A versatile, engineered cartilage platform for tissue regeneration and disease modeling

Peck, Yvonne

2015

Peck, Y. (2015). A versatile, engineered cartilage platform for tissue regeneration and disease modeling. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/65557>

<https://doi.org/10.32657/10356/65557>

**A VERSATILE, ENGINEERED CARTILAGE PLATFORM FOR TISSUE
REGENERATION AND DISEASE MODELING**

YVONNE PECK

School of Chemical and Biomedical Engineering

A thesis submitted to Nanyang Technological University
in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

2015

Preface

This thesis is submitted for the degree of Doctor of Philosophy in the School of Chemical and Biomedical Engineering (Division of Bioengineering) at Nanyang Technological University, under the supervision of Associate Professor Wang Dong-An. No part of this thesis has been submitted for other degree at other universities and/or institutions. To the author's best knowledge, all the work presented in this thesis is original unless reference is made to other works. Parts of this thesis have been published in the international journals and conferences listed in the List of Publications.

Acknowledgements

I would like to first thank my supervisor, Associate Professor Wang Dong-An for his supervision and guidance throughout my four years of study. Without his support and encouragement, this thesis would not have been completed or written. One simply could not wish for a friendlier supervisor.

I would also like to thank my senior, Ting Ting for her patience in teaching me all the relevant skills to survive in the lab. I am really thankful for your help and all the time spent working and chilling together.

In my daily work I have been blessed with a group of wonderful friends and colleagues: Wenyan, Weiching, Kaiyang, Roger, Derrick, Akhila, Padmaja, Pengfei, Jiayin, Wenzhen, Changjiang, Su Kai, Zhang Feng. I sincerely thank all of you for adding colors to my otherwise dull and boring PhD chapter.

To all the lab technicians from SCBE, thank you for your assistance and support.

To my housemates, Yen Ting and Beng Beng, thank you for lending me your ear and for the comforting words you girls always seem to be able to find.

To my dearest family, daddy, mummy, and brother, thank you for always listening to me, supporting me and encouraging me. Last but not least, Jing, thank you for coming into my life, and I look forward to a brighter future ahead, together.

~Every good and perfect gift is from above -James 1:17~

Table of content

Preface.....	i
Acknowledgements	ii
Table of content	iv
List of Publications	x
List of Figures	xii
List of Tables.....	xxi
List of Abbreviations.....	xxii
Summary	xxiv
1 Introduction.....	1
1.1 Articular cartilage: structure and composition	1
1.2 Cartilage damage: trauma and diseases	3
1.3 Tissue engineering and cartilage	4
1.3.1 Engineered cartilage for tissue restoration and replacement.....	5
1.3.2 Engineered cartilage as biological systems for <i>in vitro</i> studies	9
1.4 Motivation: Towards the generation of a versatile cartilage platform	11
1.5 Hypothesis	12
1.6 Aims and specific aims	12
1.7 Thesis outline.....	13
2 Literature Review.....	16
2.1 Engineered cartilage for tissue regeneration and replacement.....	16
2.1.1 Cartilage injuries and conventional cartilage repair strategies.....	16

2.1.2	Cartilage tissue engineering: scaffold-free strategies.....	20
2.1.2.1	Cell-sheet technology	20
2.1.2.2	Aggregate tissue engineering.....	21
2.1.2.3	Self-assembly method.....	23
2.1.3	An intermediate scaffolding system	24
2.2	Engineered cartilage for <i>in vitro</i> disease modelling: Osteoarthritis (OA)	25
2.2.1	Pathogenesis of OA.....	25
2.2.2	Role of inflammation and macrophages in OA pathology.....	26
2.2.3	Cytokines and OA.....	27
2.2.3.1	Proinflammatory cytokines.....	30
2.2.3.2	Nitric oxide (NO): a catabolic factor.....	31
2.2.3.3	Anti-inflammatory cytokines and cytokine antagonist	32
2.2.4	Cellular responses in OA cartilage.....	32
2.2.4.1	Cell proliferation and apoptosis	32
2.2.4.2	Chondrocyte activation and hypoanabolism	33
2.2.4.3	Synthesis of degradative enzymes.....	34
2.2.4.4	Chondrocyte hypertrophy-like changes in OA.....	35
2.2.4.5	Osteophyte formation.....	36
2.2.5	In vivo and in vitro osteoarthritic models.....	36
2.2.5.1	Animal/ In vivo OA models.....	37
2.2.5.2	In vitro OA models	38
2.3	Engineered cartilage for in vitro disease modelling: Rheumatoid arthritis (RA)	39
2.3.1	Pathogenesis of RA.....	39

2.3.2	Role of synovial fibroblast in RA.....	41
2.3.3	Role of macrophages in RA	44
2.3.4	Mechanisms of cartilage loss in RA.....	45
2.3.5	In vivo and in vitro rheumatoid arthritic models.....	48
2.3.5.1	Animal/in vivo RA models.....	48
2.3.5.2	In vitro RA models.....	49
2.4	Engineered 3D cartilage disease models for the evaluation of anti-arthritic inflammation drug delivery and efficacy in vitro	50
3	Optimization of chondrocyte isolation and phenotype characterization for cartilage tissue engineering.....	54
3.1	Introduction	54
3.2	Materials and Methods.....	56
3.2.1	Chondrocyte isolation and culture	56
3.2.2	Cell harvesting efficiency	56
3.2.3	Cell proliferation assay	57
3.2.4	Gene expression	57
3.2.5	Immuno-histology staining for Collagen type II and F-actin	60
3.2.6	Statistical analysis	60
3.3	Results	60
3.3.1	Cell-harvesting efficiency.....	60
3.3.2	Cell proliferation	62
3.3.3	Gene expression	63
3.3.4	ECM synthesis and cell morphology.....	65

3.4	Discussion	67
3.5	Summary.....	70
4	A preclinical evaluation of an autologous LhCG for articular cartilage repair.	71
4.1	Introduction	71
4.2	Materials and methods.....	73
4.2.1	Experimental design and study population.....	73
4.2.2	Cartilage biopsy harvesting	74
4.2.3	Autologous chondrocytes isolation and <i>in vitro</i> expansion	74
4.2.4	Autologous Living Hyaline Cartilaginous Graft (LhCG) preparation	74
4.2.5	Autologous LhCG engraftment into full-thickness chondral defects	75
4.2.6	Magnetic Resonance Imaging (MRI).....	76
4.2.7	Implanted tissue harvesting	77
4.2.8	Macroscopic examination.....	77
4.2.9	Histological evaluation	77
4.2.10	Immunohistochemical staining	77
4.2.11	Biomechanical Testing and Biochemical analyses	78
4.2.12	Statistical analysis.....	78
4.3	Results	79
4.3.1	Preparation of autologous LhCG for engraftment.....	79
4.3.2	Live MRI monitoring of articular cartilage repair in mini-pigs.....	82
4.3.3	Macroscopic examination.....	84
4.3.4	Histological and immunohistochemical evaluation	86
4.3.5	Biochemical and biomechanical analyses	93

4.4	Discussion	94
4.5	Summary.....	101
5	Development and characterization of engineered cartilage diseased models and drug testing	102
5.1	Introduction	102
5.2	Materials and methods.....	102
5.2.1	Preparation of a 3D-engineered living hyaline cartilage model.....	103
5.2.2	Establishment of an interactive co-culture platform for <i>in vitro</i> OA modeling	104
5.2.3	Establishment of an interactive tri-culture platform for <i>in vitro</i> RA modelling	105
5.2.4	Cell viability and apoptosis assay	106
5.2.5	Gene/protein expression analysis	107
5.2.6	Biochemical analysis.....	114
5.2.7	Histology and immunohistochemistry staining.....	114
5.2.8	Drug testing on the established disease model.....	115
5.2.9	Statistical analysis	115
5.3	A three-dimensionally engineered biomimetic cartilaginous tissue model for osteoarthritic drug evaluation	116
5.3.1	Results.....	117
5.3.1.1	Establishment of an inflammatory environment.....	117
5.3.1.2	Development of a 3D osteoarthritis cartilaginous tissue model.....	118
5.3.1.3	Validation of the established <i>in vitro</i> OA model as an accurate drug-screening model	125

5.3.2	Discussion	127
5.4	Establishment of a 3D <i>in vitro</i> model to investigate the effects of crosstalk between chondrocytes, synovial fibroblasts, and macrophages on the destructive processes in RA.	
	132	
5.4.1	Results.....	135
5.4.1.1	Establishment of a 3D interactive platform to study cartilage destruction in RA	135
5.4.1.2	Characterization of the <i>in vitro</i> models.....	136
5.4.2	Discussion	147
5.5	Summary.....	151
6	Conclusion and Future Work	152
6.1	Conclusions.....	152
6.2	Future work.....	153
	References	158

List of Publications

Journal Publications (accepted):

1. Lau, T.T.*, **Y. Peck***, W. Huang, and D.A. Wang, Optimization of chondrocyte isolation and phenotype characterization for cartilage tissue engineering. *Tissue Eng Part C Methods.*, 2015. 21(2): p. 105-11. doi: 10.1089/ten.TEC.2014.0159. Epub 2014 Oct 15.
2. **Peck, Y.**, L.Y. Ng, J.Y.L. Goh, C. Gao, and D.A. Wang, A three-dimensionally engineered biomimetic cartilaginous tissue model for osteoarthritic drug evaluation. *Molecular Pharmaceutics*, 2014. 11(7): p. 1997-2008.
3. Hou, L., **Y. Peck**, X. Wang, and D. Wang, Surface patterning and modification of polyurethane biomaterials using silsesquioxane-gelatin additives for improved endothelial affinity. *Science China Chemistry*, 2014. 57(4): p. 596-604.
4. **Peck, Y.** and D.A. Wang, Three-dimensionally engineered biomimetic tissue models for in vitro drug evaluation: delivery, efficacy and toxicity. *Expert Opin Drug Deliv.*, 2013. 10(3): p. 369-83. doi: 10.1517/17425247.2013.751096. Epub 2013 Jan 6.

Book Chapter:

1. **Peck, Y.** and D.-A. Wang, *Synovial Mesenchymal Stem Cells and Their Applications in Musculoskeletal Regeneration*, in *Emerging Trends in Cell and Gene Therapy*, M.K. Danquah and R.I. Mahato, Editors. 2013, Humana Press. p. 373-409.

Conferences:

1. **Y.Peck**, D.-A. Wang, An in Vitro 3D Cartilage Graft: Modeling the Inflammatory Environment of Osteoarthritis. International Conference on Materials for Advanced Technologies 2013
2. **Y.Peck**, D.-A. Wang, *Development of an In Vitro 3D Cartilaginous Tissue Model of Inflammatory Cytokine-Induced Osteoarthritis. Bone-tec 2013*
3. **Y.Peck**, D.-A. Wang, A 3D osteoarthritis (OA) model for in vitro drug evaluation. Tissue Engineering and Regenerative Medicine International Society 2014
4. **Y.Peck**, D.-A. Wang, Autologous articular cartilage repair in minipigs with scaffold-free cartilaginous grafts. Tissue Engineering and Regenerative Medicine International Society 2014

Journal Publications (submitted/in preparation):

1. **Y. Peck**, P.F. He, G.S.V.N. Chilla, C.L. Poh, D.-A. Wang, A preclinical evaluation of an autologous living hyaline-like cartilaginous graft for articular cartilage repair, **submitted to: Scientific Reports**
2. **Y. Peck**, D.-A. Wang, Establishment of a 3D in vitro model to investigate the effects of crosstalk between chondrocytes, synovial fibroblasts, and macrophages on the destructive processes in rheumatoid arthritis, **manuscript in preparation.**

List of Figures

Figure 1-1 Applications of LhCG across in vivo and in vitro systems.....	13
Figure 2-1 Differences between a normal and arthritic joints. Adapted from [108].....	26
Figure 2-2 The role of pro-inflammatory cytokines in OA pathogenesis. Briefly, the expression of proinflammatory cytokines such as IL-1 β , TNF and IL-6 increased in an OA joint. These cytokines contribute to disease progression by suppression of matrix synthesis, promotion of matrix degradation and induction of inflammatory responses. All these actions collectively result in OA joint destruction. Adapted from [117].....	31
Figure 2-3 Cascades and players involved in RA. Adapted from [157].....	40
Figure 2-4 Cartilage destruction in RA is mediated by the release of matrix-degrading enzymes such as MMPs and ADAMTS. Cytokines such as IL-1 and IL-17 are responsible for switching the metabolism of chondrocytes from an anabolic to a catabolic state. Additionally, synovial fibroblasts also contribute to the production of matrix-degrading enzymes and invade cartilage when activated by pro-inflammatory cytokines, including TNF and IL-1. Apoptosis of chondrocytes represents another pathway of cartilage destruction; it deprives cartilage of the ability to replace lost matrix components. Adapted from [162].	47
Figure 3-1. Cartilage fragments before and after each round of digestion, showing a progressive decrease in cartilage volume with increasing digestion.	61
Figure 3-2. a) Cell viability of the cells harvested at each round of digestion determined using live/dead reagent staining and subsequently analysed through flow cytometry. b) Table showing the amount of cartilage digested, total cells number and the amount of viable cells harvested at each round of digestion.....	62

Figure 3-3. Cell proliferation assay using WST-1. * indicates $p < 0.05$ while ** indicates $p < 0.01$ when compared to 1 st round of digestion.	63
Figure 3-4. Gel electrophoresis of the chondrocyte markers in cells isolated from various rounds of digestion.	64
Figure 3-5. qPCR analysis of the chondrocyte markers in the cells isolated from different rounds of digestion. * indicates $p < 0.05$ while ** indicates $p < 0.01$ when compared to the 1 st round of digestion at each respective time point.	65
Figure 3-6 a) Immune-staining of collagen type II in various samples groups at Day 4 and Day 7. b) Phalloidin staining of cytoskeleton in cells harvested from different rounds of digestion. Cell nuclei were counter-stained with DAPI.	66
Figure 3-7 Analysis of chondrocytes after 22 hours of single digestion. a) Cartilage fragments after digestion b) Cell viability of the cells harvested by flow cytometry c) Phase contrast image chondrocytes harvested showing presence of cell debris d) Table showing the amount of cartilage digested, total cells number and the amount of viable cells harvested.	67
Figure 4-1 Experimental design (a) A timeline showing the various experimental steps of this study, spanning a total period of 8 months. (b) A schematic diagram showing the detailed process flow of this study. The study began with (i) identification of donor site, followed by (ii) biopsy harvesting from the minor load-bearing femoral trochlear groove regions, and (iii) chondrocytes extraction. After one passage (P1) of <i>in vitro</i> expansion, the chondrocytes were (iv) co-suspended and then (v) co-encapsulated with gelatin microspheres in alginate hydrogel. The gel constructs were cultured at 37°C to dissolve the gelatin microspheres, resulting in micro-cavities formation. The	

resultant (vi) micro-cavitary hydrogel constructs (MCG) promoted vigorous cell proliferation and condensation into isogenic groups, eventually sprouting from the gel phase into the micro-cavities (PTCC)[103, 258]. Upon prolonged culture, each neotissue aggregate that filled the micro-cavities eventually connected to one another, resulting in (vii) a 3D interpenetrating network (IPN). Following alginate gel removal, LhCG was generated in (viii). The LhCGs were then trimmed and implanted (ix) into critical-sized defects on the femoral condyles (indicated as expt. site in (x)). (c) Histological evaluation of the autologous LhCGs, showing the typical rounded morphology of chondrocytes, as well as their GAG and type II collagen-rich ECM (scale bar: 200 μ m)..... 80

Figure 4-2 MRI and clinical evaluation of articular cartilage repair. Proton-density weighted MRI images in the (a) sagittal plane and (b) coronal plane for *LhCG+* and *LhCG-* group at two months (baseline) and six months show progressive tissue repair after *LhCG* engraftment into the full-thickness chondral defects located at the major load-bearing regions of the knee (condyles). Arrows indicate the defect sites. (c) The total average scores were derived from (a) and (b) for both experimental groups obtained using the 2D-MOCART scoring system. A high score (min. 0; max. 100) indicates native-like cartilage tissue. Two-tailed, paired t-test was used for data analysis. Data are presented in the form of mean $\pm$ SD, with N = 5. Abbreviations: *LhCG+*, LhCG-repaired cartilage; *LhCG-*, self-repaired cartilage..... 83

Figure 4-3 Macroscopic evaluation of cartilage repair at 6 months post-engraftment. (a) Representative photographs showing the macroscopic repair outcome. Full-thickness defects in the *LhCG+* group were filled with glossy white tissue while defects in the

LhCG- group remained insufficiently filled. (b) ICRS macroscopic mean scores for the two experimental groups. A high score (min. 0; max. 12) indicates native-like cartilage tissue. (c) The scores for the individual parameters in the ICRS scoring system. Nonparametric Mann Whitney test was used for data analysis. (*) indicates statistical significance difference ($p < 0.05$); (**) ($p < 0.01$). Data are presented in the form of mean $\pm$ SD, with N = 5. 85

Figure 4-4 Histological and immunohistochemical analyses of the repaired tissue in the *LhCG+*, *LhCG-* and *ADS* groups. (a) The average cartilage thickness for each group was determined at the defect regions and their surrounding, unaffected regions. Two-tailed, unpaired t-test was used for data analysis. Evaluation of the repaired cartilage phenotype was based on: (b) H&E; (c) Masson's Trichrome; (d) Safranin-O; (e) immunohistochemical staining for type II collagen; (f) immunohistochemical staining for type I collagen ((A-I) showed higher-magnification images at each specified location). Long scale bar: 2mm; short scale bar: 200 μ m. (g) The tallied histological scores based on the Wakitani scoring system. A low score (min. 0; max. 16) defines a native-like cartilage tissue with good defect filling and good integration with adjacent host cartilage. (h) Total average score for each specific parameter that contributed to cartilage restoration. The *LhCG+* group (Upper panels in (b)-(f)) was repaired with hyaline-like tissue, which integrated well with adjacent host cartilage. The untreated group, *LhCG-* (Middle panels in (b)-(f)) was filled with irregular fibrocartilaginous tissue, which stained positively for type I collagen but not for type II collagen. The defects in the *ADS* (Lower panels in (b)-(f)) group self-repaired and showed no signs of degeneration. (*) indicates statistical significance ($p < 0.05$); (***) ($p < 0.001$). Each set of

colored arrows demarcates individual defect sites. Data are presented in the form of mean $\pm$ SD, with N = 5..... 91

Figure 4-5 Evaluation of tissue ultrastructure via picrosirius polarization method. The orientation and arrangement of collagen fibers/fibrils as observed in the matrix of the (a) native articular cartilage; (b) *LhCG+*; (c) *LhCG-*; (d) *ADS* groups. Generally, green to greenish yellow represents thin or poorly packed collagen fibrils while yellowish-orange through orange to red represents thick or tightly packed collagen fibers. Scale bar: 200 μ m..... 93

Figure 4-6 Quantitative evaluation of articular cartilage repair. Biochemical analysis was performed to quantify the (a) GAG content and (b) total collagen content in all the explanted tissues. To correlate biochemical aspects with the functional aspects of the tissue, (c) water content and (d) compressive moduli were also determined. The results for the three experimental groups, namely *LhCG+*, *LhCG-* and *ADS* group were compared to native, unaffected cartilage. One-way ANOVA / Dunnett's multiple comparison test was used for data analysis. (NS) indicates no statistical significance between two groups while (*) indicates statistical significance ($p < 0.05$); (**) ($p < 0.01$). Data are presented in the form of mean $\pm$ SD, with N = 5..... 94

Figure 5-1 Modelling the inflammatory environment of OA joints via co-culturing of LhCGs with (a) LPS-activated macrophages (*LhCG* + *M Φ ++*); (b) non-activated macrophages (*LhCG* + *M Φ --*); (c) monoculture control group with only LhCGs.105

Figure 5-2 Release of pro-inflammatory cytokines by macrophages. (a) qPCR analysis of four cytokines produced by LPS-activated macrophages as compared to non-activated macrophages; (b) ELISA determination of TNF- α secretion from all experimental

groups. Time 0-- refers to TNF- α concentrations measured before LPS activation of macrophages while time 0 represents the concentrations measured post 6-hr LPS-activation for the LhCG+Mphage++ group. *Indicates $p < 0.05$, statistically significance differences between groups were observed.....118

Figure 5-3 Assessment of increased chondrocyte proliferation. (a) WST-1 cell proliferation assay; (b) Cell density normalized to dry weight of LhCG construct, obtained from DNA quantification using Hoechst 33258 assay. *Significantly different from LhCG control group at day 7 and day 14, respectively ($p < 0.05$). #Significantly different from day 0 LhCG prior to co-culture with macrophages ($p < 0.05$).....119

Figure 5-4 Side-by-side comparison of 'Live' specific staining of chondrocyte and Annexin-V/PI staining for the various groups at Day 7 and Day 14.....120

Figure 5-5 Gene expression levels of important anabolic genes quantified by qPCR. Fold values for each gene were calculated based on their respective expression value in day0-LhCG construct. *Significantly different from LhCG control group at day 7 and day 14, respectively ($p < 0.05$). #Significantly different from day 0 LhCG prior to co-culture with macrophages ($p < 0.05$).....121

Figure 5-6 Gene expression levels of important catabolic genes quantified by qPCR. Fold values for each gene were calculated based on their respective expression value in day0-LhCG construct. *Significantly different from LhCG control group at day 7 and day 14, respectively ($p < 0.05$). #Significantly different from day 0 LhCG prior to co-culture with macrophages ($p < 0.05$).....122

