The complete abolition of the hypoxia mediated induction of chondromodulin by siRNA to Sox9 and the reduced expression of GDF10 under the same conditions, suggest that these molecules are dependent on Sox9 for their hypoxic induction. It is also of note that Jelinsky et al. identified both of these molecules as specific markers of chondrocytes in their study ([76] supplementary information 4, table 3).

Lafont et al. identify mitogen-inducible gene 6 (MIG6) and inhibinA as potential chondrocyte markers that were hypoxia responsive but not regulated by Sox9. The expression levels of these markers in tendon are currently unknown, but they may represent interesting targets for investigation.

6.3.6.1.1 Potential Crosstalk

Although a number of authors have observed that hypoxia enhances the effects of 3D culture on chondrocyte phenotype the mechanism by which this occurs is currently unknown. Interestingly, Aprelikova et al. identified ROCK2 by microarray as downregulated by hypoxia in MCF-7 cells [318] (see Supplementary information Table 2). Should this prove to be replicated in chondrocytes, this would provide an additional mechanism for crosstalk between the hypoxia and cell shape signalling pathways.

The effects of a combined treatment of lovastatin and hypoxia are of particular interest since they more closely relate to the physiological situation in torn rotator cuff than either condition alone. In torn tendons the cells are subject to both hypoxia caused by the severing of blood vessels and to changes that are capable of altering cell shape. Interestingly, Pacary et al. report that treatment of MSC and PC-12 cells (a model of neuronal differentiation) with a combination of Y27632 and FG-0041 (a chemical mimic of hypoxia which functions by inhibiting prolyl-4-hydroxylases) initiates neuronal differentiation [339]. Although the authors of this paper strongly suggest in their discussion that HIF-1α may be involved in the differentiation process, the data presented does not preclude a role for HIF-2α. Indeed, the authors note a particularly strong induction of erythropoietin (EPO)

with combined Y27632 and FG-0041 treatment. EPO appears to be upregulated exclusively by HIF-2α in many tissues, including cortical astrocytes [340].

6.3.7 Conclusion

In conclusion, tenocytes respond to short term hypoxia (1% oxygen) by upregulating expression of the classical hypoxia response gene VEGF-A and the chondrocyte marker gene DEC1. There is, however, preliminary data to suggest that longer term hypoxia may alter the chondrocyte master transcription factor Sox9. Hypoxia does not appear to potentiate the action of lovastatin in a short-term treatment protocol. However, in the light of the preliminary chemically simulated long-term hypoxia data, it would be interesting to determine its effects over a longer time period. Overall the data presented in this chapter suggest that lovastatin is far more effective than conventional hypoxia treatment in inducing transformation from tenocytes to chondrocytes.

Chapter 7 - Discussion

7.1 Major Findings of this Thesis

7.1.1 Introduction

Chondroplasia, the appearance of chondrocytic features in otherwise tensional tendon tissue, is a commonly reported histological finding in torn rotator cuff tendons. The reason for the appearance of this degenerative phenotype is unknown, although a hypothetical pathogenesis for chondroplasia has been proposed [184]. Since all previous studies in this area have been descriptive in nature this pathogenesis is based primarily on histological and anatomical observations and analogy with non-pathological fibrocartilage formation in animals. To date only two studies have attempted to induce the transdifferentiation of tenocytes to a more fibrochondrocyte-like phenotype in vitro using alginate bead culture [341] and compression [342] respectively. No study to date has determined the effect of hypoxia on tenocyte phenotype with respect to the expression of chondrocytic markers, although it is frequently cited as a cause or contributing factor in tendon degeneration in general [23, 343] and in chondroplasia specifically [184]. This thesis has examined the effects of the putative drivers of chondroplasia cell shape change/actin cytoskeletal remodelling, hypoxia and a combination of the two on healthy human tenocyte phenotype using an in vitro system.

7.1.2 Restatement of Thesis Aims

The aims of this thesis as stated in the introduction were:

1. Test the prochondrogenic effects of lovastatin in the established ATDC5 chondrogenic model.
2. Assess the effects of cytoskeletal signalling disruptors on tenocyte phenotype.
3. Determine whether hypoxia can alter tenocyte phenotype towards fibrochondrocyte.
4. Test the effect of combined hypoxia and cell shape alteration on tenocyte gene expression.