Figure 5-7 Biochemical analyses for GAG and collagen content. (a) GAG produced per cell; (b) Total collagen produced per cell. *Significantly different from LhCG control group

at day 7 and day 14, respectively (p < 0.05). #Significantly different from day 0 LhCG prior to co-culture with macrophages (p < 0.05).	123
Figure 5-8 Histochemical and immunohistochemical staining comparing differences in ECM composition of LhCG in the three different groups. (a) immunohistochemical staining for collagen type I; (b) immunohistochemical staining for collagen type II; and (c) Safranin-O staining for GAG.....	124
Figure 5-9 Assessment of hyperthrophic differentiation of chondrocytes in the three experimental groups via (a) immunohistochemical staining for collagen type X; and (b) qPCR analysis of the gene expression level of collagen type X. *Significantly different from LhCG control group at day 7 and day 14, respectively (p < 0.05). #Significantly different from day 0-LhCG prior to co-culture with macrophages (p < 0.05).	125
Figure 5-10 The effect of celecoxib on (a) cell viability; (b) NO production; (c) PGE2 release; (d) gene expression of (i) MMP-1 and (ii) MMP-3. *Significantly different from the untreated group (LhCG (D)) at day 3 and day 7, respectively (p < 0.05). #Significantly different from the day 0/day 1 untreated group (p < 0.05).	126
Figure 5-11 Experimental setup for the 3D <i>in vitro</i> RA model. (A) The process flow of setting up the <i>in vitro</i> model, starting from a day before tri-culture, Day (-0). (B) The details of the four experimental groups.	136
Figure 5-12 Assessment of cell viability and proliferation. (A) Absorbance values measured from WST-1 cell proliferation assay; (B) Live/Dead staining of cells. * indicates p< 0.05 when compared to the healthy control group, L at each respective time point; # indicates p< 0.05 when LF++ group was compared to the L++ group at each respective	

time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.....137

Figure 5-13 Evaluation of chondrocyte response in terms of their matrix synthesis ability. *

indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.....139

Figure 5-14 Evaluation of chondrocyte response in terms of their production of matrix-

degrading enzymes. * indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.140

Figure 5-15 Evaluation of the inflammatory response of chondrocytes. * indicates $p < 0.05$

when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.....142

Figure 5-16 qPCR analysis of apoptosis-related genes expressed by chondrocytes. *

indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.....143

Figure 5-17 qPCR analysis of synovial fibroblast response. (A) The expression of inflammation-induced genes and (B) matrix-degrading genes. * indicates $p < 0.05$ when compared to the LF group at day 0; # indicates $p < 0.05$ when compared to the LF group at each respective time point.....	144
Figure 5-18 qPCR analysis of macrophage response. (A) Gene expression of important pro-inflammatory cytokines; (B) Gene expression of matrix-degrading enzymes. * indicates $p < 0.05$ when compared to the L++ group at day 0; # indicates $p < 0.05$ when compared to the L++ group at each respective time point.....	145
Figure 5-19 Evaluation of tissue response via biochemical analysis (A) GAG produced per cell; (B) Total collagen produced per cell. * indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.	146
Figure 5-20 Evaluation of ECM degradation via (A) immunostaining of collagen type II and (B) histological staining of GAG. For immunostaining, cell nuclei were counter-stained with DAPI.....	146

List of Tables

Table 1-1 Comparison of various cell sources for cartilage tissue engineering. Adapted from [18].	8
Table 2-1 Cartilage repair strategies. Modified from Williams et al. [54]	16
Table 2-2 Important cytokines and their role in the pathophysiology of OA.	29
Table 3-1 Primers used in polymerase chain reaction (PCR). A.T and P.S stand for annealing temperature and product size respectively.	58
Table 3-2 Primers used in quantitative polymerase chain reaction (qPCR). A.T. and P.S stand for annealing temperature, product size respectively.	59
Table 4-1 Abbreviations for the three experimental groups.	76
Table 4-2 Statistical analyses performed on the different data sets obtained in this study.	78
Table 4-3 2D MOCART evaluation of cartilage repair. Assessment of articular cartilage repair tissue in the femoral condyles by using the modified clinical 2D magnetic resonance observation of cartilage repair tissue (MOCART) score [253, 254].	83
Table 4-4 ICRS Score. Assessment of macroscopic repair by using the International Cartilage Repair Society (ICRS) score for cartilage repair [255]	86
Table 4-5 Wakitani score. Histological evaluation of the cartilage repair tissue using a scoring system developed by Wakitani et al. [256].	91
Table 5-1 qRT-PCR primer sequences for porcine, murine and human gene markers: forward (F) and reverse (R).	108
Table 5-2 The direct effects of celecoxib on OA cartilage/chondrocytes.	131

List of Abbreviations

ACL	Anterior cruciate ligament
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
BMP	Bone morphogenetic protein
COMP	Cartilage oligomeric matrix protein
ECM	Extracellular matrix
GAG	Glucoaminoglycan
IFN-α	Interferon-alpha
IKBKB	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1	Interleukin-1
iNOS	Inducible nitric oxide synthase
LhCG	Living hyaline cartilage graft
LhCG+Mϕ--	LhCG co-cultured with non-activated macrophages
LhCG+Mϕ++	LhCG co-cultured with LPS-activated macrophages
LIF	Leukocyte inhibitory factor
LPS	Lipopolysaccharide
MIA	Monosodium iodoacetate
MMP	Matrix metalloproteinase
NO	Nitric oxide
OA	Osteoarthritis
PGA	Polyglycolic acid

PGE2	Prostaglandin-E2
PGRN	Prostaglandin
PI	Propidium iodide
PLA	Polylactic acid
PLGA	Poly (lactic-co-glycolic acid)
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear neutrophils
RA	Rheumatoid arthritis
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor

Summary

The articular cartilage is a highly organized tissue with significant load-bearing and low friction capabilities but lacks intrinsic healing abilities. Despite the advancements in surgical techniques for cartilage repair, patients would benefit most from regenerative techniques aimed at restoring articular cartilage defects that result from physical trauma or degenerative joint diseases. The living hyaline cartilaginous graft (LhCG) in this thesis exhibits a pure hyaline cartilage phenotype, with abundant collagen type II and GAG while the presence of collagen type I is negligible. Having biological properties similar to native articular cartilage, the LhCG platform potentially holds great promise to regenerate cartilage with a similar structure to native cartilage, possibly preventing and/or delaying the onset of downstream issues like osteoarthritis (OA). LhCG fabrication demands both a sufficient number of chondrocytes isolated from cartilage tissues and a high success rate of the primary culture. Therefore, to enhance its practicality for potential clinical use, **Chapter 3** was dedicated to improve the efficiency of primary chondrocyte isolation by developing an improved isolation protocol, based on multiple enzymatic digestion using collagenase II. The results showed that this multiple digestion method yielded a 5-fold increase in the total number of cells as compared to any of the common isolation protocols. More importantly, a high percentage of the isolated cells remained viable and maintained chondrocyte phenotype and functionality. This improved isolation protocol was later employed in the following chapter, where chondrocytes were isolated from limited biopsied cartilage tissue samples.

As mentioned, LhCG has the potential to recreate a structure that resembles native cartilage. A preclinical study was conducted using autologous LhCG for treating cartilage lesions. Implantation of autologous LhCG was done at load-bearing regions of the knees in

skeletally mature mini-pigs for 6 months. Over the course of the study, significant radiographical improvements in LhCG treated sites were observed via magnetic resonance imaging. Furthermore, macroscopic repair was evident by LhCG at endpoint. Microscopic inspection revealed that LhCG engraftment restored cartilage thickness, promoted integration with surrounding native cartilage, produced abundant cartilage-specific matrix molecules, and re-established an intact superficial tangential zone. Importantly, the repair efficacy of LhCG was quantitatively shown to be comparable to native, unaffected cartilage in terms of biochemical composition and biomechanical properties. There were no complications related to the donor site of cartilage biopsy. Collectively, these results implied that LhCG engraftment might be viable for articular cartilage repair.

Besides being highly susceptible to traumatic injuries, articular cartilage is also affected by various diseases. OA and rheumatoid arthritis (RA) are the two most common forms of diseases affecting the joint and both conditions lead to cartilage destruction. Due to the lack of a cure for OA or RA, tremendous efforts have been put into understanding the pathogenesis and pathomechanisms. With the advent of tissue engineering, combined with the principle of 3Rs (Replacement, Reduction and Refinement) for animal studies, a new research area that focuses on the development of *in vitro* disease models has emerged. *In vitro* models of OA and RA were successfully established by varying the cell types that interact with the LhCG. More precisely, the OA *in vitro* model was established via a co-culture system of LhCG and lipopolysaccharide (LPS)-activated macrophages while the RA *in vitro* model was established by a tri-culture system comprised of LhCG, activated synovial fibroblasts and macrophages. Results indicated that both OA and RA models were able to capture important hallmarks of each disease. Cellular responses observed in both models corresponded to those observed in diseased cartilage.

Furthermore, extensive degradation of extracellular matrix was also observed in both models. Taken together, the results suggested that both models was able to sufficiently mimic the native, diseased OA/RA cartilage, while drug-testing results indicated the potential of the model to be used for testing/screening of new therapeutic strategies.

In summary, the versatility of the LhCG platform has been illustrated by the work of this thesis. While this platform is able to fulfil the conventional role of cartilage tissue engineering, which is to replace and to restore damaged tissue, it is also useful as an in vitro platform for modelling the various cartilage diseases.

1 Introduction

Joint pain has been recognized as a major cause of disability among the middle-aged and older people. It is usually related to articular cartilage degeneration due to primary osteoarthritis (OA) or physical trauma. It has been reported that 12.4 million of adults age 65 and older in the U.S. are affected by OA. In addition, cartilage injury from sports is very common. Given the debilitating nature of severe joint pain, many scientists and surgeons have focused on repairing and regenerating damaged cartilage. Despite decades of tremendous effort, cartilage remains as a particularly challenging tissue to engineer owing to its complex properties and its load-bearing function.

Hyaline cartilage, which makes up the articular cartilage in the joints, is a highly specialized tissue with low frictional coefficient and a high load-bearing capacity – two qualities that distinguish it from other types of cartilage. Although hyaline cartilage may appear as a simple tissue with only a single cell type and an avascular matrix, it possess unique properties such as resistance to compression and the ability to distribute loads, both of which are difficult to be completely replaced by any other tissues or devices designed to date.

1.1 Articular cartilage: structure and composition

The structure and composition of the articular cartilage are unique and have a direct role in its function as a load-bearing surface. It consists of mainly extracellular matrix (ECM) with a sparse population of cells known as chondrocytes. The ECM of hyaline cartilage is composed largely of water (70-80%) along with some other solid components like collagens (50-75%), glucoaminoglycans (GAG) (15-30%), and other noncollagenous proteins and glycoproteins present in lesser amounts [1, 2].

Collagens represent the major ECM protein and are uniformly distributed throughout the depth of the cartilage, with an exception in the collagen-rich superficial zone. They form a complex fibrillar meshwork that provides tensile stiffness and strength of hyaline cartilage. Collagen type II is the most abundant type found in this tissue, with collagen types I, IV, V, VI, IX, and XI also present in minor proportions. Besides collagens, another important structural component of this tissue is proteoglycan. The monomer of this macromolecule consists of a core protein joined by GAGs. The two major classes of proteoglycans found in the hyaline cartilage are the large aggregating molecules or aggrecans, and the smaller proteoglycans (including decorin, biglycans, and fibromodulin). Since proteoglycans are hydrophilic, they help to retain water as they become mechanically entrapped within the collagenous meshwork, providing articular cartilage with its compressive strength. In addition to the various ECM components, chondrocytes, which are the main resident cells in this tissue, play an equally important role in conserving the structural integrity of the articular cartilage. They act as the metabolic unit of cartilage, both synthesizing and regulating the balance between the many ECM components.

Articular cartilage is divided into four different zones, namely the superficial zone, the middle zone, the deep zone and the calcified zone. The distribution of cells and ECM components is non-homogeneous in zones of mature articular cartilage. Each zone plays a different role in contributing to the functional properties of articular cartilage. In summary, the articular cartilage has an elaborate, highly organized structure and the complex interactions between chondrocytes and the matrix help to maintain this specialized tissue [1, 2].

1.2 Cartilage damage: trauma and diseases

Focal cartilage lesions of the knee are common because of the load-bearing function of articular cartilage. Unlike other tissues, cartilage has limited regenerative capacity due to its avascular, aneural and alymphatic nature. Consequently, cartilage lesions are difficult to heal [3]. Cartilage injury can be classified into three main types, namely matrix disruption, partial thickness defects, and full thickness defects[4]. Matrix disruption is a result of blunt trauma and it involves slight ECM damage. This type of injury can usually heal by itself, where the remaining viable chondrocytes will increase their synthetic activity to replace lost ECM components. Partial thickness defects involves damage to the articular surface, but this type of defect does not extend to the subchondral bone. An injury to the cartilage surface will elicit an immediate response from adjacent chondrocytes to proliferate, but for unknown reasons, this attempt to repair lost cartilage ceases before the defect is healed. Full-thickness defects span the whole cartilage thickness and penetrate the subchondral bone. For this injury type, defects are initially filled with fibrin clot, and will eventually be replaced by tissue intermediate between hyaline and fibrocartilage. This tissue has weaker mechanical properties and higher permeability as compared to native cartilage, which contribute to its eventual degradation over time[5].

Besides being susceptible to traumatic injures, articular cartilage is also known to be affected by several diseases. Generally, articular disease can be broadly categorized into noninflammatory and inflammatory[6]. OA is a degenerative joint disease that is placed under the noninflammatory category although an intermittent inflammatory component is recognized. Some other conditions that have been included in this same category are traumatic arthritis, mechanical abnormalities such as torn menisci, aseptic necrosis, neuroarthropathy, and neoplasia. On the other hand, the inflammatory category is further divided into an infectious

section and a non-infectious section. The former comprises of articular disease caused by bacterial, fungal, viral, and protozoal infection while the latter can be either immunologic or non-immunologic. Some common examples for immune-mediated articular diseases are rheumatoid arthritis and systemic lupus erythematosus. Non-immune inflammatory conditions include crystal-induced arthritis (such as gout and pseudogout), and chronic hemarthrosis.[6]

1.3 Tissue engineering and cartilage

Currently, focal cartilage injuries in human patients are treated with several surgical techniques, such as abrasion arthroplasty with or without microfracture of the subchondral bone, osteochondral autograft/allograft transplantation, and autologous chondrocyte or mesenchymal stem cells (MSC) implantation[7]. As for patients with end-stage joint degeneration, a procedure known as total joint replacement will be performed, where the degenerated joint is removed and replaced with inert implants. Although these treatment modalities provide effective symptomatic relief, they are not long-term clinical solutions. For younger patients, this current state-of-the-art may translate into two or more revision surgeries during their lifetime. In order to develop a more permanent solution, many scientists and surgeons have turned their attention to finding a biological solution to repair damaged cartilage that can potentially produce a durable repair with long-term pain relief.[8]

Tissue engineering is a multidisciplinary field aimed at creating biological substitutes to replace, repair, and regenerate defective tissues. It has the potential to provide permanent solutions to tissue damage and tissue loss for countless of people each year[9]. The basic approach to tissue engineering involves the use of cells, scaffolds, and growth-stimulating

signals, either independently or in various combinations. Generating engineered cartilage is no exception to this approach. In past decades, the field of cartilage tissue engineering has rapidly evolved to encompass the use of a variety of cell sources (e.g., chondrocytes and stem cells), scaffolds (e.g., biodegradable, natural or synthetic materials, polymers, and nanocomposites), and bioactive signals (e.g., various growth factors and cytokines, mechanical and electrical stimuli, etc.) to engineer biomimetic tissues[10]. These tissue-engineered constructs can be used as cartilage substitutes, and also as platforms for studying various cartilage-related pathologies and drug effects.

1.3.1 Engineered cartilage for tissue restoration and replacement

Current clinical methods for cartilage repair, while having sufficient reparative potential, are still limited in their ability to regenerate functional cartilage in terms of biochemical composition and biomechanical properties. Due to these limitations, research in recent years has focused on tissue engineering solutions. The primary aim of cartilage tissue engineering research is to create functional cartilage tissue constructs that can be used to repair and restore both the structural and functional properties of an injured or degenerated cartilage. The most important parameters for cartilage tissue engineering are cell source, signalling molecules, scaffolds.

The ideal cell source for cartilage tissue engineering is one that can be easily extracted and expanded, and which produces abundant cartilage-specific matrix components like collagen type II and GAG. Chondrocytes and stem cells represent two most investigated cell sources in cartilage tissue engineering. Autologous chondrocytes are the first cell source to be clinically used for cartilage repair. They have the advantages of being able to produce, maintain and remodel the cartilage ECM *in vitro*[11]. However, their usage is often limited by donor tissue scarcity and also the inevitable dedifferentiation of chondrocytes during monolayer culture

expansion[12, 13]. To overcome the limited supply of primary chondrocytes, many researchers have started using multipotent stem cells (MSCs) derived from many tissues in the body, such as the bone marrow, adipose tissue, synovium, and umbilical cord[14-17]. MSCs are highly proliferative, and if given the correct set of conditions (combinations of growth factors such as bone morphogenic protein (BMP) and TGF- β family), are able to differentiate into chondrocytic cells [18]. Recently, pluripotent stem cells have emerged as an attractive cell source for repairing large chondral defects due to their potential of indefinite self- renewal and chondrogenic differentiation. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are the two cell types that fall into this category. A major challenge in employing pluripotent stem cells for tissue engineering is their tumorigenic potential and long-term functional stability. A detailed comparison of the various sources of cells used for cartilage tissue engineering is shown in **Table 1-1**.

Besides cells, signalling molecules also play an important role in determining the success of engineering a functional cartilage tissue. These molecules (several cytokines, hormones and growth factors) have known effect on the anabolic and catabolic activities of chondrocytes. Many in vitro studies, especially those that employ MSCs in their research have utilized growth factors like transforming growth factor (TGF- β), insulin-like growth factor (IGF-1), bone morphogenetic proteins (BMPs), and to a lesser extent fibroblast growth factors (FGFs) and epidermal growth factor (EGF) to promote chondrogenesis of MSCs and to stimulate ECM production. These growth factors are used individually or in combination to increase their impact on chondrocytes or MSCs.

Scaffolds represent another important factor for cartilage tissue engineering. The purpose of a scaffold is to provide a suitable microenvironment for the cells to reside, proliferate and

produce cartilage matrix. Additionally, it must also be able to substitute the function of the native matrix until new cartilage is formed. To meet all these requirements, a scaffold for cartilage regeneration must be biocompatible, biodegradable and possess a 3D structure with high porosity and pore-to-pore interconnectivity[11]. To date, a wide variety of natural and synthetic polymers have been used to fabricate scaffolds for cartilage repair. Silk, collagen, fibrin, agarose, alginate, hyaluronan, and chitosan are popular choices of natural polymers[19]. Many of these are hydrogels, which are water-swollen networks covalently or physically cross-linked. Hydrogels are especially attractive as they can be non-invasively injected, fill irregularly shaped defects, and can homogeneously suspend cells within a 3D environment[10]. Finally, the use of natural polymers is believed to elicit favourable biological responses, such as cell-responsive degradation and ECM re-modelling. These are important regulating factors for cartilage tissue regeneration. As for synthetic polymers, the most commonly explored ones are the poly- α -hydroxy esters, particularly polylactic acid (PLA) and polyglycolic acid (PGA), because of their biodegradability and clearance from US Food and Drug Administration (FDA) for clinical use[20]. Scaffolds made from these materials have high flexibility in terms of customizing their physical, mechanical and chemical properties. Also, they have better mechanical strength as compared to hydrogels[21]. However, the main limitation of these synthetic polymer-based scaffolds is that cells often do not retain their chondrocytic phenotype, thereby affecting the quality of the engineered tissue[22].

Although the majority of cartilage tissue engineering studies involves the use of a scaffold material for 3D attachment of cells or shaping of constructs, there is an innovative approach to generate tissue-engineered constructs without a scaffold[23]. This scaffold-free approach makes use of the natural extracellular material synthesized by the cells to provide

structural integrity and is done by seeding cells at high densities such that cells are layered on top of each other[24, 25]. This way of generating a cartilage construct is believed to mimic the developmental processes of native cartilage more closely, where a high cell density state in the developing anlagen precedes matrix production[26]. There are some other advantages of using a scaffold-free approach: 1) minimizes phenotypic alteration of cells; 2) prevent stress-shielding; 3) allows neotissue organization; 4) avoids degradation product toxicity[27].

Despite the many promising advances in this field, the field has yet to engineer replacements with functional properties that are comparable to native cartilage, particularly when engineered constructs are evaluated in relevant large animal models. Some of the challenges being reported are difficulties in maintaining hyaline phenotype post-implantation, unsatisfactory host-implant integration, inferior biochemical and biomechanical properties as compared to native, healthy cartilage. This presents a need for the development of an appropriate engineered cartilage construct that is able to address to these challenges.

Table 1-1 Comparison of various cell sources for cartilage tissue engineering. Adapted from [18].

	Cell types	Advantages	Disadvantages	References
Differentiated cells	Adult chondrocytes	<ul style="list-style-type: none"> • Autologous • Cartilage tissue specific 	<ul style="list-style-type: none"> • Limited availability • Invasive harvest procedure • Donor site morbidity • Dedifferentiate in vitro 	[28]
	Neonatal chondrocytes	<ul style="list-style-type: none"> • High proliferation rate • High collagen II gene 	<ul style="list-style-type: none"> • Limited availability 	[28]

		<ul style="list-style-type: none"> • expression and GAG production • Non-immunogenic 		
	Fetal chondrocytes	<ul style="list-style-type: none"> • High potential for cartilage synthesis 	<ul style="list-style-type: none"> • Limited availability 	[14]
Multipotent stem cells	Synovial-derived stem cells	<ul style="list-style-type: none"> • Autologous 	<ul style="list-style-type: none"> • Retain some fibroblastic characteristic after induction 	[16, 29]
	Adipose-derived stem cells	<ul style="list-style-type: none"> • Autologous • Minimally invasive harvest • Abundance 	<ul style="list-style-type: none"> • May be less robust in chondrogenesis compared with BMSCs • High total collagen synthesis but low of collagen type II 	[14, 15]
	Bone marrow-derived stem cells	<ul style="list-style-type: none"> • Autologous • High collagen II 	<ul style="list-style-type: none"> • Hypertrophic phenotype 	[15, 17]
Pluripotent stem cells	Embryonic stem cells	<ul style="list-style-type: none"> • Unlimited self-renewal 	<ul style="list-style-type: none"> • Ethical controversy • Potential tumorigenicity 	[30, 31]
	Induced pluripotent stem cells	<ul style="list-style-type: none"> • No ethical controversy • Patient-specific • Unlimited self-renewal 	<ul style="list-style-type: none"> • Safety concerns 	[32, 33]

1.3.2 Engineered cartilage as biological systems for *in vitro* studies

Although the mainstream applications of tissue engineering still focus on repairing or replacing diseased tissues or organs, namely for the purpose of regenerative medicine, another potential

use of these engineered tissues as biological systems for *in vitro* studies have been increasingly recognized[34-36]. Very often, the use of animal models and humans in research is limited by high cost, availability of test subjects, feasibility of testing protocols, and ethical issues regarding discomfort or distress caused to living subjects. Furthermore, data derived from animal studies might not reflect the actual clinical efficacy of therapeutics for some of the human tissue types. Although cadaveric tissues can qualify as an accurate test model, they are still limited by the scarcity of donor source and difficulties in preserving the viability of excised tissues. Additionally, the outcome of experiments may be affected by donor-specific factors associated with cadaveric tissue, for instance, disease or genetic variations. There is thus an obvious need for a more standardized platform for *in vitro* studies, especially for the study of human disease and drugs.

In light of these limitations, many researchers are motivated to establish *in vitro* models for the study of disease mechanisms and drug testing. The availability of accurate *in vitro* models is valuable as they allow cellular/tissue responses and structural changes of cartilage to be investigated in a systematic, repetitive and reproducible manner. These models are usually less costly and less time consuming as compared to animal models. Furthermore, they can be adapted for high throughput and low-cost preliminary screening of potential therapeutic agents to treat or prevent the development of different types of joint diseases such as OA or RA[37]. Such high-throughput testing is often not feasible with animal models.

Conventionally, many *in vitro* studies rely heavily on the use of monolayer-cultured cells[38, 39]. Although the two-dimensional (2D) model has contributed greatly to the basic understanding of cell biology, it has certain intrinsic limitations that have prompted the development of three-dimensional (3D) models[40]. An obvious advantage of the 3D models is

that they provide a cellular microenvironment that better recapitulates the microenvironment of native tissues[41]. This is especially important for drug testing since environmental cues are known to elicit behavioural and functional change of cells, which may in turn affect cellular responses to drugs. Besides being a more accurate drug-testing model, 3D models also represent a more suitable model for studying disease mechanism. This can be attributed to cellular organization, composition and structure of the surrounding ECM, which recapitulate *in vivo* situations.

On this note, some research groups have investigated the use of cartilage explants (mostly humans) for studies [42, 43]. However, this method is limited by the fact that most explants obtained from joint replacement surgery are of poor quality. Furthermore, they also show a high heterogeneity in terms of the degree of cartilage erosions, thus standardization for *in vitro* models is inherently difficult.[44] Additionally, there are also other studies that utilize biomaterials like the hydrogels or similar constraining materials for the construction of 3D *in vitro* cartilage models[45-50]. Although they have reported that chondrocytes are able to retain their morphological and functional behaviors in these models, the accuracy of these systems are still questionable as they involve biomaterials that are not found in the *in vivo* environment.

1.4 Motivation: Towards the generation of a versatile cartilage platform

To the author's knowledge, there exists no single platform that serves the two facets of tissue engineering: cartilage regeneration and *in vitro* models for disease modelling and drug testing. Therefore, this thesis is motivated to develop a versatile cartilage platform that may be used for these purposes. This may be achieved via the Living hyaline Cartilaginous Graft (LhCG), which is a scaffold-free construct that recapitulates the pure hyaline phenotype of articular cartilage (a

more detailed description of this platform can be found in Chapter 2, Section 2.1.3). In close relation to this versatile cartilage platform, efficient cell chondrocyte isolation is a particularly important step in its manufacture. As such, in order to enhance the practicality and attractiveness of the LhCG platform, there is a need to improve on the current cell chondrocyte isolation protocol.

1.5 Hypothesis

Hypothesis 1: Chondrocyte yield, viability and functionalities can be improved by multiple short digestion periods with fresh collagenase solution.

Hypothesis 2: Given its high purity, LhCG is hypothesized to be able produce reparative tissue that is biologically comparable to native hyaline cartilage and its sponge-like properties will enable *in situ* remodeling and maturation, resulting in good integration with surrounding native cartilage.

Hypothesis 3: LhCG platform can be used to model cartilage-related diseases through co-culture with relevant cell populations that are present in a diseased state.

Hypothesis 4: LhCG-based disease models can be employed as a test system to investigate the potential anti-arthritic and/or anti-osteoarthritic effects of drugs.

1.6 Aims and specific aims

This thesis aims to improve and evaluate the LhCG platform for *in vivo* and *in vitro* systems as illustrated in **Figure 1-1**. More specifically:

Specific aim 1: Develop a multiple digestion protocol for more efficient primary chondrocyte isolation from articular cartilage tissue, focusing on maximizing cell yield while maintaining quality (in terms of cell viability and functionalities) of the isolated cells.

Specific aim 2: Perform LhCG engraftment in the load-bearing regions of a skeletally mature porcine knee to investigate its efficacy in repairing full-thickness chondral defects.

Specific aim 3: Develop *in vitro* models that reflect the complex process of early-stage *in vivo* cartilage destruction in osteoarthritis (OA) and rheumatoid arthritis (RA) by simulating the inflammatory environment of OA/RA joints via interactive cell-culture systems.

Specific aim 4: Perform drug testing using commercially available osteoarthritis drugs on the newly established model to confirm its fidelity in mimicking *in vivo* responses to drugs.

Figure 1-1 Applications of LhCG across in vivo and in vitro systems.

1.7 Thesis outline

This thesis has been organized into the following six chapters:

Chapter 1, which is the current chapter, describes the unique and complex structure of articular cartilage that allows this tissue to fulfil its functional role but at the same time causing it to be highly susceptible to injuries. A high prevalence of cartilage injury necessitates cartilage tissue engineering that serves two purposes: 1) to restore and replace damaged cartilage; 2) to be used as biological systems for *in vitro* studies. Additionally, this chapter also defines the hypotheses and aims of this thesis.

Chapter 2 details the two facets of cartilage tissue engineering. This chapter begins with a review on the current cartilage repair strategies, focusing on their advantages and disadvantages, followed by the commonly used scaffold-free approaches in cartilage tissue engineering. The innovative approach using an intermediate scaffolding system, which was adopted in the work of this thesis, is also described. In the later part of this chapter, pathogenesis of OA and RA, as well as the various cytokines and cellular players involved in the pathophysiology of these diseases are reviewed.

Chapter 3 focuses on the development of a protocol for more efficient primary chondrocyte isolation from articular cartilage tissue, which would allow more efficient LhCG fabrication. The multiple digestion method described in this chapter is able to enhance chondrocyte yield while maintaining quality of the isolated cells in terms of cell viability and chondrocytic phenotype.

Chapter 4 illustrates the use of LhCG for *in vivo* cartilage repair and regeneration. In this chapter, the efficacy of an autologous LhCG in repairing full-thickness chondral knee defects in skeletally mature mini-pigs was demonstrated by the results of a 6-month preclinical study,

whereby cartilage resurfacing with hyaline-like tissue and good host-implant integration were detected.

Chapter 5 demonstrates the use of LhCG for *in vitro* cartilage disease modelling and its application as a drug-testing platform. In this chapter, focus is placed on developing the LhCG platform as *an in vitro* platform that serves two functions: (1) reflect early stages of *in vivo* cartilage destruction in cartilage-related diseases such as OA and RA; (2) allows drug testing using commercially available drugs.

Chapter 6 concludes this thesis and presents recommendations for future work.

2 Literature Review

2.1 Engineered cartilage for tissue regeneration and replacement

2.1.1 Cartilage injuries and conventional cartilage repair strategies

Owing the load-bearing function of articular cartilage, it is highly susceptible to injuries. Unfortunately, the inability of articular cartilage to repair the initial focal cartilage damage leads to abnormal compressive loading, which in turn causes increased mechanical stress in the adjacent healthy cartilage. This will progressively expand the area of cartilage damage and eventually lead to osteoarthritis.[51] During the late stages of the disease, the subchondral bone is exposed due to the destruction of articular cartilage, causing debilitating joint pain and severely reduced joint mobility[52]. Because of the potential of traumatic joint injuries to lead to early onset OA of the joint over time, and the serious clinical consequences of end-stage OA, many efforts have been put in to develop better quality repair strategies for articular cartilage repair[19].

At present, there is no conclusive evidence in the literature to show that any of the current cartilage repair strategies can effectively prevent or delay the initiation of OA progression[53]. All of them are however, effective in providing symptomatic relief.

Table 2-1 Cartilage repair strategies. Modified from Williams et al. [54]

Approach	Treatment	Repair tissue	Fill	Known durability	Grade and size of lesion
Palliative	<ul style="list-style-type: none">Arthroscopic debridement	None	None	< 2 yr	III or IV (0.5-2 cm ²)

Intrinsic repair enhancement	<ul style="list-style-type: none"> • Microfracture • Drilling • Abrasion arthroplasty 	Fibrocartilage	Partial	2–6 yr	III or IV (0.5–2 cm ²)
Whole tissue transplantation	<ul style="list-style-type: none"> • Mosaicplasty • Osteochondral autograft plug 	Hyaline Cartilage	Near total	2–10 yr 5–20 yr	Focal, traumatic lesions (1–4 cm ²)
Cell-based	<ul style="list-style-type: none"> • ACI • MACI • Hyalograft C 	Hyaline-like	Near total	2–4 yr 2 yr 3 yr	Large, uncontained lesions (2–10 cm ²)

These repair strategies include arthroscopic lavage and debridement (palliative), bone marrow stimulation techniques (intrinsic repair enhancement), cartilage tissue-based therapy (whole tissue transplantation) and cell-based therapy[55, 56] (**Table 2-1**). Arthroscopic lavage and debridement are usually used to alleviate joint pain. Lavage involves rinsing of the joint during arthroscopy, thereby removing debris that may provide pain relief. Debridement is a term used to describe the excision of damaged cartilage from the joint.[57] While these techniques are effective in relieving pain, they do not induce actual articular cartilage repair. It has been suggested that pain relief experienced by the patients following these procedures may be no more than a placebo effect[58].

Bone stimulation techniques make use of the intrinsic healing response, observed upon penetration of the subchondral bone in osteochondral defects[55]. These techniques include abrasion arthroplasty, Pridie drilling[59] and microfracture[60]. Among these, microfracture is the most favoured method of treatment since it is an improved version of the other two techniques. Microfracture is a surgical procedure that involves debridement of damaged articular cartilage down to the underlying subchondral bone-plate, followed by creation of multiple holes with a sharp awl on the subchondral bone[61]. This will result in the formation of blood clots and stimulate cartilage repair by cells from the bone marrow.[62] The resultant repair tissue is primarily fibrocartilage that contains more type I collagen than type II collagen and has inferior biomechanical properties to articular cartilage. Some clinical studies have reported improvement in knee function in 70-90% of patients after a year post-treatment[60, 63-65]. However, many studies have also reported that effect of microfracture repair is not long lasting[66]. Most patients showed clinical improvement for up to five years following a microfracture procedure but rapidly decline thereafter[67, 68].

In order to produce cartilage repair with hyaline phenotype, some other repair techniques such as cartilage tissue-based therapy and cell-based therapy have been explored. Mosaicplasty or osteochondral autograft transplantation is a tissue-based repair technique[69]. It involves the use of autogenic and allogeneic cartilage tissue to treat osteochondral defects. Unlike microfracture that is performed via arthroscopy, this technique requires an open surgery. During the surgery, cylindrical osteochondral plugs will be harvested from the periphery of both femoral condyles, or so called from the non-weight bearing regions. Then, these plugs will then be placed into an osteochondral defect in a mosaic fashion.[70] While there are studies that showed decreased pain and improved joint function for small and medium sized full-thickness

defects[71], some studies observed rapid deterioration for very small defects[72]. This technique is primarily limited by donor site morbidity, availability of donor tissue source, poor lateral tissue integration with surrounding native tissue, and difficulty in recreating the smooth articular surface of the knee joint when fitting the plugs into the defect[53, 55].

The concept of cell-based therapies for cartilage restoration is not unfamiliar. Autologous chondrocyte implantation (ACI) has been used in the clinic for almost thirty years to repair articular cartilage defects[73]. This approach involves three main steps: harvesting of small cartilage biopsies via a minimally invasive procedure from the patient, extracting and expanding chondrocytes in vitro, and implanting the cells to the debrided cartilage defect and covered by a membrane to generate new cartilage tissue. The two major benefits of this approach are potential immune complications from transplanting allogeneic cells or foreign materials can be avoided since patient's own cells are used, and as opposed to autologous osteochondral implantation, the small biopsy minimizes donor site morbidity. As with all treatment procedures, there are challenges associated with ACI. For example, limited donor tissue source, inevitable dedifferentiation of the monolayer expanded cells, graft hypertrophy, and surgical complexity. [74]

Based on a few recent reviews on current treatments for focal chondral defects, they identified that a 'gold standard' for the repair of chondral injuries is still lacking[2, 75, 76]; all existing surgical interventions produce inconsistent repair quality. This lack of effective treatment modalities has prompted research on tissue engineering since it forms the basis for next generation of cartilage regeneration and repair technology. By combining a variety of cell sources, scaffold materials and bioactive factors, a host of tissue-engineered cartilage constructs were generated to aid cartilage repair.[77-79] Despite the extensive efforts, significant challenges

remain with regards to generation of a durable hyaline or hyaline-like cartilage *in vivo*. Inevitable phenotypic alterations of cells during culture-expansion and the influence of scaffold materials on cell fate are some causes for the compromised tissue quality[80].

2.1.2 Cartilage tissue engineering: scaffold-free strategies

Through the conventional tissue engineering triad of cells, signals, and scaffolds, the field of biomedical engineering has made good progress toward addressing clinical needs [4, 11]. More recently, a new paradigm consists of only cells and signals emerged as a suitable modality to generate functional engineered tissues. This includes any platform that does not employ an exogenous, 3D material for engineering a tissue and is referred to as a scaffold-free approach. Focusing on cartilage, the techniques that are classified as scaffold-free approaches include cell-sheet engineering, aggregate engineering and the self-assembling process[80]. These approaches aim to produce tissues by mimicking the developmental process of cartilage, which usually follow a pattern of cell condensation, cell proliferation, cell differentiation, ECM production, and tissue maturation[27].

2.1.2.1 Cell-sheet technology

Cell sheet engineering was first reported by Okano et al. in 1993 and has since been widely adopted to engineer many tissue types in the regenerative medicine field[81]. To fabricate a cell sheet, cells are first expanded extensively in monolayer cultures until sufficient ECM is synthesized to maintain a cohesive layer of cells. After which, the entire cell sheet is lifted from the culture surface using temperature-responsive substrates. The retrieved cell sheets were subsequently processed into desired shape and sizes by rolling, layering, or draping over molds. Then, the sheets undergo tissue fusion, mimicking a common process in developmental biology.

During tissue fusion, isolated cell populations interact and fuse via cell-to-cell contact, cell-to-matrix contact, matrix-to-matrix contact, and also the remodelling of ECM to form continuous tissues. [82, 83]

For musculoskeletal cartilages, this cell-sheet technology has produced neotissues with clinically relevant dimensions and properties. For example, this technique has been adopted for the development of RevaFlex (formerly DeNovo ET) (ISTO Technologies, Missouri, USA), which is an engineered cartilage implant for cartilage repair and restoration. This implant is formed using sheet of expanded juvenile allogeneic chondrocytes and it is currently in Phase III clinical trials.[84] In a recent study, layered chondrocyte sheet has also been used to repair full-thickness cartilage defects in a porcine model. Results showed that the articular surface was not fully restored. However, as compared to the empty defect controls, the layered chondrocyte sheet filled up the defects adequately and it integrated with the surrounding native tissue. [85]

As with all techniques, there are some limitations associated with cell sheet engineering. Despite being able to produce multi-layered tissue, this technique is plagued by cell dedifferentiation problem since it is being constructed from monolayer-cultured chondrocytes. Chondrocytes are known to dedifferentiate during monolayer expansion, a process where they change from their rounded morphology to a spindle-shaped, fibroblastic phenotype and lose their capacity to synthesize articular cartilage-specific ECM components (type II collagen and aggrecan) and instead, produce fibroblast-specific ECM (type I collagen)[13]. Another challenge for using this technique is that the production of more complex tissues with thicker and defined shape would require extensive manipulation[86].

2.1.2.2 Aggregate tissue engineering

Besides cell-sheets, generation of cell aggregates represents another technique to produce

scaffold-free engineered tissues. Many studies made use rotational culture system to form cell aggregates by applying a rotational force to cells in suspension or other non-adherent culture[87]. In cartilage tissue engineering, aggregate culture is a common method used to produce cartilaginous microtissues[88]. It is believed that the formation of chondrocyte aggregates mimics aggregation of cells and matrix production that occur during native cartilage development since many studies have shown that the phenotype of these aggregates is similar to that of native cartilage. Therefore, aggregate culture has also been applied to differentiate stem cells into chondrocytic cells. In a recent study by Bhumiratana et al., human MSCs were used to form aggregates, where they were later induced to fuse and form cartilaginous tissue with biomechanical properties similar to that of native cartilage[78].

Besides being an effective method for differentiation, aggregate culture has also been employed redifferentiate chondrocytes that have experienced phenotypic change during monolayer expansion[89]. For instance, aggregate redifferentiation applied to articular chondrocytes and meniscus cells was shown to enhance biochemical and biomechanical properties of tissue-engineered constructs[90]. Aggregate engineering has also yield some commercial products such as Chondrosphere (co.don AG, Teltow, Germany), which is currently in phase III investigation in Europe. This product consists of autologous cell aggregates or spheroids and is used to treat traumatic cartilage injuries.[91]

Although aggregate engineering can partially overcome diffusion limits faced by other tissue-engineering methods, this technique is not without flaws. During the initial period where cells remain in suspension prior to cell interaction and coalescence, viability of cells may be affected due to lack of substrate contact. Additionally, it is also a challenge to control the shape and uniformity of the aggregates formed.[92, 93] Aggregates that are too big in size still

experience decreased cell viability due to nutrient or gas diffusion limits. [80]

2.1.2.3 Self-assembly method

Self-assembly is a tissue-engineering technique that does not rely on any external forces to form engineered tissues. It differs from the previously discussed aggregate culture in the way the tissues are formed, as well as the resulting tissue properties. With this method, tissue formation depends solely on the non cell-adhesive nature of agarose that drives cell coalesce.[80] The resulting tissues have organizational and functional properties closer to that of native tissue[94]. In contrast to aggregate engineering, this method offers more precise control over the shape and sizes of the tissues formed. Therefore, this method has been employed to engineer not only articular cartilage, but also meniscus and temporomandibular joint (TMJ) disc[95, 96]. For articular cartilage, a study has reported that the self-assembled constructs derived from bovine primary chondrocytes displayed histological, biochemical, and biomechanical properties resembling those of native tissue[27]. As for fibrocartilage engineering, tissue that expressed cartilage-specific genes (cartilage oligomeric matrix protein (COMP), collagen type I and II, and Sox9) was also produced via the self-assembly method using a relatively less explored stem cell source known as dermis isolated adult stem cells (DIAS cells)[97]. In another study, self-assembled co-culture constructs consisted of both fibrochondrocytes and chondrocytes were found to have similar properties as native meniscus in terms of their robust gross characteristics and ECM make up[98]. This self-assembly co-culture method was also extended to engineer TMJ discs with a specific shape and to promote matrix anisotropy within the discs[99].

While the self-assembling method recapitulates developmental processes and allows better control of tissue geometry, it presents some shortcomings. For example, this method requires very high cell numbers (10-100 million of cells per mL) and the cells must be able to

adapt to minimal cell-substrate interactions while waiting for the ECM to accumulate during the early phase of the self-assembling process[100, 101]. Similar to other tissue formation techniques, the size of the resulting tissue is limited by diffusion. [101]

2.1.3 An intermediate scaffolding system

In order to provide chondrocytes with a 3D environment for proliferation in a manner that biomaterials are traditionally able to, while preventing their long-term involvement in the remodelling process in order to preserve tissue purity, a unique methodology was developed recently[102]. This methodology combined the use of an intermediate scaffolding system with a 3D cell culture strategy known as Phase Transfer Cell Culture (PTCC). The intermediate scaffolding system comprised of a microcavitary hydrogel (MCG) model. This model basically made use of gelatin microspheres (which readily dissolve at elevated temperatures) to create cavities within a cell-laden alginate hydrogel-based construct. This is believed to solve the nutrient/gas diffusion problem that has plagued many other scaffold-free techniques[80]. As a result, this environment promoted vigorous chondrocyte growth and abundant cartilage-specific extracellular matrix (ECM) production. The scaffolding material phase was completely removed from the MCG construct once the cells have produced enough ECM to maintain the structural integrity of the construct. [102] The final 3D, scaffold-free construct is known as the Living Hyaline Cartilage Graft (LhCG), and had been demonstrated to recapitulate characteristic markers of an articular cartilage *in vitro*. Furthermore, in a leporine model for articular cartilage repair, results strongly demonstrated the advantage of the LhCG in mediating efficacious repair *in vivo*. [103]

2.2 Engineered cartilage for *in vitro* disease modelling: Osteoarthritis (OA)

2.2.1 Pathogenesis of OA

Owing to its complex composition and highly organized structure, it is particularly challenging to repair or replace the hyaline cartilage once it is damaged or lost[2]. Additionally, unlike most tissues in the body, the articular cartilage has only a minimal potential for self-repair and regeneration attributed to its avascular nature [103].The damaged areas of this tissue are usually replaced by fibrocartilage, another type of cartilage that is both mechanically and biochemically inferior to hyaline cartilage. Therefore, chondral lesions often result in progressive deterioration of this tissue that would inevitably develop into joint diseases such as osteoarthritis (OA). The irreversible destruction of this tissue can cause pain, reduced mobility and ultimately leads to disability.