7.1.3 The Prochondrogenic Effects of Lovastatin in the ATDC5 Chondrogenic Model.

To gain further insight into the recently described prochondrogenic effects of statins lovastatin was added to ATDC5 cells cultured in pellets. A relative decrease in DNA concentration was noted which may be attributable to the lovastatin mediated inhibition of cell cycle at the G_1/S transition. This is a well known effect of statins including lovastatin in other cell types but has not been described in cartilage. Such an inhibition of cell cycle, if it occurs, may support chondrogenesis [270].

Lovastatin treatment also caused a dose dependent increase in sGAG expression and resulted in a strong and accelerated increase in collagen II expression. This both confirms the published literature and previous work undertaken by our group

and extends the data available by demonstrating that the resultant tissue has the collagen II structure of normal cartilage.

These data suggest that lovastatin treatment, which causes cell rounding and changes to the actin cytoskeleton could be capable of enhancing, possibly even inducing chondrogenesis in a chondrocytic cell line. Lovastatin was therefore included in the panel of cytoskeletal signalling disruptors used on the tenocytes.

7.1.4 Assessment of the Effects of Cytoskeletal Signalling Disruptors on Tenocyte Phenotype

A panel of five cytoskeletal signalling disruptors which have been previously demonstrated to promote expression of chondrocytic markers in chondrocytes was assembled. The components of the panel were confirmed to induce actin cytoskeletal disruption, and in some cases gross cell shape change, in tenocytes. The effects of five disruptors of cytoskeletal signalling on normal tenocyte phenotype in short and long term culture were determined by QPCR. While a number of the disruptors upregulated chondrocytic markers at 72h, only lovastatin maintained expression of Sox9 and Sox5 in long term culture. Further investigation was therefore performed using this inhibitor. Lovastatin was found to dose dependently increase the mRNA levels of the chondrocytic markers Sox5 and Sox9 while simultaneously decreasing the mRNA levels of the tenocytic markers COL1A1 and COL3A1. This effect was reversible on addition of the downstream metabolite mevalonate. Additionally, long term treatment of tenocytes with lovastatin induced the expression of collagen II protein. This

suggests that lovastatin may be capable of altering cell phenotype from tenocytic to more chondrocytic.

7.1.5 Assessment of the Effect of Hypoxia on Tenocyte Phenotype

The effect of range of different oxygen concentrations on the expression of chondrocytic markers was determined by QPCR. Tenocytes were found to upregulate expression of the classical hypoxia response gene VEGF-A and the hypoxia responsive pro-chondrocytic marker DEC1 after 72h in hypoxic conditions. The expression of the other chondrogenic markers was unaffected, with the possible exception of Sox9 which showed a slight upward trend. Since only short time periods were examined in this study it is not possible to eliminate hypoxia as a pro-chondrogenic paradigm. The conditions within the cuff may more closely resemble a long term culture under less stringent hypoxia. Indeed, preliminary data using the hypoxia mimic cobalt chloride in long term culture suggest that Sox9 gene expression may be HIF responsive in tenocytes. Further experiments using a hypoxic workstation would be needed to confirm this finding.

7.1.6 Assessment of the Effect of Combined Hypoxia and Cell Shape Alteration on Tenocyte Gene Expression.

Since the conditions in the torn rotator cuff are likely to involve both hypoxia and altered matrix composition with altered mechanical loading, the effects of a combination of hypoxia and lovastatin were examined. No synergistic effect was observed for any of the assayed markers. However, lovastatin was noted to

upregulate DEC1, an unexpected and, as far as we have been able to determine, completely novel observation. Once again, the short term nature of this study and the unknown oxygen concentration to which the cells are exposed in vivo means that it is not possible to exclude a prochondrogenic interaction between hypoxia and cell shape change in the process of chondroid metaplasia.

7.2 Future Work

7.2.1 RhoA – A Phenotype Switch?

From the data presented in this thesis it appears that cell shape change, as induced by treatment with lovastatin, is the stronger of the two paradigms for the induction of the fibrocartilaginous phenotype. Lovastatin inhibits prenylation which disrupts the intracellular location of small GTPases and prevents their involvement in signalling pathways. Although this study has not confirmed that disruption of small GTPase signalling is important to the upregulation of chondrocytic marker expression by lovastatin it is not unreasonable to presume that this is indeed the mechanism of action. Since lovastatin disrupts a number of different small GTPase signalling pathways it is also impossible to say with certainty which pathway(s) is likely to be important in the upregulation of chondrocytic markers. However, inhibition of RhoA has been demonstrated to upregulate expression of Sox5 and Sox9 and to downregulate the expression of COL1A1 and COL3A1 in chondrocytes. It is therefore possible the effects of lovastatin were caused by the inhibition of RhoA signalling. Further experiments using inhibitors of specific prenyl transferases, the Rho subfamily and possibly

the use of one or both of the dominant negative forms of RhoA would be necessary to confirm this hypothesis.

It is possible that RhoA may act as a phenotype switch between tenocytes and a more chondrocyte-like phenotype, with active signalling resulting in a tenocytic phenotype while inactive signalling yields a chondrocytic phenotype.

7.2.2 Tenascin-C and Chondroplasia

Tenascin-C has previously been hypothesised to play a role in the development of chondroplasia [160, 184]. In chondroplastic regions tenascin-C is concentrated in the matrix immediately surrounding the fibrochondrocytes [160, 184]. Tenascin-C is reported to have anti-adhesive properties. In vitro it promotes cell rounding [344, 345], induces a cortical actin structure [345] and promotes chondrogenesis [344] in chick wing bud cells [344] and adult human chondrocytes [345]. Studies of wound healing have found that culture of fibroblasts on a matrix which contained tenascin-C suppressed the activation of RhoA [346] (although it should be noted that both culture on a tenascin-C containing matrix and inhibition of Rho signalling induced the production of filopodia in theNIH-3T3 cells used rather than the cell rounding observed in chondroplasia). In the light of the published literature and the current study it would be interesting to determine if culture of tenocytes on tenascin-C upregulates chondrocytic markers and, if so, if the upregulation correlates with RhoA activity.

7.2.3 The Relationship of BMP-2 Expression to Sox Gene Expression

Lovastatin additionally dose dependently upregulated BMP-2 mRNA expression in treated tenocytes. Some studies report that BMP-2 is capable of upregulating chondrogenic markers, including Sox9 [347]. The relationship between the lovastatin induced upregulation of BMP-2 and the upregulation of Sox9 and Sox5 in this study is not clear. Further experiments using the inhibitor of BMP-2 signalling, noggin, would determine if the upregulation of Sox9 and Sox5 is BMP-2 mediated or likely to be a direct effect of the statin treatment.

7.3 Weaknesses of this study

7.3.1 Disadvantages of Cell Culture - Mechanical

The experiments in this thesis were conducted in vitro, a method which, whilst it enables the use of human material has a number of disadvantages. The mechanical environment to which the cells are subjected differs from that to which they would be exposed in vivo. Particularly, unlike the native environment, these cultured tenocytes were not exposed to tensile loading. This may affect the phenotype of the tenocytes and may promote dedifferentiation. It may also alter the response of the tenocytes to the stimuli used in this study.

To consider the effect of the mechanical environment on tenocyte phenotype first - lack of tension is known to downregulate expression of tenocyte matrix molecules [224]. There are also some hints that scleraxis expression may be influenced by tensile loading [348], however no study has attempted a

comprehensive examination of the relationship. It should, however, be noted that the expression of tenocyte markers in the cells used in this study was stable until passage 5.

Aside from the effect of mechanical loading on their differentiation status the alteration of mechanical signals may have an effect on the way in which cells respond to other stimuli including hypoxia and cytoskeletal disruption. Different stimuli are known to use common signalling components, with certain proteins appearing to form convergence points for a number of different pathways creating the potential for synergistic effects. There certainly appears to be potential for adaption of the way in which tenocytes respond to hypoxia in a situation in which the cells are subject to tensile strain, since cyclic uniaxial strain has been shown to stabilise expression of HIF-1α protein [349].

7.3.2 Disadvantages of Cell Culture - Matrix

Cells are known to be sensitive to the composition of the ECM in which they reside. Cell culture removes them from the ECM and their behaviour may consequently be altered. Linked to this loss of interaction with ECM components is the monolayer nature of the work undertaken in this study. This involves a significant alteration in cell shape for the tenocytes. Monolayer culture is well known to induce dedifferentiation of chondrocytes. It should be noted, however, our tenocytes maintained expression of tenocytic markers until passage 5.