Osteoarthritis (OA), a prevalent joint disease caused by the compromised quality of hyaline cartilage, is a multifactorial degenerative disorder characterized by a progressive degradation of ECM components, cartilage cell destruction, subchondral bone remodelling and synovitis.[104] This disease affects not only the articular cartilage but also almost all the other joint tissues including bone, synovium, ligaments and muscle.[105, 106] It can be categorized into primary and secondary OA. Primary OA mostly affects older individuals (age 55 or 60) and is associated with aging and gradual wear and tear. As for secondary OA, it does not specifically affects any single age group and this type of OA has a specific cause such as a traumatic joint injury, an effect of obesity, genetics or due to other diseases.[107] The distinctive features of arthritic joints (both OA and RA) are compared to a normal healthy joint in **Figure 2-1**.

Figure 2-1 Differences between a normal and arthritic joints. Adapted from [108].

2.2.2 Role of inflammation and macrophages in OA pathology

In spite of the numerous investigations, the pathogenesis of OA has not been fully elucidated. Biomechanical stress, specifically static compression appears to be an important factor in OA pathogenesis, but this factor alone cannot provide an adequate explanation to the disease process. OA has often been referred to as a non-inflammatory disease to differentiate it from other form of inflammatory arthritis such as rheumatoid arthritis (RA) or seronegative spondyloarthropathies. However, this description of OA is inaccurate since synovial inflammation is now a well-documented phenomenon at the clinical stage[109]. This phenomenon is increasingly recognized to be an important contributing factor to OA pathology. For instance, some of the OA symptoms like the swelling of joint and effusion are associated with an inflamed synovium.[110] Furthermore, it is also highly probable that synovial inflammation contributes to disease progression based on the correlation between the

inflammatory biologic markers, such as C-reactive protein and cartilage oligomeric matrix protein (COMP), with the progression of structural changes in OA. However, the cause of synovial inflammation has not been identified, but it was suggested that materials released from damaged cartilage might trigger it since these materials are commonly present in the synovial fluid of osteoarthritic joints. The degradative products of cartilage, such as cartilage fragments and soluble proteoglycans and type II collagen, can activate macrophages and cause them to release a variety of inflammatory mediators.[110-112]

The involvement of macrophages in OA pathology has been demonstrated in many experimental OA studies[113]. In order to probe the role of macrophages in this disease, a study has selectively depleted this population of cells from the murine knee joints before inducing OA by intra-articular injections of collagenase. The results showed the pivotal role of macrophages in mediating osteophyte formation and causing fibrosis in early stages of experimental OA[114]. In another study, the synovial macrophages were also shown to be crucial in early MMP activity where they mediate MMP production in the synovium [115]. Macrophages are thus believed to be the main cell type that is responsible for maintaining synovial inflammation. Furthermore, they are also believed to drive subsequent destructive responses in OA by activating synovial fibroblasts and chondrocytes.

2.2.3 Cytokines and OA

It is generally accepted that cartilage integrity is maintained by equilibrium between the cytokine-driven anabolic and catabolic processes. Any disruptions to this delicate balance will lead to the development and progression of OA. Therefore, cytokines and growth factors are believed to play an important role in OA pathophysiology. They are known to cause functional changes in synovium, cartilage and subchondral bone, and their synthesis can occur

spontaneously or following stimulation by the joint tissue cells. They appear to be first produced by the synovial membrane and reach the cartilage via diffusion through the synovial fluid. They subsequently cause the activation of chondrocytes, which could in turn synthesize catabolic factors such as proteases and proinflammatory cytokines. [116, 117] The major cytokines, both pro- and anti-inflammatory and antagonists that have been reported to be involved in OA pathophysiology are being listed in **Table 2-2** [117]. Besides that, there are also a number of growth factors that appear to be involved in this disease such as transforming growth factor (TGF)-beta, bone morphogenetic proteins (BMPs), and insulin-like growth factor 1 (IGF-1)[118].

Table 2-2 Important cytokines and their role in the pathophysiology of OA.

Cytokine	Activities in OA
TNF	Elevated in OA synovial fluid, synovial membrane, subchondral bone and cartilage Suppresses the synthesis of proteoglycan, link protein and type II collagen in chondrocytes ¹⁸ Stimulates the release of MMP-1, MMP-3 and MMP-13 ^{27,28} Induces the production of IL-6 ³³ and chemokines such as IL-8, ³⁴ MCP-1 ³⁵ and CCL5 (also known as RANTES) ³⁶
IL-1 β	Elevated in OA synovial fluid, synovial membrane, subchondral bone and cartilage Suppresses type II collagen ^{20,21} and aggrecan expression ²² Stimulates the release of MMP-1, MMP-3 and MMP-13 ^{27,28} Induces the production of IL-6 ³³ and chemokines such as IL-8, ³⁴ MCP-1 ³⁵ and CCL5 ³⁶
IL-6	Elevated in synovial fluid and sera from patients with OA ⁸¹ Upregulates MMP-1 and MMP-13 expression in combination with IL-1 β and oncostatin ^{82,83} Reduces expression of type II collagen ⁸⁵
IL-15	Elevated in synovial fluid in early OA Suggested association with MMP-1 and MMP-3 ¹⁰⁴
IL-17	Induces IL-1 β , TNF and IL-6 production Upregulates NO and MMPs ¹⁰⁵ Downregulates proteoglycan levels ¹⁰⁶
IL-18	Elevated in human OA chondrocytes Structurally similar to the IL-1 protein family Acts through both IL-1-dependent and IL-1-independent pathways in OA ¹⁰⁷
IL-21	Elevated in synovial fluid in early OA
LIF	Elevated in OA synovial membrane and fluid Produced in response to IL-1 β and TNF Stimulates cartilage proteoglycan degradation, MMP synthesis and NO production ¹⁰⁸
Chemokines (CCL5, IL-8, GRO α and MCP-1)	Elevated in OA tissues Induce iNOS, MMP-1 and IL-6 Stimulate proteoglycan depletion ^{36,108}
Anti-inflammatory cytokines (IL-4, IL-10 and IL-13)	Elevated in OA tissues Inhibit IL-1 β , TNF and proteases Upregulate IL-1Ra and TIMP production ³⁹
Abbreviations: CCL, CC-chemokine ligand; GRO, growth-related oncogene; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; iNOS, inducible nitric oxide synthase; LIF, leukemia inhibitory factor; MCP, monocyte chemotactic protein; MMP, matrix metalloproteinase; NO, nitric oxide; OA, osteoarthritis; RANTES, regulated upon activation, normal T cell expressed and secreted; TIMP, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor.	

2.2.3.1 Proinflammatory cytokines

Current evidence indicates that proinflammatory cytokines are responsible for the catabolic process observed in diseased tissues. Among them, TNF- α and IL-1 β have been specifically suggested to play a crucial role in OA pathogenesis, as they are believed to be the initiators of disease process (**Figure 2-2**)[117]. They are capable of stimulating their own production and are responsible for synovial inflammation by inducing synovial cells and chondrocytes to release other regulatory cytokines such as IL-6, IL-8, and leukocyte inhibitory factor (LIF). Regulatory cytokines like IL-6 contributes to the pathological process of OA by increasing the number of inflammatory cells in the synovial membrane, stimulating the proliferation of chondrocytes, as well as enhancing the production of proteases while inhibiting proteoglycan synthesis. As for IL-8, it is a potent chemotactic cytokine for polymorphonuclear neutrophils (PMN), stimulating their chemotaxis and generating reactive oxygen metabolites.[119] It has been suggested that IL-8 plays an important role in the acute inflammatory reaction. LIF is a cytokine of the IL-6 family that stimulates proteoglycan resorption, MMP and NO production. However, it is unclear if they have a direct or indirect role in the development of cartilage destruction and joint inflammation. The end result of these cascaded events mediated by the proinflammatory cytokines is the deterioration of cartilage matrix quality. [120]

Figure 2-2 The role of pro-inflammatory cytokines in OA pathogenesis. Briefly, the expression of proinflammatory cytokines such as IL-1 β , TNF and IL-6 increased in an OA joint. These cytokines contribute to disease progression by suppression of matrix synthesis, promotion of matrix degradation and induction of inflammatory responses. All these actions collectively result in OA joint destruction. Adapted from [117].

2.2.3.2 Nitric oxide (NO): a catabolic factor

Another potential promoter of cartilage catabolism in OA is the inorganic free radical NO. A significantly higher level of NO has been found in OA cartilage as compared to the healthy state, under both spontaneous and proinflammatory cytokine-stimulated conditions[121]. An enzyme known as inducible form of NO synthase (iNOS) is responsible for the synthesis of NO. NO contributes to disease progression by inhibiting synthesis of cartilage matrix macromolecules such as collagen and proteoglycan, increasing the level of MMP activity and also inducing apoptosis of chondrocytes. [120, 122] Interestingly, a number of studies have demonstrated that the inhibition of iNOS caused reduced progression of experimental OA in vivo. [123, 124]

2.2.3.3 Anti-inflammatory cytokines and cytokine antagonist

Besides the release of regulatory cytokines during the inflammatory process, another group of cytokines with inhibitory effects were also found in the OA joint. Of this group, three different anti-inflammatory cytokines namely IL-4, IL-10, IL-13, were detected in increased levels in the synovial fluid of OA patients. They exert their anti-inflammatory effect by decreasing the synthesis of IL-1 β , TNF- α and MMP while increasing the production of IL-1Ra and TIMP-1. They also inhibit the release of PGE₂. [120]

2.2.4 Cellular responses in OA cartilage

Chondrocytes are undoubtedly the most crucial cells involved in the progression of the osteoarthritic process. It was found in human and animal studies that chondrocytes in osteoarthritic cartilage display more than one abnormal metabolic feature. These abnormal features can be summarized into five categories, namely increased levels of cell proliferation and apoptosis, metabolic activation and hypoanabolism, synthesis of degradative enzymes, phenotypic changes of the chondrocytes and also formation of osteophyte. [125]

2.2.4.1 Cell proliferation and apoptosis

Chondrocytes have been known to be metabolically inert cells where almost no cell proliferative activity can be detected in the healthy cartilage. However, the inflammatory environment in osteoarthritic joints causes a change in their proliferative activity. Many studies observed notable increase in cell proliferation among osteoarthritic chondrocytes. [126, 127] This can be attributed to the disruptions in the collagen network or compromised matrix quality allowing the cells to have a better access to the various proliferative factors from the synovial fluid. This sudden surge

in their proliferative activity most probably contributes to chondrocytes clustering, which is a characteristic feature of OA cartilage. [128]

Besides that, many research studies have suggested that apoptosis is another important feature in the degeneration of osteoarthritic cartilage[129]. This is hypothesized based on the correlation between reduction of chondrocytes and increase of degenerative changes in osteoarthritic cartilage, and also the higher number of apoptotic chondrocytes found in pathological tissue than in normal tissue[130]. Although almost all studies have agreed that programmed cell death is one of the features associated with cartilage destruction, they have different opinions on how dominant this event is in mediating the morphological changes of the tissue. Some studies suggest that it is a central feature as evidenced by the high percentage of cell death observed in cartilage samples (about 5 to 11%) and in OA patients (about 22 to 51%) of all cells. [131] However, some researchers believed that there is an overestimation in the extent of apoptosis in OA cartilage since such a high percentage of cell death would undoubtedly hinder cartilage matrix turnover[127]. This is because chondrocytes are the only cell source responsible for the synthesis of cartilage matrix components and there is no renewal of these cells due to the lack of blood supply, lymphatic drainage, and innervations in mature cartilage. Thus, they believe that apoptosis has only a limited impact on OA pathology.

2.2.4.2 Chondrocyte activation and hypoanabolism

A number of studies that focus on biochemical analysis of the OA cartilage have reported an increased rate in the production of extracellular matrix components[132-134]. This is seen as an attempt by the chondrocytes to repair the damaged matrix by increasing their synthetic activities. However, this increased anabolic activity eventually loses out to the overall enzymatic degradation of the matrix components, resulting in a net loss of proteoglycan content. A previous

study has pointed out that this hyperactivity of matrix synthesis is limited to the chondrocytes in the middle and deeper zones of osteoarthritic cartilage[135]. Therefore, the loss of proteoglycan is most prominent in the superficial zone. [128]

2.2.4.3 Synthesis of degradative enzymes

The main enzymes responsible for the initial digestion of ECM are metalloproteinases (MMPs)[136]. They are a family of enzymes that are important in regulating the degradation of ECM and play a pivotal role in many physiological and pathological processes of different tissues. Undeniably, the degradation of ECM at an appropriate time is necessary for embryonic development, morphogenesis, reproduction, and tissue resorption and remodeling[43]. However, under pathological conditions, their production is mainly induced by the exposure of cells to inflammatory cytokines. An amplifying cascade sequentially activates this family of degradative enzymes. Their biologic activity is partially regulated by specific tissue inhibitors of MMPs (TIMPs), whose synthesis is low relative to MMP production in OA cartilage[137]. Additionally, other activators such as other MMPs or plasmin also affect their activity.

In almost all OA cells, the levels of MMP-3 (stromelysin), MMP-8 (collagenase-2), and MMP-13 (collagenase-3) were found to be elevated. MMP-3 activates MMP-1 (collagenase-1) and mediates the cleaving of a broad range of matrix proteins. MMP-1 is an interstitial collagenase while MMP-3 are among the most abundantly expressed MMPs.[138] MMP-3 has the ability to cleave in the nonhelical telopeptide of type II and type IX collagens, resulting in the cleavage of collagen crosslinks. This could lead to a disrupted fibril structure, consequently affecting the fibril function. A previous study has validated this by showing that treatment of cartilage explants with MMP-3 causes significant swelling of the tissue while this phenomenon was not observed in trypsin-treated explants[139]. As for MMP-8 (neutrophil collagenase), it has

been shown to be capable of cleaving the aggrecan molecule at the aggrecanase site [140]. Another key enzyme in OA pathogenesis is MMP-13, which is believed to be a major enzyme that is responsible for degrading the cartilage. Compared to other MMPs, MMP-13 has a more restricted expression pattern where it is often expressed within connective tissues [138]. Its targets for degradation include type II collagen, proteoglycan, types IV and type IX collagen, osteonectin and perlecan in cartilage[42]. Clinical studies consistently showed that OA patients have high MMP-13 expression, suggesting that upregulation of MMP-13 may be associated with cartilage degradation[141]. Furthermore, previous studies have also demonstrated that a transgenic mouse model that overexpressed MMP-13 developed a spontaneous OA-like articular cartilage destruction phenotype[142].

2.2.4.4 Chondrocyte hypertrophy-like changes in OA

To date, the exact pathomechanism of OA is still not clear. However, phenotypic changes in the chondrocytes represent another likely reason for their anabolic failure in osteoarthritic cartilage[143]. It has been proposed that articular chondrocytes lose their differentiated phenotype and follow a differentiation route resembling that of growth plate chondrocytes, expressing hypertrophy-like changes. [144]

Hypertrophic chondrocytes are indicated by the upregulation of several genes; with type X collagen and MMP13 being the most widely used [143, 145]. Hypertrophic chondrocytes also expressed osteopontin, osteocalcin, Indian Hedgehog, Runx2, VEGF, HtrA1 and transglutaminase-2 (TG-2). This phenotypic change is accompanied by acquisition of an autolytic phenotype by the chondrocytes, which will result in the degradation of the surrounding cartilage. Chondrocyte hypertrophy eventually leads to calcium deposition. However, this state is not seen in all cases of OA.[128, 144]

2.2.4.5 Osteophyte formation

One of the most prominent pathological features that distinguish OA from other arthritis is the formation of osteochondral nodules known as osteophytes. They are found mostly at the margins of the joint, initially as cartilage outgrowths and then undergo endochondral ossification. Growth factors such as transforming growth factor beta (TGF β) potentiate their formation. [146]

These fibrocartilage-capped bony outgrowths can form in the early stages of OA and can be seen prior to joint space narrowing. They can have a significant clinical impact and can be a source of pain and dysfunction. The exact relationship between osteophyte growth and OA progression remains unclear although it has been suggested that osteophytes might help to stabilize joints affected by OA. [147]

The cellular responses in OA is complex, but understanding it is crucial to the development of a disease model that can replicate important features observed in the progression of OA. The proposed disease model is expected to be valuable in the effort to probe the pathomechanism of OA, but it will also be equally suitable as a drug screening model which provides accurate predictions of drug responses.

2.2.5 In vivo and in vitro osteoarthritic models

An *in vivo* or an *in vitro* osteoarthritic model generally can be used for two different purposes in OA studies: as a study model to probe the pathogenesis of cartilage degeneration or as a drug test model to identify potential therapeutic agents with anti-arthritic properties or disease modifying effects.

2.2.5.1 Animal/ In vivo OA models

Animal models that are commonly used in osteoarthritic research include spontaneous models in aging animals, genetically modified mice, as well as surgically, enzymatically or chemically induced models[122, 148]. Spontaneous OA is detected in various inbred strains of mice, guinea pigs and non-human primates.[123] Among them, Duncan-Hartley guinea pigs are popularly used since OA developed in them are well characterised. As for the genetic models of OA, they are developed by knocking outs genes encoding important matrix proteins or some other signaling molecules crucial for cartilage homeostasis. Surgical induction is one of the most widely used methods in establishing osteoarthritic animal models. The underlying initiating mechanism is altered mechanical loading, which is also a common cause for the development of secondary OA in humans. The aim of the surgical procedures is mostly to mimic the common injuries in humans, such as anterior cruciate ligament (ACL) tear and meniscal injury (partial or complete meniscectomy, meniscal transection and meniscal destabilization). Another relatively convenient way to induce OA in animal models is by intra-articular injection of a variety of agents, including enzymes (papain, collagenase, trypsin, hyaluronidase), cytokines (interleukin (IL)-1, transforming growth factor (TGF- β) and chemicals (monosodium iodoacetate (MIA)[149]. These agents are capable of inducing an acute local inflammation at the site of injection, which would subsequently lead to the degeneration of cartilage.

Although there are numerous animal models available for OA, each model of induction and species has distinct advantages and disadvantages [122]. For example, both spontaneously developed and transgenically induced models require extended time frame for disease progression, and hence prolongs the time for drug testing and studies of pathogenesis. Intra-articular injection of various agents to initiate cartilage degeneration also often raises concerns about its relevancy to modeling human OA. This is because this method that stimulate local

acute inflammation may not replicate the naturally occurring sequence in the human OA disease process. Thus, there is currently no single gold standard model that can truly represent human OA.[123] Another limitation of using animal models is the lack of reliability inherent to interspecies comparisons. The data obtained from animal models cannot always be extrapolated to humans due to distinct anatomical and physiological differences. Moreover, animal experiments are often costly, inefficient and associated with ethical issues.

2.2.5.2 In vitro OA models

Besides animal models, *in vitro* models also play an important role in OA-related studies. One of the most popularly used *in vitro* models is the 2D chondrocyte monolayer culture. A large number of studies have utilized monolayer cultures of chondrocytes isolated from OA-affected joints to evaluate the therapeutic potential and cytotoxicity of various potential therapeutic agents [104, 124, 131, 138, 141, 142]. This model is heavily favoured owing to its simplicity and low-cost. However, studies have shown that chondrocytes expanded in monolayer cultures will inevitably undergo dedifferentiation into fibroblastic cells [49, 50, 150, 151]. Although a re-differentiation of the expanded chondrocytes is possible but it is difficult to achieve, especially for long-term cultures. More importantly, this simplistic model is unable to replicate the intercellular environment of the native cartilage that is crucial for the understanding of OA pathogenesis [152]. The lack of fidelity in mimicking the native microenvironment causes the accuracy of this *in vitro* model in predicting the clinical effect of a novel therapeutic agent to be questionable.

2.3 Engineered cartilage for in vitro disease modelling: Rheumatoid arthritis (RA)

2.3.1 Pathogenesis of RA

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is associated with chronic synovitis and progressive joint damage[153]. In most RA patients, the presence of a spectrum of autoantibodies can be detected in their serum and synovial fluid as a manifestation of systemic autoimmunity. The two most prominent ones are rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs), with the latter being especially specific for RA[154]. The exact cause of this disease is still unclear but its initiation is known to involve a complex interplay among genetic factors, environmental factors, and chance. It is well established a few key genetic factors, such as the presence of shared epitope human leukocyte antigen (HLA) alleles and PTPN22 polymorphism increase susceptibility and severity to RA[155]. Furthermore, environmental triggers, such as smoking were also demonstrated to increase the risk for this disease[156]. The events and players involved in RA are illustrated in **Figure 2-3**.

Figure 2-3 Cascades and players involved in RA. Adapted from [157].

At a local joint level, the pathology in RA is established by the conversion of a normal quiescent synovial membrane into a highly metabolic, tumor-like pannus tissue [158]. In order to better understand this prominent change of the synovium, its structural and functional properties need to be studied. In a healthy joint, synovium is a thin delicate lining that produces nutrients for cartilage since cartilage itself is avascular. Additionally, it also acts as a source of lubricating molecules for the joint such as hyaluronic acid, as well as collagens and fibronectin that make up the structural framework of the synovial interstitium. The synovium comprised of two distinct layers, namely the synovial lining (or intimal layer) and the sublining (or subintimal area of synovium).