7.3.3 Limitations of the Hypoxia Study

One of the difficulties inherent in this work is an uncertainty over the precise oxygen concentration to which the tenocytes are subject in the torn cuff. Indeed, involvement of this paradigm as an enhancer of chondrogenesis may well be dependent on local conditions. Another weakness of this work is the short term nature of most the experiments, particularly those in the hypoxia section. This resulted from the lack of a hypoxia workstation necessary for uninterrupted long-term exposure to hypoxia and from a reduced time frame due the need to repeat experiments caused by the discovery of the mycoplasma infection.

7.3.4 Disadvantages of QPCR

The primary assay used in this study for the expression of chondrogenic markers in the tenocytes was QPCR. This assays mRNA and thus provided a good 'snap shot' of the immediate transcriptional response of the cell to the treatment paradigms. However while increased mRNA levels often correspond to increased protein levels this is not universally true. Ideally a protein assay such as Western blot should be carried out to confirm the mRNA data. These were not untaken for the current study for various reasons. Matrix molecules such as collagen I and III are difficult to assay due to their insolubility. It additionally proved impossible within the time frame to locate reliable antibodies for other chondrogenic markers such as DEC1, although several were tested.

7.4 Challenges within this field

7.4.1 Chondroplasia and Transcription Factor Expression

Chondroplasia itself is poorly defined. The published literature principally contains descriptive accounts based on histological examination and focuses on presence/absence and physical location of the molecules examined. With the notable exception of the staining for S100 undertaken by Gigante et al. [184] all of the molecules examined to date have been components of the ECM. This thesis has considered the presence of high levels of the chondrogenic Sox trio in chondroplasia highly likely given the importance of these transcription factors in activating and enhancing collagen II transcription, a protein which is known to be present in chondroplastic regions. It should, however, be noted that no study has been made of the expression of any transcription factor, chondrocyte associated or otherwise, in chondroplastic regions. In the light of the findings in this thesis, it would be interesting to investigate the expression of Sox9, Sox5 and DEC1 in samples of torn rotator cuff tendon. The correlation of such expression with changes in cell shape, markers of hypoxic insult such as HIF-1α and changes in matrix composition such as the accumulation of tenascin-C or a switch in expression from tenomodulin to chondromodulin could be particularly useful in understanding the chondroplastic process. Indeed, such a study was planned but we were unable to optimise antibodies within the given time period.

7.4.2 Fibrocartilage and Chondroplasia

The relationship of chondroplastic regions to fibrocartilage, a tissue with which they appear to share many characteristics, is also uncertain. For example,

chondroplastic regions are known contain a large quantity of sGAG including chondroitin sulphate [184]. Fibrocartilage is known to contain aggrecan and it seems likely that the accumulation of sGAG seen in chondroplasia is due to the presence of aggrecan, however precisely which protein(s) in chondroplastic regions are modified by the attachment of these carbohydrate side chains is currently unknown. There is no comprehensive survey of the components of either fibrocartilage or chondroplastic regions and no published comparison of the relative levels of the components.

The lack of clarity on the issue of the similarity of fibrocartilage to chondroplasia is significant since the process of fibrocartilage formation is likely to be important in understanding the chondroplastic process. Understanding the similarities and differences in the matrix components, transcription factors and tissue structure may assist in understanding which drivers they share and which they do not.

7.4.3 Non-Pathological Fibrocartilage Formation and Chondroplasia

The work within this field is severely limited by a poor understanding of the molecular processes involved in the formation of non-pathological fibrocartilage within the tendon. Although many good studies have been published by Vogel's group, many of these pre-date the discovery of the master transcription factor Sox9 and are therefore limited to observations concerning matrix molecules alone. Those studies which have been undertaken by other groups since the elucidation of the role of Sox9 in chondrogenesis have primarily focused on the role of mechanical loading in the development of fibrocartilage, examining the

development of the specialised fibrocartilaginous matrix from a biomechanical perspective [350]. No attention appears to have been paid to the role of transcription factors or their upstream signalling pathways in fibrocartilage formation. Even simple descriptions of the timing of Sox9 expression in relation to fibrocartilage development at entheses or in compressed regions are absent from the current literature. This follows the general pattern of a poor understanding of the basic biology of tendons. Such descriptive studies are required, however, in the light of attempts at regenerative medicine. It is very difficult to regenerate normal or near-normal tissue when the process by which this normal tissue is formed natively is not understood. Advances in molecular biology, including the generation of knock-out mice, GFP mice etc. have proven to be of great value in the understanding of the development and maintenance of hyaline cartilage. Currently existing models could be used for such studies and should yield much useful data.

7.4.4 Dedifferentiated Chondrocytes and the Tenocytic Phenotype

Studies which use dedifferentiated adult chondrocytes are of particular value when considering chondroplasia. The use of adult rather than foetal or progenitor cells seems a priori to be a better match for the situation found within the degenerate cuff, where the majority cell population subject to the pro-chondrogenic microenvironment is adult, differentiated tenocytes, not progenitor cells. The chondrocyte dedifferentiation model itself is of particular interest to the tendon field since dedifferentiated chondrocytes are known to assume a fibroblastic morphology, accumulate actin stress fibres and to express molecules

associated with tendon ECM including collagen I, collagen III and versican [351]. This dedifferentiated phenotype has been poorly characterised, but the similarities to tenocytes are notable. It is tempting to speculate that dedifferentiated chondrocytes have a very similar phenotype to tenocytes. Chondrocytes are known to express both Sox9 and scleraxis and to downregulate expression of Sox9 as they dedifferentiate. No one has determined the levels of expression of scleraxis during dedifferentiation, although a recent microarray examining early changes in gene expression during the dedifferentiation process did not identify scleraxis as showing a greater than two fold difference in expression between differentiated and dedifferentiated foetal human chondrocytes [352]. If scleraxis expression is unaffected by dedifferentiation the resultant cell could easily resemble a tenocyte. Indeed, a recently published study using horse tissue found that while cartilage had higher levels of Sox9 mRNA than tendon, there were no statistically significant difference in scleraxis mRNA levels between tendon and cartilage [67]. CD90 (also called Thy-1) is a membrane associated glycoprotein which has been reported to be expressed by human tenocytes [353] but not by differentiated chondrocytes. Several recent studies have shown the de novo expression of CD90 by dedifferentiating articular chondrocytes [354, 355]. Whilst this molecule is by no means a tenocyte specific marker, its upregulation by monolayer culture in chondrocytes is interesting. A detailed comparison of freshly isolated chondrocytes, dedifferentiated chondrocytes and tenocytes should reveal the nature of their relationship, which, in turn, should allow for further comment on the plasticity of cell phenotype in response to their immediate environment.

7.4.5 Summary

This thesis has attempted to further elucidate the process of chondroid metaplasia with special reference to the degenerate rotator cuff, specifically the role(s) of cell shape change, hypoxia and a combination of these two treatments. The data presented suggest that cell shape change, particularly that induced by lovastatin treatment, is far more effective than hypoxia in inducing transformation from tenocytes to chondrocytes. Moreover, lovastatin induces this upregulation of chondrocyte genes and downregulation of type I and III collagens in monolayer culture. This may be a useful approach to generating better quality cartilage in applications like autologous chondrocyte implantation where chondrocytes are expanded in monolayer prior to implantation in patients. Further, rotator cuff tendons typically rupture at the osteotendinous junction and repair involves stitching tendon directly onto the bone. While this has obvious mechanical benefits, it is questionable whether the torn tendon has the ability to recreate the natural architecture of the enthesis. It might be possible to improve repair using local application of agents such as statins capable of generating a more normal fibrocartilage enthesis.

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Appendix A - Papers, Publications and Presentations of the Work Contained in this Thesis

A.1 Papers

Hannah R Cornell, Andrew J Carr, Philippa A Hulley, "Cell shape change and actin cytoskeleton remodelling drives human tenocytes towards a chondrocytic phenotype" *Manuscript in preparation*

Min Liang, **Hannah Cornell**, Nasim Zargar Baboldashti, Mark S Thompson, Andrew J Carr, Philippa A Hulley, "Regulation of hypoxia-induced cell death inhuman tenocytes," *Submitted to J Cell Physiol.*

Isabelle Gennero, Sally C. Dickinson, Trevor J. Sims, **Hannah Cornell**, Michele Nieto, Anthony A. Hollander, R. Graham G. Russell and Philippa A. Hulley, "Lovastatin stimulates cartilage matrix production by adult primary articular chondrocytes," *Manuscript in Revision*.