In a diseased state, the synovial lining surrounding the synovial fluid compartment undergoes hyperplasia, causing it to thicken from 1-3 cell layers to 10-15 cell layers due to the overgrowth of both macrophage and fibroblast populations[159]. The sublining layer of the synovial membrane also experience hyperplasia caused by the influx of inflammatory cells such as macrophages, mast cells, T cells, B cells and plasma and dendritic cells[160]. The hyperplastic synovial tissue eventually forms a tissue structure known as pannus, which invades and destroys adjacent articular cartilage and bone[161]. This shift towards cartilage and bone destruction is driven by a proinflammatory cascade of cytokines, which are predominantly synthesized by macrophage- and fibroblast-like cells of the synovial lining. These cytokines include tumor necrosis factor alpha (TNFa) and interleukin-1 (IL-1), and the various matrix-degrading enzymes such as collagenase, aggrecanase, stromelysin, and cathepsin[162].

2.3.2 Role of synovial fibroblast in RA

Synovial fibroblasts (SFs), otherwise known as fibroblast-like or type B synoviocytes, are the main resident cell type in the synovial tissue. In a healthy joint, the physiological function of SFs is to produce synovial fluid for the joint cavity. The synovial fluid contains nutrients and lubricants that are crucial for normal joint function. SFs also play a role in matrix remodeling via synthesis of matrix components such as, collagen and hyaluronan as well as the various matrix-degrading enzymes, such as MMPs. In RA synovium, RASFs developed properties that differ considerably from the normal SFs. Fassbender first discovered their altered phenotype in 1983. Since then, a large amount of data has been collected that allow us to better understand the active role of RASFs in establishing RA. [159]

RASFs are implicated in the pathogenesis of RA at two fundamental levels[163]. Firstly, they are known as the effector cells in inflammation. They produce a wide spectrum of chemotactic proteins that cause an influx of immunomodulatory cells, such as neutrophils and B and T lymphocytes into the RA synovium. For instance, CXCL16[164], IL-16, and stromal cell derived factor-1 (SDF-1) secreted by RASFs are known to enhance the influx of CD4+ T cells into the proliferating synovial tissue. Some other chemokines produced include macrophage inflammatory protein (MIP), IL-8, CCL5, and CXCL1.[165] In addition to cell recruitment, RASFs also produce constitutive chemokines, such as CXCL 12, CXCL13, and CCL 21 to prevent infiltrating leukocytes from leaving the synovial tissue. RASFs were also reported to produce survival factors such as, type I interferon, IL-15 and BAFF to prevent apoptosis of the leukocyte subpopulations, which would usually happen during the resolution phase of an inflammatory response. In short, synovial fibroblast activation in RA results in the buildup, retention and survival of leukocytes at disease sites, simulating the microenvironment observed in lymphoid tissues, and thus preventing the resolution of chronic inflammation.

Besides actively participating in fuelling inflammation, RASFs are also known to be the key mediators of joint destruction. At sites of invasion, they are directly involved in degrading the cartilage via secretion of MMPs, aggrecanases, cathepsins and inflammatory cytokines. Specifically, RASFs secrete MMP-1, -3, -9, and -10, and the release of these MMPs correlates with invasion[166]. Their active participation in cartilage destruction has been demonstrated in severe combined immunodeficient (SCID) mice, where *in vitro* cultured RASFs were reported to attach and invade co-implanted human cartilage while normal and osteoarthritic SFs did not show such behaviors[167]. The results from animal studies thus suggest that the aggressive feature of RASFs is independent of the cells from the immune system and also is

disease-specific. Moreover, RASFs maintain this destructive phenotype even after several passages in vitro. An in vitro study further confirmed that RASFs is more invasive than SFs from OA patients and this feature is strongly associated with clinical joint destruction[166].

Having known that they are the key effectors in the pathogenesis of RA, many studies were dedicated to further characterize the RASF phenotype with an aim to find the triggers that initiate their aggressive behavior. It was revealed recently that the Toll-like receptors (TLRs), a class of proteins that play a vital role in the innate immune system, might be involved at an initial stage of synovial activation[168]. Hypothetically, RASF activation is mediated by the presence of microbial components or endogenous ligands[169] (eg. RNA from necrotic cells) within the synovial fluid via TLR signalling, which leads to an increased production of pro-inflammatory cytokines and chemokines[170]. As previously mentioned, the release of these signaling molecules would in turn cause influx and accumulation of immune cells in the synovium and, via a stimulatory loop, to chronic inflammation. In addition, activation of RASFs is also known to be associated with molecular crosstalk between these cells and other cell populations in the synovial microenvironment. Prominent examples include pro-inflammatory cytokines like the tumour necrosis factor alpha (TNF α) and interleukin (IL)-1, and also some other mediators like IL-15, IL-21receptor[171] and IL-22[172]. To date, there is still no conclusive evidence about the exact activation pathway of RASFs. Most likely, a collection of different factors at the initial stage of synovitis or even before the adapted immune system is involved is required to perpetually activate SFs in RA.[159]

2.3.3 Role of macrophages in RA

Macrophages play an important role in RA. More specifically, they have been shown to massively infiltrate the inflamed synovium and the cartilage-pannus junction in RA, and the degree of infiltration corroborated well with radiological damage [173] and with joint pain and inflammation[174]. Their obvious activation status and their responsiveness to anti-rheumatic therapy continue to make them an attractive research focus.

Synovial macrophages are mainly derived from circulating monocytes. In RA tissue, macrophages are known to overexpress major histocompatibility complex class II molecules, which is an indicator of their activation status. Once activated, macrophages can produce a variety of pro-inflammatory or regulatory cytokines and growth factors, such as TNF- α , IL-1, IL-8, MIF, IL-15, IL-18 and TGF- β , for the establishment of an inflammatory joint environment. In addition, they are also known to play a part in chemotaxis and angiogenesis, which is a result of positive feedback between macrophage-derived cytokines and chemotactic factors (eg. IL-8, and monocyte chemoattractant protein-1). IL-8 secreted by synovial macrophages is a strong promoter of angiogenesis, hence providing a link between macrophage activation and the obvious neovascularization of the RA synovial tissue[175].

Besides their inflammatory role, macrophages also interact with other cells in the joint microenvironment, such as fibroblasts and endothelial cells to effect cartilage matrix destruction through MMP synthesis. Janusz and Hare demonstrated this combined effect in an *in vitro* study, where cartilage degradation was observed in cocultures of murine fibroblasts and macrophages[176]. Similar cartilage destructive process was also found with purified human synovial fibroblasts and myelomonocytic cells[177]. Notwithstanding the information provided earlier, activated macrophages also perform the following roles in an RA joint: 1) activate

incoming inflammatory cells via cytokine production; 2) activate adjacent inflammatory cells mediated by cell contact; 3) transdifferentiate of macrophages into osteoclasts involved in subchondral bone destruction. In a nutshell, the multitude of mediators derived from macrophage in RA and their paracrine and autocrine effects suggest that these cells serve as amplifiers of disease severity and perpetuation. [178]

2.3.4 Mechanisms of cartilage loss in RA

For bone remodelling, non-resident cells from the blood and marrow are recruited to the tissue surfaces to remodel the bone matrix. Contrastingly, cartilage remodelling involves only a single cell type, the chondrocytes. Chondrocytes are, so far, the only known resident cell type of the cartilage.[179] They are responsible for the synthesis of cartilage matrix components while they also produce proteinases that degrade the damaged matrix to permit repair. In contrast to the continuous remodelling process of bone, cartilage turnover is comparatively limited, resulting in its restricted capacity to self-repair once damaged.[158] Many factors released from the inflamed RA synovial tissue are involved in regulating cartilage remodelling. These factors normally promote matrix degradation while suppressing matrix synthesis and repair. Among the synovial products that are associated with cartilage loss, IL-1 and TNF- α represent the two most important and well-characterized factors. IL-1 has been shown to stimulate the chondrocytes to upregulate their expression of MMPs and other degradative factors such as, nitric oxide[180, 181]. As for TNF- α , both in vitro and in vivo studies have suggested that this molecule has effects similar to, or synergistic with IL-1[162, 182]. Besides causing an increased production of degradative factors, these two cytokines further contribute to cartilage matrix depletion by reducing the expression of cartilage-specific matrix components like collagen type II and proteoglycans.

Generally, cartilage destruction in RA is mediated by synovial tissues and can occur via two principal mechanisms: direct and indirect mechanism[158]. For direct mechanism, RA synovial tissues are producing products that directly degrade the cartilage matrix. Some of the factors involved are MMPs and cathepsins. Due to this mechanism, cartilage damage occurs predominantly in areas adjacent to the proliferating synovial pannus[183, 184]. At the cartilage-pannus junction, fibroblast-like and macrophage-like synovial cells have been found to attach and actively producing proteinases that digest the cartilage matrix components. There is a distinctive fibroblast-like cell population, known as pannocytes, which display anchorage-independent growth and these cells are able to invade cartilage even in the absence of inflammation[185]. As for indirect mechanism, RA synovium produces cytokines and other mediators that induce chondrocytes to produce matrix-degrading products. IL-1 and TNF α represent two of the synovial factors that function in this manner. This indirect involvement of RA synovium is the main cause of cartilage matrix loss at sites not directly associated with the pannus. For example, proteoglycan loss that occur throughout the cartilage matrix[186], and selective degradation of collagen type II detected in middle and deep zones of cartilage[187], suggesting that the chondrocytes were induced to participate in degrading its own matrix.

Among the MMPs that participate in cartilage matrix degradation in RA, MMPs of the collagenase and stromelysin families have been given the most attention due to their specificity in digesting cartilage specific collagens and proteoglycans [188]. MMP-1, MMP-8, and MMP-13 (or collagenase 1,2 and 3, respectively), gelatinases like MMP-2 and MMP-9, MMP-3 or stromelysin-1 are several examples of the enzymes present in RA synovium[189, 190]. While some of the MMPs like -3, -8 and -14 are capable of digesting proteoglycans, ADAMTS (ADAM with thrombospondin-1 domains)-4 and ADAMTS-5 are currently regarded

as the primary mediators of aggrecan degradation[191]. The former is expressed constitutively while the expression of the latter is regulated by inflammatory cytokines[192]. A summary of the mechanisms of cartilage loss in RA can be found in **Figure 2-4**.

Nature Reviews | Immunology

Figure 2-4 Cartilage destruction in RA is mediated by the release of matrix-degrading enzymes such as MMPs and ADAMTS. Cytokines such as IL-1 and IL-17 are responsible for switching the metabolism of chondrocytes from an anabolic to a catabolic state. Additionally, synovial fibroblasts also contribute to the production of matrix-degrading enzymes and invade cartilage when activated by pro-inflammatory cytokines, including TNF and IL-1. Apoptosis of chondrocytes represents another pathway of cartilage destruction; it deprives cartilage of the ability to replace lost matrix components. Adapted from [162].

2.3.5 In vivo and in vitro rheumatoid arthritic models

2.3.5.1 Animal/in vivo RA models

Animal models are being heavily dependent on in RA research. In spite of the inherent limitations in all animal models, several rodent models have provided useful insights to the fundamental mechanisms of this systemic inflammatory disorder, leading to a number of major advances in therapy. Generally, there are two main categories of RA animal models: induced RA models and spontaneous RA models[193]. Rodent models are the most common for RA studies and have been developed in both mice and rats. They are preferred over other animal species due to a lower cost, genetic homogeneity, and the capacity to use genetically modified strains for mice[194].

For induced RA models, the disease is introduced into animals by treatment with an inducing agent. One of the most extensively studied models under this category is collagen-induced arthritis (CIA). This model shares many similarities with human RA in terms of its immunological and pathological features[195]. Specifically, the CIA model demonstrates breach of tolerance and production of autoantibodies toward self and collagen, which make this model the gold standard in vivo model for RA research[194]. DBA/1 mice are the most popular choice among the other CIA models. Another animal RA model can be established by transferring serum containing anti-type II collagen IgG antibodies from an immunized mouse into a non-immunized recipient. The model set up using this method is known as the collagen-antibody-induced arthritis (CAIA) model[196]. In addition, some other inducing agents used are zymosan (causes activation of macrophages via TLR signalling)[197], pristane (largely T-cell dependent)[198], and human cartilage-derived proteoglycans[199].

The spontaneous RA models comprised of genetically modified animals, such as transgenic and knockout mice. For instant, a transgenic mouse model was established to overexpress human TNF- α [200]. The chronic progressive nature of the arthritis in this model mimics the human disease more closely than the CIA model that is acute. Furthermore, some studies have shown that this model is an important tool for investigating the distinct role of effector cytokines in inflammation and tissue destruction. The K/B $\times$ N mice represent another model under this category[201]. This model is useful for studying the role of antibodies in the development of RA[202]. Other spontaneous transgenic models of arthritis include NZB/NZW mice, Human/SCID chimeric mice[203], Human DR4-CD4 mice[204], and IL-1RA $-/-$ mice. For all the animal models described so far, each of them has its pros and cons in mimicking human pathology. Although none of these models is able to completely recapitulate clinical pathology, they remain very valuable in providing information relating to the pathogenesis of RA.

2.3.5.2 In vitro RA models

As compared to OA, there are many more studies on the establishment of 3D *in vitro* model for rheumatoid arthritis (RA). There are several studies that have worked on the development of *in vitro* models that mimic the human joint microenvironment[161, 205-207]. Besides differences in the cell types and purity used for coculture (eg. whole synovial tissue, isolated synovial fibroblasts, macrophages, T- or B-cells), these studies also differ widely in the type of cartilage (-like) matrix they employed[207]. Some used cartilage explants obtained either from human patients[177, 208] or other animal species[207] while some used in vitro generated, cell-containing matrices, such as high-density chondrocyte pellets[161, 209], chondrocyte-laden alginate beads[210], and chondrocyte/RA synovial cell encapsulated in fibrin matrices[206].

2.4 Engineered 3D cartilage disease models for the evaluation of anti-arthritis inflammation drug delivery and efficacy in vitro [211]

Three-dimensionally (3D) engineered biomimetic tissue models are sought after due to their high fidelity in mimicking various native tissues of the human body, this quality of which gives them an important role at the forefront of drug discovery and development. A multitude of studies have consistently indicated that gene expression profiles, cellular phenotypes, differentiation capabilities and functionalities are all affected by tissue architecture. Thus, the drug evaluation process will stand to gain immense benefits from the fairly accurate predictions of cellular responses displayed by 3D-engineered tissue models when exposed to the drugs of interest in vitro. Stemming from this fact, many studies have set out to capitalize on developing tissue models that are tailored to specific aspects of drug evaluation.

Drug evaluation is generally assessed in terms of three key areas: drug delivery methods, drug efficacy and drug toxicity. These parameters are important because it is imperative to develop an efficient drug delivery method that can maximize drug efficacy whilst minimizing toxic side effects. Conventionally, human or mammalian cells cultured in monolayers are employed extensively for *in vitro* drug evaluations upon the delivery methods, effectiveness and toxicity. However, in recent years there are increasingly more attempts to create three dimensional (3D) biomimetic engineered tissue models that can better fulfill the role in drug evaluation.[212] These attempts are aimed at recapitulating some of the important human native tissue structures and functions so that the responses to therapeutic administrations may be predicted more accurately in an *in vitro* setting. This paradigm shift from two-dimensional (2D) cell-based model to 3D tissue model is essential because cells cultured on cell culture plates or

micro-wells are incapable of providing a realistic model of the intercellular environment[152]. On the other hand, animal experimentation, which is extensively relied upon for drug evaluation, remains costly and the data derived from animal models in preclinical trials often cannot be extrapolated to humans. For instance, animal models may fail to predict human responses to some pathogens that are species specific (such as hepatitis C). Gaps in understanding like this could have dangerous results such as liver toxicity. This is one of the primary causes for the failure of novel drugs in clinical trials.[213, 214] Therefore, in order to bridge the knowledge gaps, the development of more realistic and physiologically relevant 3D tissue models *in vitro* are necessary.

As previously mentioned, OA is a degenerative joint disease that leads to eventual cartilage destruction. To date, a major portion of research has been dedicated to the pursuit of effective regenerative therapies for OA-afflicted joints [215-217]. There are not many studies that focus on establishing 3D models for drug screening purposes. One of the few is a study by Cortial et al., they have attempted to develop a 3D *in vitro* model that may be used to investigate the potential anti-arthritic effects of drugs. Their approach of setting up the model was by seeding primary bovine chondrocytes onto a collagen sponge, followed by interleukin (IL)-1 β activation. Results showed that inflammation-induced genes such as, MMP-1, -3, -13, ADAMTS-4, -5 and IL-1Ra were up-regulated while the genes responsible for ECM production (COL2A1, aggrecan) were down-regulated[218]. In another recent study by Sun et al, a human-based 3D inflammatory cartilage model was developed for the modelling of OA disease since inflammation plays a significant role in OA progression. This human cartilage model was constructed by seeding human primary chondrocytes onto a silk protein-derived porous scaffold. There were two different approaches in inducing inflammation within the model; the first by

supplementing proinflammatory cytokines (IL-1 β and TNF- α) and the second by using the macrophage conditioned medium (MCM). By comparing these two approaches, MCM was shown to perform better at inducing immune-related features of OA. This is because MCM not only able to stimulate features achieved by using cytokines, but it also introduced additional features such as expression of collagen type X mediating chondrocyte hypertrophy and induction of apoptosis in chondrocytes, both of which are common in OA.[219]

RA represents another disease commonly affecting the joints. With the emergence of a plethora of potential therapeutic agents coupled with the increasingly prevalent cases of RA-related cartilage destruction, many researchers have heightened their efforts in developing more relevant *in vitro* RA models for effective drug screening. These models must be able to mimic the microenvironment of the diseased cartilage accurately. For this purpose, there is a need to simulate the inflammatory environment of RA joints. There are a number of studies that have attempted to induce inflammation in their *in vitro* 3D models by utilizing co-culture systems with RA synovial fibroblasts (RASFs). As shown in a study, high-density *in vitro* chondrocyte pellets were directly co-cultured with RASFs by overlaying these cells on the pellets. The invasion of the RASFs into the cartilage matrix was revealed by histological and immunohistochemical stainings. Furthermore, degradation of the cartilage pellets was also observed after 14 days of co-culturing with the RASFs as evidenced by the decreased gene expression of collagen type II.[220] This is consistent with the results from an earlier study[221]. It is also possible to induce RA-related cartilage destruction by co-culturing the two types of cells without direct cell contact. In a study by Kristin et al., they have demonstrated the use of alginate beads for *in vitro* 3D human chondrocyte cultures. These cell-encapsulated beads were activated by supernatants of RASF, indicating that these fibroblasts released soluble mediators to direct catabolic gene

expression by the chondrocytes, which ultimately impaired their function. Some of these crucial regulatory molecules mediating RA-related cartilage destruction have been identified in this study[205].

The establishment of such *in vitro* models not only allow a more in-depth understanding on the pathomechanism of RA. Additionally, these models may also serve as pathological models for the elucidation of the key mechanisms of antirheumatic drug action on human chondrocytes.[205] In their follow-up study, Kristin et al. further demonstrated the feasibility of using these models to test for the efficacy of various drugs from three different categories, namely DMARD, NSAID and SAID. By examining the chondrocyte gene expression using both microarray analysis and enzyme-linked immunosorbent assay, the different antirheumatic drugs tested were shown to exert different molecular effects on the human chondrocytes. The results have indicated that SAIDs displayed the highest potency in treating RA since the usage of this class of drugs have reverted many genes to a normal expression level. [222]

Collectively, the studies discussed thus far have demonstrated the feasibility and importance in developing *in vitro* 3D diseased cartilage models for OA and RA. These models can be very useful in the effort to supplement and reduce our dependency on animal experiments in validating the efficacy of various anti-arthritis drugs. Additionally, they may also help in the acceleration of drug discovery and development process since they can be adapted to enable high-throughput screening[223].

3 Optimization of chondrocyte isolation and phenotype characterization for cartilage tissue engineering

3.1 Introduction

As mentioned earlier in Chapter 1 of this thesis, damaged articular cartilage lacks the ability to self-repair due to its avascular nature[224]. In recent years, cell-based repair procedures are widely adopted to aid in the repair of cartilage defects[225]. Among the cell sources available for cell-based repair procedures, primary chondrocytes still remain a popular option despite the scarcity of donor tissue, as they are the only clinically approved cell source. Additionally, chondrocytes are readily producing cartilage-specific ECM. Although stem cells have become increasingly popular, widespread use of these cells is still not seen due to some inherent limitations and technical challenges. For example, heterogeneity of stem cells (especially those from the bone marrow) can lead to variable results and there is still lack of a standardized protocol relating to the optimum growth factors and culture conditions needed to drive differentiation of stem cells to a stable chondrocyte phenotype.

A very common cell-based therapy known as autologous chondrocyte implantation (ACI) involves isolating chondrocytes from biopsied cartilage samples, followed by *in vitro* expansion and transplantation to cartilage defect sites [226, 227]. Alternatively, these isolated chondrocytes will be used for the construction of implantable tissue grafts in the field of cartilage tissue engineering[228]. The success of these procedures is very much dependent on the number and the quality of chondrocytes isolated[229]. Hence, in line with the first specific aim of this thesis, a high efficiency cell isolation method is required to maximize the yield of chondrocytes from

the limited cartilage tissue samples. [230]

The commonly used protocols for harvesting these cells are based on enzymatic digestion of the extracellular matrix (ECM) with different proteinases, mainly targeting the extensive collagen network by using collagenases[225, 229, 231]. According to protocols published, the concentrations and incubation times of digestion enzymes vary significantly. A combination of trypsin and collagenase or just collagenase alone have been used; in addition, the concentration of the digestion enzyme such as collagenase may also differ from 0.05% to 2% w/v depending on the incubation length used [225]. Despite variations in chondrocyte isolation protocols, chondrocyte isolation efficiencies remained unsatisfactory[225, 232]. Typically, only $1-5 \times 10^6$ cells out of 1×10^8 of the total chondrocytes present in each gram of cartilage were successfully isolated. A large proportion of cells remained entrapped within the cartilaginous matrix, suggesting an incomplete digestion of the ECM, leading to suboptimal isolation efficiency. Thus, there is a need for an improved isolation protocol to optimize harvesting of viable cells.