A.2 Presentations

Hannah R Cornell, Andrew J Carr, Philippa A Hulley, "Factors Contributing to Chondroplasia in Degenerate Rotator Cuff Disease" *Nuffield Department of Orthopaedic Surgery (NDOS) Research Day* 2008

Hannah R Cornell, Andrew J Carr, Philippa A Hulley, "Factors Contributing to Chondroplasia in Degenerate Rotator Cuff Disease" *Nuffield Department of Orthopaedic Surgery (NDOS) Postgraduate student oral presentation* 2007

A.3 Oral Posters and Posters

Hannah R Cornell, Andrew J Carr, Philippa A Hulley, "Lovastatin Upregulates Chondrocyte Marker Expression in Tenocytes," Poster *4th International Workshop on Advances in the Molecular Pharmacology and Therapeutics of Bone Disease*, 2009

Hannah R Cornell, Andrew J Carr, Philippa A Hulley, "Lovastatin Upregulates Chondrocyte Marker Expression in Tenocytes" Oral Poster *Bone Research Society and British Matrix Biology Society Joint Meeting*, 2009

Hannah R Cornell, Min Liang, Andrew J Carr, Philippa A Hulley "Cell shape change and actin cytoskeleton remodeling drives human tenocytes towards a chondrocytic phenotype" Poster *2nd Joint Meeting of the International Bone & Mineral Society and the Australian & New Zealand Bone and Mineral Society*, 2009

Hannah R Cornell, Andrew J Carr, Philippa A Hulley, "Cell shape change and actin cytoskeleton remodeling drives human tenocytes towards a chondrocytic phenotype" Poster *Oxford Medical Sciences Division DPhil Day* 2009

Hannah R Cornell, Andrew J Carr, Philippa A Hulley, "Lovastatin modulates Chondrocyte Cell Cycle and Matrix Output" *Calcified Tissue International*, vol. 83, pp. 1-33, Poster no. BRS-P35 2008

Appendix B - Notes Added in Corrections

B.1 Chapter 1

B.2 Pain and Tendinopathy

Rotator cuff disease is the most common cause of shoulder pain in the elderly population [2, 3]. The precise cause of this pain is uncertain, particularly since studies indicate that a surprisingly large proportion of rotator cuff ruptures are apparently asymptomatic [9]. Tendons are innervated and the neurotransmitter and neuromodulator substance P has been detected in medial and lateral epicondylitis [356]. A study by Gotoh et al. found higher levels of substance P in the subacromial bursa of patients with tendinopathy than in normal controls [357]. Blood vessel and nerve ingrowth is associated with the healing process in ruptured tendons [358]. It has been suggested that neurotransmitters secreted by these nerves may be responsible for the pain felt in tendinopathy [358].

B.3 Chapter 2

B.4 Composition of Media for Cell Culture

B.4.1 ATDC5 Maintenance Medium

The components of the media used for the culture of the ATDC5 cell line in this thesis are those used by Shukunami et al. [262] which have become widely accepted for the ATDC5 cell line. A "back-of-the-envelope" study by Altaf et al. [359] in 2006 of studies examining hypertrophy using the ATDC5 model found that "most" used the media compositions advocated by Shukunami et al. A close examination of the reasoning behind the inclusion of some of the components of the maintenance medium does shed doubt on the importance of their continued

presence as a part of the standard make-up of this medium. It is, however, important to note that their inclusion in this particular thesis does ensure that the work presented here is comparable to the published literature in this area.

The medium used to maintain the ATDC5 cell line in the original isolation paper by Atsumi et al. was known as 3F and contained a 1:1 mix of DMEM:Ham's F12, $10\mu gml^{-1}$ transferrin, 3×10^{-8}M sodium selenite and $10\mu gml^{-1}$ insulin supplemented with 5% FCS [246]. The cell line from which ATDC5s were isolated (AT805) was maintained in α-MEM supplemented with 8% FCS [360]. The reason for the change in culture medium is not given by Atsumi et al. but it seems likely that it is due to prevailing beliefs concerning the use of foetal calf serum in mouse teratoma cell line cultures. For some time prior to the publication of Atsumi et al's original ATDC5 isolation paper [246] there had been interest in the potential of mouse embryonic teratoma cells as models for examining in vitro the development of various tissues. However there was concern that the presence of serum in the culture medium of these cells introduced artefacts into the differentiation process and might even be acting to inhibit differentiation of some cell lines [361]. Atsumi and Ikawa (the first and last authors respectively on Atsumi et al. 1990 [246]) regarded the production of serum-free media as "essential for use in the study of the cellular mechanisms of induction of differentiation in teratocarcinoma cells" in their 1985 paper [362]. The success of Gordon Sato's group in replacing serum with insulin, transferrin and some cell type specific supplements for many cell lines and the production of an improved differentiation medium for the teratoma cell line 1003 by the removal of serum [361] probably made this particular media recipe an appealing starting point for