In this Chapter, we developed a protocol for more efficient primary chondrocyte isolation from articular cartilage tissue, focusing on maximizing cell yield while maintaining quality of the isolated cells. It is well known that serum is a necessary medium component to ensure cell viability during isolation process. However, it also contains factors that would inhibit collagenase activity, thereby reducing cell isolation efficiency. Hence, instead of employing a single long digestion time of 10 to 22 hours as seen in many current protocols, multiple short incubation periods is used. Fresh collagenase solution would be replenished at the end of each digestion round to ensure the continuous activity of collagenase throughout the entire isolation process. [230]

3.2 Materials and Methods

3.2.1 Chondrocyte isolation and culture

Unless stated otherwise, all cell culture related items used were supplied by Invitrogen. Cartilage was extracted from the patellar articular surface of the femur in 5-month-old domestic pigs (approximately 3.5g each, n = 3). All cartilage pieces were washed with phosphate buffer saline (PBS) before they were cut into smaller pieces (~ 2×2 mm in size) in a 100mm petri dish and immersed in 15ml of filtered collagenase containing media for digestion. The collagenase containing media was prepared by dissolving collagenase type II at the concentration of 1mg/ml in Dulbecco's Modified Eagle Medium (DMEM) glutamax, 10% fetal bovine serum (FBS). A total of three rounds of digestions were carried out and their duration were 14 hours (1st digestion), 5 hours (2nd digestion), and 3 hours (3rd digestion) respectively. The remaining cartilage pieces were rinse with PBS before addition of collagenase containing media for subsequent round of digestion. Additionally, a single digestion period of 22 hours was also carried out as a comparison.

3.2.2 Cell harvesting efficiency

After each digestion, supernatant were collected and total cell number was determined using haemocytometer before centrifuging at 1100 rpm to remove collagenase. To calculate the efficacy of cell harvesting during each digestion round, total cell number was counted and normalized by the weight of cartilage digested.

To determine the cell viability of the harvested chondrocytes, live/dead staining was carried out immediately after digestion. Chondrocytes from the same round of collagenase digestion were pooled together to be used for Live/Dead dye staining (Molecular Probes, Invitrogen). Briefly, 0.5 µl of calcein-AM and 2 µl ethidium homodimer were added to every milliliter of cell

suspension. After 30 minutes of incubation at 37°C, the cells were loaded into the flow cytometry (BD LSR II) for analysis. The result presented is based on a record of 10 000 events.

3.2.3 Cell proliferation assay

Cell proliferation capacity was evaluated using WST-1 assay. Briefly, 40 000 cells from each sample were seeded into each well in 96-well plate. At designated time points, media were removed and replaced with a mixture containing 100µl of media and 10 µl of WST-1 reagent. The mixture was incubated with cells for 1.5 hours before reading absorbance at wavelength 450nm with reference wavelength at 620nm.

3.2.4 Gene expression

At designated time points, RNA from chondrocytes was extracted using TRIzol® (Invitrogen). The acquired RNA samples were reverse transcribed to cDNA by M-MLV reverse transcriptase (Promega, Singapore) before the subsequent polymerase chain reaction (PCR). For semi-quantitative PCR, cDNA were amplified by respective primers via the following PCR parameters: 94°C for 60s followed by 35 cycles of 94°C for 30s, 58°C for 30s and 72°C for 30s and finally 72°C for 5min. The amplified product was loaded into 1.5% agarose gel and a constant voltage of 100V was applied for 50min. The gel was then stained ethidium bromide solution before viewing. Real-time quantitative PCR (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad) and gene expression was calculated with the comparative $2^{-\Delta\Delta C_T}$ method, using β -actin and TATA box protein (TBP) as the reference genes and the values were further normalized to that of the 1st digestion group of respective time points. All the primers used in PCR and qPCR were synthesized by AIT Biotech (Singapore) and their sequences were presented in **Table 3-1** and **3-2** respectively.

Table 3-1 Primers used in polymerase chain reaction (PCR). A.T and P.S stand for annealing temperature and product size respectively.

Gene	Gene ID/ Reference	Sequence	A. T.	P. S.
GAPDH	41	Forward: ACCCCTTCATTGACCTCCAC Reverse: ATACTCAGCACCAGCATCGC	58	179
Collagen Type II (Col II)	AF201724.1	Forward: TGTTCTGAGAGGTCTTCCTGGCAA Reverse: AGTCAGACCTCTTCCGCCGTCTTT	58	487
Aggrecan (Agg)	41	Forward: CGAGGAGCAGGAGTTTGTCAAC Reverse: ATCATCACACGCAGTCCTCTC	58	177
COMP	AB086984.1	Forward: TTGTGACAGCGATCAAGACCAGGA Reverse: AAGATGAAGCCCGCATAGTCGTCA	58	516
Collagen Type X (Col X)	AF222861.1	Forward: TGCCAACCAGGGAGTAACAGGAAT Reverse: AAGCCTGATCCAGGTAGCCTTTGA	58	287
Collagen Type I (Col I)	AK223175.1	Forward: ATACGCGGACTTTGTTGCTGCTTG Reverse: TGTCCCTTCAATCCATCCAGACCA	58	556

Table 3-2 Primers used in quantitative polymerase chain reaction (qPCR). A.T. and P.S stand for annealing temperature, product size respectively.

Gene	Gene	ID/	Sequence	A. T.	P. S.
	Reference				
TATA	NM_001172		Forward: AACAGTTCAGTAGTTATGAGCCAGA	58	152
Box	085.1		Reverse: AGATGTTCTCAAACGCTTCG		
protein					
(TBP1)					
Collagen	41		Forward: GCTATGGAGATGACAACCTGGCTC	58	256
Type II			Reverse: CACTTACCGGTGTGTTTCGTGCAG		
(Col II)					
Aggrecan	41		Forward: CGAGGAGCAGGAGTTTGTCAAC	58	177
(Agg)			Reverse: ATCATCACCCACGCAGTCCTCTC		
COMP	NM_007112.		Forward: GGCACATTCCACGTGAACA	58	127
	3		Reverse: GGTTTGCCTGCCAGTATGTC		
Rho A	NM_001664.		Forward: AGCTGGGCAGGAAGATTATG	58	200
	2		Reverse: TGTGCTCATCATTCCGAAGA		
Sox 9	NM_000346.		Forward: GCTGGCGGATCAGTACCC	58	165
	3		Reverse: CGCGGCTGGTACTTGTA		
Collagen	40		Forward: ATACGCGGACTTTGTTGCTGCTTG	58	84
Type I			Reverse: TGTCCCTTCAATCCATCCAGACCA		
(Col I)					

3.2.5 Immuno-histology staining for Collagen type II and F-actin

Primary cells harvested were seeded directly in 24-well plate at a cell density of 200 000 cells/well. Cells at each time point were fixed in 4% (w/v) paraformaldehyde for 1 day. They were gently rinse with PBS before permeabilized with 1% Triton-X for 10min. To prevent non-specific binding of IgG, incubation with 2% bovine serum albumin was carried out for 30min at room temperature before staining. Cells were incubated with Col II (2 µg/ ml in PBS, Chemicon) primary antibody at 4°C overnight and washed with PBS. Subsequently, they were incubated with Anti-IgG (5 µg/ml in PBS, Invitrogen AlexaFluor, 488) at room temperature for 1 hour in the dark. Nuclei were then counterstained by 4',6-diamidino-2-phenylindole (DAPI). The samples were rinsed with PBS washing for 3 times before viewing using fluorescent microscope.

3.2.6 Statistical analysis

Where appropriate, ANOVA is used to analyze results using Tukey or Games-Howell as the post hoc test. Data are presented as mean $\pm$ SD, calculated from 3 specimens from each sample group, and any statistical significance is denoted in the figures.

3.3 Results

3.3.1 Cell-harvesting efficiency

We investigated the effect of multiple enzymatic digestions on cartilage digestion. After each digestion, the volume of cartilage pieces decreased and most of them were completely digested after the third round (**Figure 3-1**). In order to evaluate the cell harvesting efficiency for each digestion, the yield of chondrocytes after each digestion was determined and expressed as the total number of cells harvested per gram of cartilage. As seen in **Figure 3-2b**, the total number of cells per gram of cartilage collected from both second and third digestion was approximately 2.5-

fold higher than the first digestion. Additionally, we also evaluated cell viability via flow cytometry analysis: ethidium homodimer (EthD-1) was used to stain for dead cells while calcein was used to stain for live cells (**Figure 3-2a**). Similarly, the second and third digestion yielded a much higher number of viable cells as compared to the initial round of digestion (2.5-fold and 3.4-fold, respectively). It is interesting to note that although the total number of cells collected during the additional two digestions was similar, the third digestion yielded the highest percentage of isolated viable cells (as high as 76.8%). Although a single 22h digestion was shown to digest 2.22g of cartilage, only about 4 million cells were harvested and of which, 2.34 million of them were viable from each gram of cartilage (**Figure 3-7**). Compared to the proposed multiple digestions method, a single long digestion period yielded 7 times less total viable chondrocytes.

Figure 3-1. Cartilage fragments before and after each round of digestion, showing a progressive decrease in cartilage volume with increasing digestion.

Digestion round	Cartilage digested (gram)	Total cells harvested (10 ⁶)	Total cell number per gram of cartilage (10 ⁶)	Cell viability (%)	No. of viable cell per gram of cartilage (10 ⁶)
1st	0.82±0.29	3.75±1.40	4.55±0.78	56.0	2.55±0.44
2nd	1.29±0.41	14.54±4.10	11.38±0.86	55.8	6.37±0.48
3rd	1.40±0.09	15.57±1.82	11.14±1.21	76.8	8.56±0.93

Figure 3-2. a) Cell viability of the cells harvested at each round of digestion determined using live/dead reagent staining and subsequently analysed through flow cytometry. **b)** Table showing the amount of cartilage digested, total cells number and the amount of viable cells harvested at each round of digestion.

3.3.2 Cell proliferation

Besides determining cell-harvesting efficiency, it is also important to characterize the harvested cells to ensure that the introduction of two more digestion steps does not affect the cells, both structurally and functionally. We first examined cell proliferative capacity after the cells were plated and cultured for 1, 4 and 7 days using WST-1 proliferation assay (**Figure 3-3**). As compared to the first digestion, the two additional digestions did not affect the capacity of the cells to proliferate. In fact, cells harvested from the second digestion consistently showed the

highest cell proliferation as indicated by the highest absorbance value among the three but the difference was especially significant on day 4 and day 7. At day 4, cells from the third digestion also showed significantly higher cellular proliferative activity as compared to the first digestion. Although the same phenomenon was not observed on day 7, their proliferative activity still remained comparable to that of the control group.

Figure 3-3. Cell proliferation assay using WST-1. * indicates $p < 0.05$ while ** indicates $p < 0.01$ when compared to 1st round of digestion.

3.3.3 Gene expression

In order to evaluate the effect of multiple digestions on cartilage-specific phenotype, both PCR and qPCR were performed to investigate the expression of chondrocyte markers. As shown by the PCR results in **Figure 3-4**, all important chondrocyte markers tested were expressed, except for some irregularities observed for collagen type II on early time points (day 1 and day 4). Of

particular importance was the expression of collagen type I, which was not observed throughout the entire experimental period. It is also interesting to note that the expression of collagen type X was high initially, but its expression was attenuated on day 7, as suggested by the decrease in band intensity. As for the qPCR results (**Figure 3-5**), cells from the third digestion generally showed an upregulation for all the anabolic genes tested (col II, Agg, COMP, Sox 9). The increase in gene expression levels was most significant on day 4 for all the important cartilage matrix components tested, namely Col II, aggrecan and COMP ($p<0.01$). The cells isolated from the second digestion also showed a marked increase in all their anabolic gene expressions at day 4, except for COMP. Besides the anabolic genes, we also analysed the expression of Rho A and collagen type I. The expression levels of Rho A for both the second and third digestion were comparable to, if not higher, than the control group. As for collagen type I, we observed that cells from the two additional digestions showed significantly lower expression for this gene as compared to the first digestion towards the end of the experimental period ($p<0.05$).

Figure 3-4. Gel electrophoresis of the chondrocyte markers in cells isolated from various rounds of digestion.

Figure 3-5. qPCR analysis of the chondrocyte markers in the cells isolated from different rounds of digestion. * indicates $p < 0.05$ while ** indicates $p < 0.01$ when compared to the 1st round of digestion at each respective time point.

3.3.4 ECM synthesis and cell morphology

After examining the transcriptional levels of collagen type II, immunofluorescent staining was also performed on the harvested cells for this protein (**Figure 3-6a**). Regardless of digestion round, all three groups shown to produce comparable amount of collagen type II. Apart from protein expression, the cell morphology was also analysed by phalloidin staining (**Figure 3-6b**).

Over the 7-day culture period, the cell appearance took on the usual polygonal shape as seen in typical monolayer-cultured chondrocytes. There was no observable difference in cell spreading area among the three groups.

Figure 3-6 a) Immune-staining of collagen type II in various samples groups at Day 4 and Day 7. b) Phalloidin staining of cytoskeleton in cells harvested from different rounds of digestion. Cell nuclei were counter-stained with DAPI.

Figure 3-7 Analysis of chondrocytes after 22 hours of single digestion. a) Cartilage fragments after digestion b) Cell viability of the cells harvested by flow cytometry c) Phase contrast image chondrocytes harvested showing presence of cell debris d) Table showing the amount of cartilage digested, total cells number and the amount of viable cells harvested.

3.4 Discussion

Chondrocyte isolation from cartilage is one of the most critical steps in determining the success of cell-based repair procedures such as autologous chondrocyte implantation (ACI) and cartilage tissue engineering. To date, the efficiency of cell isolation from native cartilage still remains suboptimal. As reported by previous studies, common isolation protocols only yield about 1-5 million viable chondrocytes per gram of cartilage, which is well below the total number of available cells in the native cartilage[225, 229]. In light of this, an improved protocol with enhanced cell isolation efficiency was developed. Instead of performing the usual one round of digestion in collagenase for a prolonged period of time (10-22 hours)[233-236], this new protocol essentially involves three consecutive digestions that comprises of 14 h, 5 h and 3 h

respectively. For each round of digestion, freshly-prepared digestion medium containing 0.1% (w/v) of collagenase type II is employed.

The chondrocyte isolation efficiency in this proposed protocol is evaluated based on two criteria: cell yield and cell quality, which are the two major concerns in the development of any cell isolation protocol. From the results obtained in this study, we improved cell yield by more than 5-fold: we successfully isolated a total of 1.75×10^7 cells per gram of viable chondrocytes as compared to the 2.34×10^6 cells per gram of cartilage in a single 22 hour digestion (**Figure 3-7**). Higher cell yield is a direct result of the multiple digestion method employed, allowing more complete cartilage digestion, as evidenced by the greater reduction in volume of the digested cartilage fragments (**Figure 3-1**). Furthermore, the use of fresh digestion medium at each digestion also helped to ensure consistently high proteolytic activity from the collagenase, thus resulting in the release of more cells from the collagen fibril network[225]. Interestingly, it is also noted that the two additional digestions yielded much more viable cells than the first digestion even though the digestion periods are much shorter. This observation can be attributed to the first digestion that acted as a priming step for the initial disruption of the extensive collagen networks in cartilage ECM, thereby facilitating subsequent cell retrieval. Another possible reason is the collection of cells at each round of digestion reduced the collagenase exposure in the isolated chondrocytes, leading to higher cell viability. This is evident from the 22 hours digestion data where substantial amounts of small cell debris were observed (**Figure 3-7 (c)**). It is hypothesized that this is a result of apoptosis of the isolated cells due to long hours of continuous collagenase digestion.

Besides cell viability, successful cell-based repair procedures also require high cell quality[237, 238]. In other words, isolated cells must retain their proliferative capacity and

tissue-specific phenotype when cultured *in vitro*. Therefore, the effect of additional digestions on the proliferative capacity, gene expression, ECM synthetic activity, and cell morphology were assessed. WST-1 proliferation assay (**Figure 3-3**) and gene expression analysis of Rho A (**Figure 3-5**) collectively showed that the newly developed protocol had no adverse effects on the proliferative capacity of the isolated cells. In fact, we observed that cells obtained from the additional rounds of digestion generally proliferated at a higher rate.

To evaluate the maintenance of phenotype in the chondrocytes isolated from all three digestion rounds, gene expression of various hyaline cartilage markers were examined. The gel electrophoresis results confirmed that cells from all three digestions strongly expressed collagen type II, aggrecan and COMP (**Figure 3-4**). As mentioned, there were some irregular expressions of collagen type II. We believed that such irregularities are due to the constant exposure to high collagenase activity. Chondrocytes from the second and third digestions experienced a total of 19 and 22 hours of collagenase exposure respectively and require time for recovery[232] and thus did not express any Col II on Day 1 but only on Day 4. Collagen type I, a typical dedifferentiation marker for chondrocytes, remained undetected. To quantify the gene expression among different sample groups, qPCR was carried out. From the results obtained, the cells harvested from the third digestion were observed to have the highest expression levels for the anabolic genes examined. The zonal structures and depth-dependence on collagen crosslinking extensiveness were previously mentioned[239-241], which may be a possible explanation for the results observed. It is hypothesized that the chondrocytes retrieved from the third digestion may contain a higher proportion of cells collected from the deeper zones of the native cartilage, which were reported to have greater synthetic capabilities for the main ECM components of cartilage than the superficial zone cells[242].

Although collagenase digestion may cause severe disruptions to cellular structure, especially after prolonged treatment[232], cells harvested in from the additional digestion protocol have no observable morphological difference for the cells collected as compared to those from the first digestion, suggesting that the additional rounds of digestion did not cause any cell behavioural change. The results of this study generally suggested that the cells retrieved using this new protocol retained their chondrocytic phenotype and are manifested in their ability to synthesize cartilage-specific ECM. This is particularly important for the construction of functional tissue-engineered constructs since the production of cartilaginous materials is very much dependant on the cells seeded in the construct[232].

3.5 Summary

In conclusion, this study suggests that a more efficient protocol for isolating chondrocytes is established. This protocol has greatly improved cell yield for primary chondrocyte isolation by more than 5-fold, and also yielded cells with good quality. Therefore, we suggest that neither a longer digestion time nor a higher collagenase concentration that is necessary; instead, multiple short digestions with freshly prepared digestion medium (0.1% w/v of collagenase) may be the technique to be used. The use of this isolation protocol is recommended especially for isolating cells from biopsied cartilage tissue samples in adult species for efficient utilization of the limited sample source.

4 A preclinical evaluation of an autologous LhCG for articular cartilage repair.

4.1 Introduction

Chapter 1 of this thesis introduced some of the biochemical and biomechanical properties and functions of the articular cartilage. As mentioned, articular cartilage is a highly organized connective tissue with poor reparative capacity due to its low metabolic activity and avascular nature[243]. Owing to its load-bearing function, focal cartilage lesions of the knee are common and are typically located on the femoral condyles[3]. Inappropriate management of knee-joint related injuries may lead to serious downstream problems like osteoarthritis (OA), a cartilage degenerative disease that severely affects patient quality-of-life[244]. Currently, a variety of surgical techniques have been developed for treating cartilage-related injuries, including microfracture, mosaicplasty and autologous chondrocyte implantation (ACI)[245].

Microfracture is a marrow stimulation technique and is popularly employed as a first-line treatment for articular cartilage defects in the knee[62]. It usually results in fibrocartilaginous repair tissue, which is mechanically inferior to the native hyaline cartilage. This technique provides effective short-term symptomatic relief and functional improvement up to 2 years post-treatment but its long-term performance is debatable as the repaired tissue often shows signs of deterioration.[62, 66, 246, 247] Osteochondral transplantation (or mosaicplasty) is another long-standing surgical option. However, it suffers from limited donor source and inadequate host-implant integration[248]. Among the clinically approved procedures, ACI and its derivatives are the most hopeful for restoring structural properties of the native hyaline cartilage. However, reports have shown that the clinical outcome of ACI is merely comparable to those achieved by

microfracture, with no obvious functional improvements and pain relief[249, 250]. This cell-based technique is plagued with problems related to the dedifferentiation of cells and graft hypertrophy [251]. Hence, it is clear that there is an unmet need for a truly effective treatment modality that provides both short-term relief and long-term repair.

The tissue engineering approach offers hope to restore articular cartilage, and has since evoked widespread interest over the past decade [252]. Its principle involves combining a variety of cell sources, scaffold materials and bioactive factors to aid cartilage repair [77-79]. This appears to be advantageous over the direct usage of cells, which may be implicated by cell handling and delivery issues[76]. However, significant challenges remain with regards to the generation of a durable articular cartilaginous graft *in vivo*. This is largely due to an impure cartilage matrix, which compromises neotissue quality[252]. In order to achieve a pure cartilage matrix with hyaline-like phenotype, scaffold-free approaches are attractive as they minimize the introduction of any exogenous materials from scaffolds into the neotissue[80]. Additionally, a three-dimensional (3D) environment is crucial to prevent chondrocyte dedifferentiation, a process that causes them to lose their differentiated phenotype. In view of these requirements, a unique methodology that allows chondrocytes to form neocartilage through an intermediate scaffolding system was developed [102]. The transient use of scaffolds prevents the long-term involvement of exogenous scaffold materials to preserve tissue purity. In addition, this adopted, non-cell adhesive 3D culture strategy hinders focal cell adhesion, thus avoiding dedifferentiation of chondrocytes induced by monolayer culture expansion[12]. The end product is a 3D, scaffold-free construct known as the Living Hyaline Cartilage Graft (LhCG). As demonstrated in Chapter 3, this thesis has further improved on the LhCG platform by enhancing cell chondrocyte isolation

efficiency through a multiple digestion method. This chapter employs chondrocytes isolated using this method to fabricate LhCG constructs.

In this chapter, a preclinical study was conducted in skeletally mature mini-pigs with the eventual aim of achieving clinical translation of LhCG. This 6-month study was designed to validate the performance of autologous LhCG engraftment. Skeletally mature mini-pigs were chosen as their joint sizes and cartilage thickness are amenable to clinically relevant manipulations and outcome assessments[253]. The major load-bearing regions located on the femoral condyles were selected as the experimental site for creation of full-thickness chondral defects followed by LhCG engraftment while the minor load-bearing regions located on the femoral trochlear groove areas were adopted as the donor site for autologous chondrocytes. The study duration was deliberately designed to determine the suitability of LhCG for *in situ* cartilage repair studies with the knowledge that fibrotic scar tissue becomes detectable in the same amount of time[254]. Live magnetic resonance imaging (MRI) was performed at stipulated time-points to assess cartilage repair. At the study endpoint, gross examination and histological outcomes of the repair were assessed. Additionally, repair tissue was quantitatively analysed in terms of its biochemical composition and biomechanical properties, and compared to native articular cartilage.

4.2 Materials and methods

4.2.1 Experimental design and study population

Animal surgeries were performed in line with the guidelines for the care and use of laboratory animals, and protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Five skeletally matured mini-pigs (15-17 months of age; mean body weight, 29kg)

were used for this study. They were supplied by at PWG Genetics Pte Ltd (GLP certified contract research organization), Singapore and were maintained under specific-pathogen-free conditions. Two surgeries were performed: the first surgery was for cartilage biopsy harvesting and the second surgery was performed after two months for LhCG engraftment.

4.2.2 Cartilage biopsy harvesting

During the first surgery, small cartilage discs (eight per knee, each with a diameter of 4mm) were harvested for cell isolation and expansion from a minor load-bearing region of the host medial and lateral upper femoral trochlear area using a biopsy punch.

4.2.3 Autologous chondrocytes isolation and *in vitro* expansion

Unless stated otherwise, all cell-culture reagents were supplied by Invitrogen, Life technologies. About 300-400mg of cartilage discs were harvested from each mini-pig. After which, the cartilage discs were rinsed in sterile Phosphate Buffered Saline (PBS), followed by thorough washing in penicillin (100 units /mL) and streptomycin (100 mg/ mL). The discs were then minced into fragments using tweezers and surgical blades and subsequently being digested in culture media containing collagenase II at a concentration of 1 mg/mL. Following enzymatic digestion, chondrocytes isolated were plated individually (according to different pigs) in tissue culture flasks and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units /mL) and streptomycin (100 mg/ mL).

4.2.4 Autologous Living Hyaline Cartilaginous Graft (LhCG) preparation

The fabrication and characterization of LhCG were previously done and the protocol for establishing LhCG was used this study[103]. Unless specifically mentioned, all chemicals used were purchased from Sigma Aldrich, Singapore while all cell-culture reagents were purchased from Invitrogen, Life Technologies. Briefly, gelatin microspheres with a porogenic role were

fabricated using a oil-in-water-in-oil double-emulsion system and sieved into a size range of 150 to 180 μm . Subsequently, the microspheres were sterilized in PBS containing 1000 units/mL of penicillin and 1000mg/mL of streptomycin overnight and stored in sterile PBS at 4°C. Upon reaching 80% confluence, autologous chondrocytes (described in previous section) were individually trypsinized, washed and centrifuged before being co-suspended (1×10^7 cells/mL) with gelatin micropsheres in chilled alginate solution (1.5% w/v in 0.15M NaCl, 4°C). The co-suspension was later transferred into a 35mm petri dish and 102 mM aqueous calcium chloride was added gently for gelation. Each individually matched LhCG was then trimmed into dimensions of circa 6 mm x 5 mm x 1.5 mm and cultured on agarose gel-coated tissue culture plates in chondrocyte culture (CC) medium that composed of Dulbecco's modified Eagle medium (DMEM) supplemented with 20% (v/v) fetal bovine serum (FBS, Invitrogen), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES, 0.01 M), non-essential amino acids (NEAA, 0.1 mM), proline (0.4 mM), vitamin C (0.05 mg/mL), penicillin (100 units/ mL), and streptomycin (100 mg/mL). The LhCGs were maintained on an orbital shaker at 50 rpm in a humidified incubator at 37°C with 5% CO₂. When the LhCGs reached maturity after 35 days, they were immersed in sodium citrate (55 mM in 0.15 M NaCl) for the removal of alginate bulk (10 mins, at room temperature) and were cultured for another ten days prior to implantation.

4.2.5 Autologous LhCG engraftment into full-thickness chondral defects

All surgeries were performed under general anaesthesia, which was induced by intramuscular administration of Atropine (0,05mg/kg) followed by xylazil (2.5mg/kg) and ketamine (10mg/kg). Additionally, the animals were intubated using an appropriate size cuffed endotracheal tube and anaesthesia was maintained by inhalation of 1-3% of isoflurane. Full thickness chondral defects of 6.0-6.3 mm in diameter and 1.5-2.0 mm in depth were created on both condyles using biopsy

punches. LhCGs were punched out using the same biopsy punches and were press-fitted into the defect either on the medial or lateral condyles (randomly assigned) while the other defect was left empty. This interference fit, coupled with the nature of LhCG, which is sponge-like, no further fixation was needed to secure the graft. The mini-pigs were allowed to move freely after the surgery. The three experimental groups designed for this study are given in **Table 4-1**.

Table 4-1 Abbreviations for the three experimental groups.

Experimental group	Abbreviation
LhCG-repaired tissue	<i>LhCG+</i>
Untreated control	<i>LhCG-</i>
Donor site for autologous cells	<i>ADS</i>

4.2.6 Magnetic Resonance Imaging (MRI)

The mini-pigs were imaged under general anesthesia at 2 months and 6 months post-surgery. MRI images of the entire knee joints were obtained using an 18-channel body matrix coil on a clinical 3T MRI Scanner (Siemens, Skyra). A fat-saturated proton density-weighted MR imaging sequence was used, without any contrast agent. All three planes were acquired. Imaging parameters were as follows: repetition time (TR) = 3800 ms, echo time (TE) = 39 ms, field of view (FOV) = 130 mm, matrix size = 384×384 , slice thickness = 2 mm, slice gap = 0.2 mm. MRI images were independently scored by an experienced radiologist, who specializes in musculoskeletal imaging and is blinded to experimental data, using a modified clinical 2D MOCART scoring system [255, 256]

4.2.7 Implanted tissue harvesting

Six months following engraftment, the mini-pigs were sacrificed by intravenous injection of pentobarbital (150 mg/kg). The entire knee joints were collected, exposed, and photographed. A surgical saw was used to collect an area of surrounding host tissue containing the implant and underlying bone from each defect site.

4.2.8 Macroscopic examination

The repaired cartilage was examined macroscopically and scored using International Cartilage Repair Society (ICRS) scoring system for cartilage repair [257]. After macroscopic evaluation, half of the samples were used for histological and immunohistochemical staining while the remaining samples were used for biomechanical testing followed by biochemical analysis.

4.2.9 Histological evaluation

Collected samples were fixed in 10% buffered formalin, and decalcified, followed by standard histological processing. Histology slides were stained with H&E, Safranin-O, Masson's Trichrome, and picrosirius red according to standard protocols. Regenerated cartilage was scored using the scoring system described by Wakitani and colleagues[258]. The criteria considered by this histological scoring system include cell morphology, matrix staining, surface regularity, cartilage thickness, and integration with host adjacent cartilage. Samples were scored independently by three evaluators who were blinded to experimental data. The scores of the three observers were averaged to obtain a representative score for each criterion.

4.2.10 Immunohistochemical staining

For immunohistochemical detection of type I and II collagen, tissue sections were deparaffinized and rehydrated and stained using UltraVision Quanto Detection System HRP DAB kit (Thermo Scientific) according to the manufacturer's protocol.

4.2.11 Biomechanical Testing and Biochemical analyses

Normal articular cartilage in the same weight-bearing region from mini-pigs served as a normal control in the biomechanical testing. For the analysis of biomechanical properties, the compressive modulus of the collected samples were measured as described by Cui et al.. Briefly, LhCG-repaired tissue and auto-regenerated tissue were punched out for biomechanical analyses (diameter, 4mm) using biopsy punches. A constant compressive strain of 1 mm/min was applied until reaching the maximal force at 400 N and a force–displacement curve was obtained. The compressive modulus of the tested tissue was automatically calculated by the auxiliary software in the equipment (Instron, USA).

After biomechanical testing, the samples were used for biochemical analysis. Briefly, samples were frozen at -20°C and freeze-dried for 24 h, followed by overnight digestion in papain buffer consisting of 0.3 mg/ml papain dissolved in 0.2 mM dithiothreitol and 0.1 mM disodium ethylene diamine tetraacetic acid. Chondrocyte density was extrapolated from DNA content measured by the fluorometric Hoechst 33258 assay. Glycosaminoglycan (GAG) content was measured by 1,9-dimethylmethylene blue (DMMB) dye binding assay while total collagen content was quantified by using proline/hydroxyproline assay from acid hydrolyzed samples.

4.2.12 Statistical analysis

All data presented are in the form average $\pm$ standard deviation. **Table 4-2** describes the statistical analyses used to determine statistical significance in this study (all at 95% confidence interval).

Table 4-2 Statistical analyses performed on the different data sets obtained in this study.

Statistical Test type / Post-hoc (if any)	Data set
Two-tailed, paired t-test	2D MOCART score
Nonparametric Mann Whitney test	Macroscopic repair score
Two-tailed, unpaired t-test	Cartilage thickness measurement
One-way ANOVA / Dunnett's Multiple Comparison Test	Biochemical and biomechanical analyses

4.3 Results

4.3.1 Preparation of autologous LhCG for engraftment

The preparation of autologous LhCG for engraftment into the load-bearing regions of the knee joints was performed according to the timeline presented in **Figure 4-1(a)**. The whole process began with harvesting articular cartilage biopsies from the minor load-bearing femoral trochlear groove regions, from which autologous chondrocytes were then extracted (**Figure 4-1(b)-i~iii**). To allow spontaneous healing, the defect size at these autologous donor sites (*ADS*) was 4mm in diameter, which is below the reported critical sized defect of 6 mm in adult pigs [259]. Upon cell extraction and one passage (P1) of *in vitro* expansion, the chondrocytes were then used to construct LhCGs.

Figure 4-1 Experimental design (a) A timeline showing the various experimental steps of this study, spanning a total period of 8 months. (b) A schematic diagram showing the detailed process flow of this study. The study began with (i) identification of donor site, followed by (ii) biopsy harvesting from the minor load-bearing femoral trochlear groove regions, and (iii) chondrocytes extraction. After one passage (P1) of *in vitro* expansion, the chondrocytes were (iv) co-suspended and then (v) co-encapsulated with gelatin microspheres in alginate hydrogel. The gel constructs were cultured at 37°C to dissolve the gelatin microspheres, resulting in micro-cavities formation. The resultant (vi) micro-cavitory hydrogel constructs (MCG) promoted vigorous cell proliferation and condensation into isogenic groups, eventually sprouting from the gel phase into the micro-cavities (PTCC)[103, 260]. Upon prolonged culture, each neotissue aggregate that filled the micro-cavities eventually connected to one another, resulting in (vii) a 3D interpenetrating network (IPN). Following alginate gel removal, LhCG was generated in (viii). The LhCGs were then trimmed and implanted (ix) into critical-sized defects on the femoral condyles

(indicated as expt. site in (x)). (c) **Histological evaluation of the autologous LhCGs, showing the typical rounded morphology of chondrocytes, as well as their GAG and type II collagen-rich ECM (scale bar: 200 μ m).**

The protocol for LhCG preparation has been established in our previous work [103] and is depicted in **Figure 4-1(b)-iv~viii**. Briefly, autologous chondrocytes (P1) were co-suspended and then co-encapsulated with gelatin microspheres (~200 μ m in diameter) in alginate hydrogel. When the gel constructs were cultured at 37°C, the gelatin microspheres rapidly dissolved and leached out of the constructs, leaving behind micro-cavities of the same size throughout the cell-laden gel phase. These resultant micro-cavitary hydrogel constructs (MCG) allow cells to proliferate vigorously and condense into isogenic groups, eventually sprouting from the gel phase into the micro-cavities (Phase Transfer Cell Culture or PTCC) [103, 260]. Following prolonged culture, the micro-cavities were filled with cartilaginous neotissue aggregates formed by the outgrowing chondrocyte isogenic groups. The aggregates in each micro-cavity eventually connected to one another, resulting in a 3D interpenetrating network (IPN) of the neotissue and the alginate gel phase. Upon complete removal of the alginate gel with citrate buffer, LhCG was generated. The whole process lasted two months, which is inclusive of the initial monolayer cell expansion (7~10 days), PTCC in MCGs (35 days) and the final maturation phase of LhCG (10~14 days).

Prior to engraftment, autologous LhCGs were evaluated histologically to confirm their hyaline-like phenotype. As seen in **Figure 4-1(c)**, the staining results demonstrated that the chondrocytes maintained their typical rounded morphology and were surrounded by abundant ECM, which consists of glycosaminoglycan (GAG) and type II collagen. The presence of type I collagen, a fibrocartilage-related marker, remained undetectable. The results suggested that the autologous LhCGs that were produced are of hyaline-like phenotype. The engraftment surgery

for autologous LhCG was performed after two months, calculated from the point of articular cartilage biopsies harvesting to LhCG maturation. During the surgery, full-thickness critical-sized defects (6mm in diameter) were created on the femoral condyles. The autologous LhCG were then trimmed to be press-fitted into the freshly created defects (**Figure 4-1(b)-ix~x**). These LhCG-engrafted sites were labelled as *LhCG+*. In parallel, 6mm defects that were kept empty were used as negative controls and labelled as *LhCG-*.

4.3.2 Live MRI monitoring of articular cartilage repair in mini-pigs

In order to monitor the progress of repair in a non-invasive manner, magnetic resonance imaging (MRI) was performed at 2-month and 6-month post-engraftment (**Figure 4-2**). Subsequently, the MRI images were scored using the 2D MOCART scoring system shown in **Table 4-3**. At 2 months, the *LhCG+* group performed comparably to the *LhCG-* group. However, at 6 months, the *LhCG+* group showed a significant improvement (p value < 0.05) in the total score (**Figure 4-2(c)**). More specifically, *LhCG+* out-performed *LhCG-* in terms of cartilage resurfacing, where 4 out of the 6 surfaces were restored in the former while all surfaces were damaged in the latter. Furthermore, 5 out of the 6 of the repaired tissue in the *LhCG+* group had a homogeneous structure while all self-repaired tissue in the *LhCG-* group showed inhomogeneous structure or cleft formation.

Figure 4-2 MRI and clinical evaluation of articular cartilage repair. Proton-density weighted MRI images in the (a) sagittal plane and (b) coronal plane for *LhCG+* and *LhCG-* group at two months (baseline) and six months show progressive tissue repair after *LhCG* engraftment into the full-thickness chondral defects located at the major load-bearing regions of the knee (condyles). Arrows indicate the defect sites. (c) The total average scores were derived from (a) and (b) for both experimental groups obtained using the 2D-MOCART scoring system. A high score (min. 0; max. 100) indicates native-like cartilage tissue. Two-tailed, paired t-test was used for data analysis. Data are presented in the form of mean $\pm$ SD, with N = 5. Abbreviations: *LhCG+*, LhCG-repaired cartilage; *LhCG-*, self-repaired cartilage.

Table 4-3 2D MOCART evaluation of cartilage repair. Assessment of articular cartilage repair tissue in the femoral condyles by using the modified clinical 2D magnetic resonance observation of cartilage repair tissue (MOCART) score [255, 256].

2D MOCART score		
Parameter	Item	Points
Defect fill	Subchondral bone exposed	0
	Incomplete < 50%	5
	Incomplete > 50%	10
	Complete	20
	Hypertrophy	15
Cartilage interface	Complete	15
	Demarcating border visible	10
	Defect visible < 50%	5
	Defect visible > 50%	0

Surface	Surface intact	10
	Surface damaged < 50% of depth	5
	Surface damaged > 50% of depth	0
Adhesions	Yes	5
	No	0
Structure	Homogeneous	5
	Inhomogeneous or cleft formation	0
Signal intensity	Normal	30
	Nearly normal	10
	Abnormal	0
Subchondral lamina	Intact	5
	Not intact	0
Subchondral bone	Intact	5
	Granulation tissue, cyst, sclerosis	0
Effusion	No effusion	5
	Effusion	0
Total points		100

4.3.3 Macroscopic examination

Upon completion of the study at 6 months post-engraftment, the animals were sacrificed and their knees were excised and examined. In the *LhCG+* group, full-thickness defects were repaired with glossy white tissue, which was comparable to normal articular cartilage in appearance. Contrastingly, defects in the *LhCG-* group remained insufficiently filled (**Figure 4-3 (a)**). The average ICRS (International Cartilage Repair Society) macroscopic score based on **Table 4-4** [257] of the *LhCG+* group (8.11 ± 1.96) was significantly higher than that of the *LhCG-* group (4.11 ± 2.85) (Fig. 3(b), $p < 0.05$). The individual breakdowns of the score are shown in Fig. 3(c). Mean scores for the *LhCG+* group in terms of defect filling ($p < 0.01$), integration to surrounding host cartilage ($p < 0.05$), and macroscopic appearance ($p < 0.05$) were all significantly higher than those of the *LhCG-* group. In line with the MRI results, macroscopic evaluations also suggested positive repair in the *LhCG+* group while such observations were lacking in the *LhCG-* group.

Figure 4-3 Macroscopic evaluation of cartilage repair at 6 months post-engraftment. (a) Representative photographs showing the macroscopic repair outcome. Full-thickness defects in the *LhCG+* group were filled with glossy white tissue while defects in the *LhCG-* group remained insufficiently filled. (b) ICRS macroscopic mean scores for the two experimental groups. A high score (min. 0; max. 12) indicates native-like cartilage tissue.

(c) The scores for the individual parameters in the ICRS scoring system. Nonparametric Mann Whitney test was used for data analysis. (*) indicates statistical significance difference ($p<0.05$); (**) ($p<0.01$). Data are presented in the form of mean $\pm$ SD, with N = 5.

Table 4-4 ICRS Score. Assessment of macroscopic repair by using the International Cartilage Repair Society (ICRS) score for cartilage repair [257]

Cartilage repair assessment ICRS	Points
Degree of defect repair	
In level with surrounding cartilage	4
75% repair of defect depth	3
50% repair of defect depth	2
25% repair of defect depth	1
0% repair of defect depth	0
Integration to border zone	
Complete integration with surrounding cartilage	4
Demarcating border < 1 mm	3
3/4th of graft integrated, 1/4th with a notable border > 1 mm width	2
1/2 of graft integrated with surrounding cartilage, 1/2 with a notable border > 1 mm	1
From no contact to 1/4th of graft integrated with surrounding cartilage	0
Macroscopic appearance	
Intact smooth surface	4
Fibrillated surface	3
Small<comma> scattered fissures or cracs	2
Several<comma> small or few but large fissures	1
Total degeneration of grafted area	0
Overall repair assessment	
Grade I: normal	12
Grade II: nearly normal	11-8
Grade III: abnormal	7-4
Grade IV: severely abnormal	3-1

4.3.4 Histological and immunohistochemical evaluation

In order to identify the quality of the repaired tissue, *LhCG+*, *LhCG-*, and *ADS* group were histologically evaluated. Using the histological images, the average cartilage thickness for each group was determined at the defect regions and their surrounding, unaffected regions. As

presented in **Figure 4-4 (a)**, the thickness of the *LhCG+* group was found to be the closest to that of its surrounding cartilage and also the highest among all groups. Similar results were obtained for the *ADS* group. In contrast, the thickness value measured from the *LhCG-* group was found to be significantly lower than its surrounding tissue, particularly at the central regions of the defects ($p < 0.001$).

As shown in (**Figure 4-4 (b) ~ (f)**, upper panel, centered at location (B)), the repaired cartilage generated in the *LhCG+* group displayed tissue morphology similar to normal hyaline cartilage. At higher magnification, the cells appeared like well-differentiated chondrocytes and were surrounded by a matrix that is comparable to those of the adjacent host cartilage. More specifically, glycosaminoglycans (GAG, Safranin-O stains) and type II collagen (**Figure 4-4 (d) & (e)**, upper panel, centered at location (B)) were evident throughout the tissue while type I collagen (**Figure 4-4 (f)**, upper panel, centered at location (B)) was minimal. In contrast, untreated defects in the *LhCG-* group (**Figure 4-4 (b) ~ (f)** (middle panel, centered at location (E))) were filled mostly with disorganized fibrocartilage that did not restore a continuous articular surface with adjacent host cartilage. Furthermore, there was reduced staining for GAG (**Figure 4-4 (d)**, middle panel, centered at location (E)) and type II collagen (**Figure 4-4 (e)**, middle panel, centered at location (E)) while type I collagen staining was intense (**Figure 4-4 (f)**, middle panel, centered at location (E)), suggesting that the self-repaired tissue was not healthy hyaline cartilage.

In terms of cellular organization, the *LhCG+* group showed a greater extent of columnar organization than the *LhCG-* group. Additionally, repaired cartilage generated in the *LhCG+* group was also observed to integrate well with the surrounding cartilage without gaps or discontinuities. As far as the *ADS* group is concerned (**Figure 4-4 (b) ~ (f)**, lower panel, centered

at location H), in which the defects were created for autologous cell extraction and left untreated for eight months, the defects showed significantly better self-repair as compared to the *LhCG*- group. Upon closer examination, the *ADS* group showed a more fibrous structure in the matrix and reduced staining for GAG as compared to the surrounding, unaffected cartilage (**Figure 4-4 (c) & (d)**, lower panel, centered at location H). Additionally, the immunostaining results indicated that both type I and type II collagen were present in deeper parts of the self-repaired tissue. At the articulating surface, however, type I collagen was particularly abundant while type II collagen was minimally present (**Figure 4-4 (e) & (f)**, lower panel, centered at location H). All these observations suggested that fibrocartilage was predominantly lining the surface of the self-repaired tissue while hyaline-like cartilaginous tissue was generated in deeper parts.