the development of the ATDC5 cell line. Indeed, Ikawa's group had already had some success with the use of a modified form of 4F medium (the medium used by Gordon Sato's group in 1981 [361]) known as 3F in the complete removal of serum from PCC3/A/1 chondrogenic cultures in 1985 [362] (although feeder cells were still necessary in this system). The addition of 5% FCS to the 3F medium for ATDC5 culture probably reflects a failure on the part of Ikawa's group to establish a serum-free culture protocol for these cells, although, happily, the presence of serum does not inhibit differentiation.

B.4.1.1 Selenium

Selenium is an essential component of glutathione peroxidase, the first enzyme in the glutathione peroxidase cycle [363]. This cycle is one of two enzymatic mechanisms used by cells to metabolise hydrogen peroxide, a harmful by-product of cellular processes. Selenium is a component of many recently formulated cell culture media, although DMEM/Ham's F12 without serum supplementation does not contain any selenium.

Sodium selenite was used by Darmon et al. to prevent massive lysis in 1003 cells grown in serum-free medium [361]. Ikawa's group appear to have regarded sodium selenite as an essential component of their media, since they did not determine the effects of sodium selenite removal on either the viability of the ATDC5 cells or their capacity to differentiate [246] (this belief is also apparent in their previous 1985 paper [362] where all media contain sodium selenite). The reasoning behind the continued presence of sodium selenite in medium which is

supplemented with foetal calf serum (a source of selenium) is not clear from the initial ATDC5 description paper.

B.4.1.2 Transferrin

In contrast to their position on sodium selenite, Atsumi et al. did investigate whether transferrin was a necessary component for ATDC5 differentiation, finding that its addition to cultures did not affect chondrogenesis [246]. It is therefore unclear why Shukunami et al. choose to add it to their cultures and why it has become a standard component of ATDC5 maintenance media.

The continued presence of these two components of ATDC5 maintenance medium is particularly strange given that neither reagent is advocated by either the Health Protection Agency cell bank in the culture instructions for the ATDC5 deposits in their care [364].

B.4.1.3 Ascorbate

Ascorbate was added to the ATDC5 cultures as a component of the chondrogenic medium (see Section 2.1.1). Unlike humans, mice are known to possess a functional copy of the enzyme responsible for synthesising ascorbate, gulonolactone oxidase [365]. In adult mice gulonolactone oxidase activity is confined to the liver, however, other organs are capable of synthesising ascorbate in embryonic mice, gradually losing this ability as the organism develops [366]. It is not known whether embryonic cartilage or, indeed, ATDC5s possess the capacity to synthesise ascorbate. However, the addition of exogenous ascorbate has been shown enhance chondrogenesis in ATDC5 cultures [359]. Indeed, the

addition of 50μgml^{-1} of ascorbic acid was recently found to be sufficient to induce expression of collagen II mRNA even in the absence of insulin [367] (the effects of concentrations lower than 50μgml^{-1} on collagen II production were not examined by this study). Ascorbate is a cofactor for the prolyl-hydroxylases responsible for the modification of the proline residues in collagen into hydroxyproline [368]. The addition of ascorbate to the ATDC5 cultures thus should also ensure the correct deposition of a collagen containing ECM.

B.5 Protein Quantification Techniques

The Bradford assay [369] was introduced in 1976 as an improvement on the then currently available methods for protein quantification, namely the Lowry method (which was subject to interference from various substances including carbohydrates [370], Tris [371] and EDTA [372]) and the biuret method (a relatively less sensitive method that is also subject to interference from compounds including Tris [373]). The active component of the Bradford assay is the dye Coomassie Brilliant Blue G-250 [369, 374]. When dissolved in acidic conditions Coomassie Brilliant Blue G-250 exists as three differently charged dye species (an anion, a neutral species and a cation) in dynamic equilibrium [374]. Each of the dye species absorbs at a different wavelength, with the anion species having a maximum absorbance at 595nm [374]. Protein interacts with the anion dye species, stabilising it [374]. This disrupts the equilibrium, pulling the reaction in favour of the anion species, resulting in an accumulation of the anion dye species which is proportional to the concentration of protein added [374]. Although the mechanism by which this occurs is not completely understood [375],

protein stabilisation is believed to occur primarily via the formation of ion pairs with the positively charged amino acids arginine and lysine [376].

The composition of the reagent used in the Protein 660 is not available in the public domain, however, from the information provided by the manufacturers it would appear that the assay functions in a similar manner. As with the Bradford assay a change in dye absorbance values is induced by a deprotonation of the dye reagent by interaction with protein under acidic conditions. As with the Bradford assay the interaction is stated to primarily occur with the basic amino acids arginine, lysine and histidine, and to a lesser extent with tyrosine, tryptophan and phenylalanine [377]. It is possible to speculate that Protein 660 is a modification of the Bradford assay in which the dye, Coomassie Brilliant Blue G-250, has been modified to incorporate at least one metal atom (the reagent is stated to be " a proprietary dye-metal complex" [378]).

The presence of a number of compounds in sample buffer, most notably for this study SDS [379] and urea [379] (as present in the HIF buffer see Section 2.4.1.3), interferes with the Bradford assay. The Protein 660 assay is marketed as having a greater tolerance for detergents and other reducing agents. It is also marketed as generating a more linear standard curve than the Bradford assay. Our group began to use Protein 660 in preference to the Bradford assay because of this linearity and with the aim of replacing the more time consuming BCA protein quantification protocol (necessitated by intolerance of the Bradford assay to components present in the HIF buffer). Unfortunately, while the Protein 660 is less sensitive to urea than the Bradford it is still sensitive to high SDS

concentrations making it an unsuitable replacement for the BCA assay without the addition of extra reagents.

B.6 Dimethylmethylene Blue Assay

The functional component in the DMB assay is the DMB dye itself [249]. Pure dye solution produces an absorption spectrum with three peaks (600, 650 and 760nm). Addition of sGAG reduces the absorption strength at 600 and 650nm while a specific absorption peak at 530nm appears. The 530nm absorption peak is produced with a variety of sGAG including dermatan-sulphate and chondroitin-6-sulphate. An increase in absorption at 530nm is also noted for keratan sulphate, although the response is markedly lower than for other sGAG. Modifications to the original protocol [249] include measuring absorption at 525nm since the blank absorption is lower at this wavelength [380] (this was not possible for this thesis since the filter set in the spectrophotometer did not include a 525nm filter). Other modifications to the original DMB assay protocol have been made in order to reduce interference from other common sample components. The pH was decreased from 3.5 to 3.0 and the salt concentration increased, both of which reduced interference from hyaluronic acid and DNA [250]. Subjecting the samples to papain digestion was also found to decrease interference from proteins and glycoproteins [250].

B.7 Chapter 5

The experiment determining the effects of the panel of inhibitors of cytoskeletal signalling (Figure 5.2) was only performed once in the exact format presented in this thesis. An earlier run used tenocytes from another patient and medium

containing 10% FCS instead of 5% FCS. The results from this were substantially similar to those presented in this thesis. The experiment was not repeated because the study was regarded as initial pilot data with claims of potential pro-chondrogenic activity on the part of lovastatin backed-up by further experiments with multiple repeats.

Appendix C - Correspondence with Epitomics

Hi Hannah,

Thank you for your inquiry with Epitomics, and I sincerely apologize for the delay in response.

We currently do not determine the specific isotype for our rabbit monoclonal antibodies, and all of our antibodies including the anti-FOXO3a; cat.#2071-1are simply considered to be Rabbit IgG.

Please let me know if I can be of further help!

Best regards,
Cynthia

Cynthia Morimoto
Marketing Associate
Epitomics, Inc.
Phone: (650) 583-6688 ext. 245
cynthia.morimoto@epitomics.com

From: Hannah Cornell [mailto:hannah.cornell@wolfson.ox.ac.uk]
Sent: Tuesday, November 23, 2010 7:27 AM
To: info
Subject: Isotype of 2071-1 - Anti-FOXO3a

Dear Sir/Madam,

I would be very grateful if you would be able to tell me which isotype your rabbit monoclonal antibody to FOXO3a (product number 2071-1) is. Thank you for your time.

Hannah