Based on the histological and immunohistochemical staining outcome, mean histological scores for all three groups were obtained using Wakitani scoring system as shown in **Table 4-5** [258]. As presented in **Figure 4-4(g)**, the overall mean score for the *LhCG*+ group (1 , no standard deviation) was significantly lower than that of the *LhCG*- (9 ± 1) and the *ADS* group (3 ± 0.58), indicating a hyaline-like repair. The scores for five key parameters of chondral restoration, which contribute to the overall histological scores of each sample, were displayed in **Figure 4-4(h)**. The parameters that best distinguished the *LhCG*+ group from the other two groups were: tissue morphology, matrix staining, and tissue integration.

Figure 4-4 Histological and immunohistochemical analyses of the repaired tissue in the *LhCG+*, *LhCG-* and *ADS* groups. (a) The average cartilage thickness for each group was determined at the defect regions and their surrounding, unaffected regions. Two-tailed, unpaired t-test was used for data analysis. Evaluation of the repaired cartilage phenotype was based on: (b) H&E; (c) Masson's Trichrome; (d) Safranin-O; (e) immunohistochemical staining for type II collagen; (f) immunohistochemical staining for type I collagen ((A–I) showed higher-magnification images at each specified location). Long scale bar: 2mm; short scale bar: 200µm. (g) The tallied histological scores based on the Wakitani scoring system. A low score (min. 0; max. 16) defines a native-like cartilage tissue with good defect filling and good integration with adjacent host cartilage. (h) Total average score for each specific parameter that contributed to cartilage restoration. The *LhCG+* group (Upper panels in (b)-(f)) was repaired with hyaline-like tissue, which integrated well with adjacent host cartilage. The untreated group, *LhCG-* (Middle panels in (b)-(f)) was filled with irregular fibrocartilaginous tissue, which stained positively for type I collagen but not for type II collagen. The defects in the *ADS* (Lower panels in (b)-(f)) group self-repaired and showed no signs of degeneration. (*) indicates statistical significance ($p<0.05$); (*) ($p<0.001$). Each set of colored arrows demarcates individual defect sites. Data are presented in the form of mean $\pm$ SD, with N = 5.**

Table 4-5 Wakitani score. Histological evaluation of the cartilage repair tissue using a scoring system developed by Wakitani et al. [256].

Cell morphology	
Hyaline cartilage	0
Mostly hyaline cartilage	1
Mostly fibrocartilage	2
Mostly non-cartilage	3
Non-cartilage only	4
Matrix staining (metachromasia)	
Normal (compared with host adjacent cartilage)	0
Slightly reduced	1
Markedly reduced	2
No metachromatic stain	3
Surface regularity (total smooth area compared with entire area of cartilage defect)	
Smooth ($> 3/4$)	0
Moderate ($> 1/2-3/4$)	1
Irregular ($1/4-1/2$)	2
Severely irregular ($< 1/4$)	3
Thickness of cartilage (compared with that of surrounding cartilage)	
$>2/3$	0
$1/3-2/3$	1
$<1/3$	2

Integration of donor with host adjacent cartilage	
Both edges integrated	0
One end intergrated	1
Neither edge integrated	2

Besides compositional analysis of the matrix, a more detailed analysis was also performed to evaluate the ultrastructure of the repaired tissue generated in all groups. Tissue ultrastructure is mainly reflected by the orientation and arrangement of collagen fibres, which was visualized via picrosirius polarization. In general, thin collagen fibers or poorly packed collagen display green to greenish yellow polarizing colors, whereas thick or tightly packed fibers show yellowish-orange through orange to red polarizing colors [261]. In addition, type I collagen may show up as thick (2-10 μ m in diameter), strongly birefringent, yellow or red fibers whereas type II collagen forms fibrils (20-30nm in diameter) and shows weak birefringence of a varying color[262].

From the orientation of the collagen (**Figure 4-5**), the superficial tangential zone (STZ, indicated as T) was clearly delineated in the *LhCG+* group (**Figure 4-5(b)**), but not in the *LhCG-* group (**Figure 4-5(c)**). From the perspective of collagen arrangement, *LhCG+* group displayed predominantly thin, loose fibrils that are representative of type II collagen. On the other hand, *LhCG-* group predominantly exhibited thick, packed fibers that indicate type I collagen. In the *ADS* group, the STZ comprised of thick, tight type I collagen fibers, while the deeper zones exhibited thin, loose type II collagen fibrils.

Figure 4-5 Evaluation of tissue ultrastructure via picrosirius polarization method. The orientation and arrangement of collagen fibers/fibrils as observed in the matrix of the (a) native articular cartilage; (b) *LhCG+*; (c) *LhCG-*; (d) *ADS* groups. Generally, green to greenish yellow represents thin or poorly packed collagen fibrils while yellowish-orange through orange to red represents thick or tightly packed collagen fibers. Scale bar: 200 μ m

4.3.5 Biochemical and biomechanical analyses

Biochemical and biomechanical properties of articular cartilage tissue are closely linked[243]. As indicated in **Figure 4-6(a)**, GAG content of the *LhCG+* group was comparable to native cartilage, whereas the *LhCG-* and *ADS* groups exhibited significantly lower GAG contents ($p < 0.01$). When assessed for their total collagen content (**Figure 4-6(b)**), both *LhCG+* and *ADS* group were found to be significantly higher than the native cartilage ($p < 0.05$), which could be attributed to the remodelling process as a form of compensation. The water content results

revealed that only *ADS* group possessed significantly lower water content than that of native cartilage (**Figure 4-6(c)**, $p<0.01$). The mechanical (compressive) results indicated that *LhCG+* group performed significantly better than all other groups (**Figure 4-6(d)**), ($p<0.05$).

Figure 4-6 Quantitative evaluation of articular cartilage repair. Biochemical analysis was performed to quantify the (a) GAG content and (b) total collagen content in all the explanted tissues. To correlate biochemical aspects with the functional aspects of the tissue, (c) water content and (d) compressive moduli were also determined. The results for the three experimental groups, namely *LhCG+*, *LhCG-* and *ADS* group were compared to native, unaffected cartilage. One-way ANOVA / Dunnett's multiple comparison test was used for data analysis. (NS) indicates no statistical significance between two groups while (*) indicates statistical significance ($p<0.05$); (**) ($p<0.01$). Data are presented in the form of mean $\pm$ SD, with N = 5.

4.4 Discussion

The capacity of cartilage to self-heal after injury is known to be very limited, predisposing it to downstream issues like OA. ACI treatment and its derivatives are currently the most hopeful option for restoring the structural properties of native hyaline cartilage [73, 76]. The ACI

treatment procedure consists of three main steps: (1) harvesting of autologous cartilage from minor load-bearing sites via minimally invasive surgery; (2) *in vitro* extraction and culture expansion of chondrocytes; and (3) re-implantation of the chondrocytes into the defect either with or without scaffolds [74].

In the current study, the outcome from the analysis of *ADS* samples in terms of histology, biochemistry and biomechanics (**Figure 4-4 and 4-6**) verified that donor-site morbidity, induced by the harvesting of autologous chondrocytes for the LhCG approach could be excluded by and large. This is similar to step (1) of the ACI approach. In order to obtain sufficient number of cells for treatment, ACI approach requires extensive cell expansion in monolayer culture (step (2)), which inevitably causes phenotypic alterations of chondrocytes [90]. Contrastingly, the chondrocytes used for *LhCG* fabrication experienced only one passage of monolayer expansion followed by a 35-day culture (PTCC) in 3D alginate-based MCG constructs (Fig. 1 (b) Step iii~viii) [102]. This strategy allows cells to maintain their differentiated phenotype, as shown through the retention of their characteristic rounded morphology and capacity to synthesize cartilage-specific molecules, which is in agreement with other reports [263-265]. Additionally, the prolonged culture expansion in a non-cell adhesive 3D environment purifies the isolated cell population, as it is strictly selective for non-anchorage dependent cells like the chondrocytes to survive and proliferate [266]. This ensures high purity of the end product.

Following the PTCC process, chondrocytes were pre-formed into centimeter-sized LhCG constructs with a defined shape and structure. Coupled with its biological resemblance to native articular cartilage (abundant type II collagen and GAG) (**Figure 4-1(c)**), this strategy of re-implanting an engineered living tissue instead of dissociated cells (as per 1st and 2nd generation ACI approach, step (3)), circumvents issues regarding cell retention and a non-homogeneous cell

distribution at the recipient site [76]. In comparison to the third generation of ACI, which involves the use of scaffolds in step (3), the LhCG is scaffold-free, and the adoption of this approach avoids the contamination of its purity by any exogenous material. Furthermore, from a biological perspective, the purity of both LhCG and ACI is also enhanced by the use of a homogeneous population of autologous chondrocytes. In contrast to stem/progenitor cells, chondrocytes are more attractive since no further manipulation is required (such as providing the appropriate growth factors, signaling molecules, or physical influences for precise control of differentiation)[267].

Besides the advantages of the LhCG platform in cell expansion and maintaining cartilage phenotype *in vitro*, it is also important to address two key requirements in *in vivo* cartilage repair [55]: to restore the articular surface with the best possible repair tissue that has similar biological and biomechanical properties as articular cartilage; and to promote successful integration of the repair tissue with the adjacent native articular cartilage. To investigate the capacity of LhCGs in fulfilling these requirements, they were implanted into critical sized defects created on the femoral condyles. The *in vivo* results after six months were promising. From a radiographic perspective (MRI), the *LhCG+* group was observed to restore the articular cartilage sufficiently, as compared to the *LhCG-* group (**Figure 4-2**). At the study endpoint, macroscopic observations presented similar findings, wherein the *LhCG+* group achieved good defect filling as compared to the *LhCG-* group. Scoring of macroscopic repair corroborated well with these observations, with the degree of repair and integration to host cartilage in *LhCG+* out-performing *LhCG-* ($p<0.01$) (**Figure 4-3**).

Upon histological analysis of tissue harvested at 6 months post-engraftment (**Figure 4-4**), hyaline-like cartilaginous tissue formation was apparent in the *LhCG+* group, as evidenced by

the presence of lacunae and cells that were embedded within basophilic ground substance that was rich in GAG and collagen type II. In the case of the *LhCG*- group, fibro-cartilaginous repair tissue was observed, as indicated by the presence of more type I collagen while less proteoglycans were present as compared to native articular cartilage. As mentioned in the preceding text, the use of autologous cell source is ideal to enhance clinical translation, but it raises concerns about donor site morbidity [268]. Therefore, in addition to the two experimental groups (*LhCG*+ and *LhCG*-), the donor site conditions (*ADS*) at endpoint were also deliberately assessed. The histology results for the *ADS* group showed that the defects located at minor-load bearing areas were capable of self-repair, as seen by obvious wound healing and minimal scar tissue formation. Collectively, these results indicated that *LhCG* engraftment was able to withstand load bearing and had sufficiently repaired the defect site with cartilaginous tissue that exhibits similar characteristics to native hyaline cartilage. Thus far, a major challenge has been addressed [85, 269, 270], which is in maintaining hyaline-like phenotype post-implantation; the lack of a true hyaline quality of the repaired cartilage tissue escalates the development of OA.

After being shown to produce repaired cartilage with hyaline-like phenotype, the ability of *LhCG* to integrate with the host articular cartilage was also evaluated. Poor integration has traditionally plagued many cartilage repair approaches due to the low metabolism of cartilage and its dense, anti-adhesive ECM [2, 271]. In the case of the *LhCG*+, the ECM produced by *LhCG* integrated adequately into the surrounding cartilage resulting in significantly better macroscopic and microscopic healing of the cartilage defects when compared to untreated controls (*LhCG*-). No signs of cleft, delamination, and fissuring were observed at the boundary between host and repair tissue (**Figure 4-4(b) ~ (f)**). As suggested before, this can be attributed to the sponge-like properties of *LhCG* [103]. Its lower mechanical stiffness compare to native

cartilage enabled *in situ* remodeling and maturation, resulting in good integration with surrounding native cartilage. Further benefit of host-implant integration was manifested in terms of cartilage thickness restoration. In the *LhCG+* group, cartilage thickness of the defect regions and also of the adjacent host cartilage regions was thicker than those in the *LhCG-* and *ADS* groups (**Figure 4-4(a)**). This was due to more uniform load distribution arising from better host-implant integration[2]. To sum up, these results demonstrated that the LhCG was able to sufficiently address host-implant integration.

In order to evaluate cartilage regeneration more extensively, the biochemical make-up and biomechanical responses of explanted tissues were also determined at endpoint (**Figure 4-6**). The *LhCG+* group showed similar GAG and water content to those of native cartilage, while out-performing it in terms of total collagen. As mentioned earlier, staining results confirmed that the matrix formed by LhCG displayed strong immunopositivity for type II collagen while the expression of type I collagen was negligible, suggesting that the increase in total collagen refers predominantly to type II collagen. As demonstrated by Garnero et al.[272], water and type II collagen make up the majority of the biochemical aspects of articular cartilage, and the results here resonate well with these data. The biochemical content corresponded well with their functional aspects, which may be validated through water retention by GAG and biomechanical properties. On this note, there was a corresponding increase in water content with GAG content in all groups. The same relationship was observed between compressive moduli and total collagen content, in accordance with the role of collagen in providing structural support in cartilage[243].

Further investigation was carried out on tissue ultra-structure, which also plays an integral role in the regenerative process by transferring compressive loads from the directly-

loaded regions to the adjacent zones. In particular, Hosseini et al.[273] reported that the STZ is very important for this purpose. The STZ restored by LhCG was continuous and well-defined, as observed through the picrosirius red stain (**Figure 4-5(b)**). Closer observations revealed that the collagen fibrils superficial to the articular surface were arranged in a tangential direction, before subsequently being arranged transversely. This hierarchy of structures was previously reported by Kaab et al.[274] and was observed in our study, in the native cartilage and *LhCG+* groups. The observable difference between native cartilage and the *LhCG+* group lies in the depth of the tangential region, where it was found to be much thicker in the latter. On this note, we believe with progressive remodeling of the repaired cartilage, the STZ may eventually become thinner when the deep zone of the cartilage is fully regenerated. According to the recommendations of ICRS, a longer recovery period is required for tissue structure to be regained, and this duration can be up to 12 months [275].

Many natural and synthetic materials have been employed to fabricate scaffolds for cartilage tissue engineering (as detailed in Chapter 1, Page 7). Some examples of natural materials include collagen, fibrin, agarose, silk and chitosan. As for synthetic polymers, the most commonly explored ones are the poly- α -hydroxy esters, particularly polylactic acid (PLA) and polyglycolic acid (PGA), mainly due to their biodegradability and clearance from US Food and Drug Administration (FDA) for clinical use. In the process of fabricating LhCG, alginate hydrogel was used as an intermediate scaffold. The rationale of using alginate hydrogel to support the initial stage of tissue formation and maturation is due to ease of removal of this material. Sodium citrate treatment used for alginate removal is mild and has minimal effect on cell viability. The end product, LhCG itself is a scaffold-free engineered graft, containing no exogenous materials.

In recent years, scaffold-free techniques have gained interest in cartilage tissue engineering so as to mimic the developmental processes of cartilage and also to mitigate the challenges related to the use of scaffolds [80, 276, 277]. Chondrocyte sheets and chondrocyte spheroids are among other scaffold-free engineered tissues. A recent study has investigated the efficacy of layered chondrocyte sheets in treating full-thickness cartilage defects in mini-pigs [85]. Good defect filling and integration were reported; however, in contrast to LhCG engraftment of this study, predominant expression of type I collagen was observed in the repaired cartilage. Overexpression of type I collagen indicates that the defects were filled with fibrocartilage, which is both biochemically and mechanically inferior to hyaline cartilage. Chondrocyte dedifferentiation may be the main contributing factors for this phenomenon since fabrication of the chondrocyte sheets requires monolayer expansion of chondrocytes on a temperature responsive substrate. On the other hand, chondrocytes were maintained in a 3D environment throughout the LhCG fabrication process. This minimized any phenotypic changes of chondrocytes and maintained hyaline-like quality of the end product. In addition, the surgical procedures for LhCG engraftment are relatively simpler since it can be press-fitted into the defects; no suturing was needed for fixation. As for chondrocyte spheroids (the components of a commercial product called Chondrospheres®), it is challenging to control the shape and uniformity of the spheroids formed and the final product size is limited by nutrients/gas diffusion problem. This problem is mitigated in LhCG by incorporation of gelatin microspheres into the alginate bulk during the initial process of LhCG fabrication to allow more efficient nutrient/gaseous exchange. Although LhCG have some advantages over chondrocyte sheets and spheroids, it also present some limitations such as a more technically challenging fabrication process and an open surgery is required for LhCG implantation.

Having shown that LhCG survived the load-bearing conditions *in vivo* in this study, longer-term studies (12-18 months [254]) are warranted to evaluate the durability of LhCG-mediated repair. Lastly, it should be noted that the mini-pigs in this study returned to full activity immediately after the implantation surgery. In the clinic, however, the human patients are advised against engaging in strenuous activities in the early days/weeks after surgery, followed by a gradual rehabilitation process to improve load-bearing on their joints to provide appropriate mechanical stimuli [278, 279]. The same routine may be applicable for future LhCG studies in preclinical models, in order to minimize/avoid the induction of post-traumatic OA.

4.5 Summary

This study has demonstrated that the LhCG platform may be extended to autologous chondrocytes originated from mature cartilage, with encouraging cartilage repair observed after six months in a skeletally mature mini-pig model. Particularly, the LhCG exhibited good macroscopic repair and good integration with host cartilage, manifested in the maintenance of cartilage thickness and the restoration of an intact articular surface. More significantly, the reparative tissue generated by LhCG was biologically comparable to native hyaline cartilage, suggesting the potential to result in a more durable repair. These encouraging results and clinically relevant surgical techniques potentiate the development of the LhCG beyond a preclinical setting.

5 Development and characterization of engineered cartilage diseased models and drug testing.

5.1 Introduction

The increasing prevalence of cartilage destruction in OA and RA has entailed an intensified demand for *in vitro* cartilage diseased models for the investigation of the pathophysiological processes leading to cartilage degradation in these two diseases. Current methods of generating 3D *in vitro* models are heavily dependent on scaffolds from synthetic and natural biomaterials^{13, 14, 17-20}. However, they are not native to articular cartilage tissue, and thus do not sufficiently represent *in vivo* microenvironment. As presented in the preceding chapters of this thesis, the LhCG platform fabricated through the use of cells obtained from the multiple digestion method (Chapter 3) recapitulated characteristic features of the pure hyaline phenotype, and comes across as a suitable tissue engineered construct for studying its accuracy in mimicking the early stages of cartilage-related diseases such as OA and RA. In this chapter, focus is placed on developing the LhCG platform as an *in vitro* platform that serves two functions: (1) reflect early stages of *in vivo* cartilage destruction in cartilage-related diseases such as OA and RA; (2) allows drug testing using commercially available drugs, as mentioned in Specific Aims 3 and 4. This chapter will be divided into two sub-sections for the purpose of delineating the characteristics and pathogenesis of OA (**section 5.3**) and RA (**section 5.4**).

5.2 Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich (Singapore) and all cell-culture reagents, as well as supplements were obtained from Invitrogen, unless otherwise stated.

5.2.1 Preparation of a 3D-engineered living hyaline cartilage model

The 3D-engineered living hyaline cartilage model used throughout this study was based on the LhCG prototype prepared according to our previous studies[103, 280]. Briefly, gelatin microspheres were fabricated via oil-in-water-in-oil double-emulsion method and were sieved to obtain microspheres of the desired size range (150-180 μm). Subsequently, the microspheres were sterilized by immersing them overnight in 1000 units/ml penicillin and 1000 mg/ml streptomycin solution, before storing them in phosphate buffer saline (PBS) at 4°C. Primary chondrocytes were harvested from the articular cartilage of 5-month old pigs and expanded in culture flasks. Later, passage one porcine chondrocytes (10 million cells/ml) were co-suspended with the microspheres (0.3g/ml) in 1.2 ml of alginate solution (1.5% w/v in 0.15 M NaCl, 4°C). The co-suspension was transferred into a 30mm petri dish, where 102 mM calcium chloride solution was then added gently for gelation to occur[103, 280, 281]. All 3D-constructs were cultured in chondrocyte culture (CC) medium, which comprised of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 20% (v/v) of fetal bovine serum (FBS), 0.1 mM nonessential amino acids (NEAA), 0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), 0.05 mg/ml vitamin C, 0.4 mM proline and 100 units/ml penicillin and 100 mg/ml streptomycin. The constructs were kept on an orbital shaker (with gentle shaking provided at every alternate 12 h at 50 rpm) in a humidified incubator at 37°C with 5% CO₂. Upon culturing at this temperature, the encapsulated gelatin microspheres dissolved, thus created cavities within the constructs. Chondrocytes within the alginate bulk then actively proliferated into the cavities, occupying and eventually forming neo-tissue within them. The constructs were cultured for 35 days to reach maturation and this was followed by the removal of the alginate bulk in sodium

citrate (55 mM in 0.15 M NaCl) solution (10 min at 25°C). The removal of gel bulk produced LhCG constructs that served as a 3D-engineered hyaline cartilage model in this study.[103, 281]

5.2.2 Establishment of an interactive co-culture platform for *in vitro* OA modeling

The day on which the alginate bulk was removed is denoted as “Day -7” and “Day 0” denotes the beginning of the co-culture period. Following the removal, the LhCG constructs were cultured for another week before the start of co-culture so as to ensure their structural integrity. A day before the co-culture, 2×10^6 murine macrophages (RAW 264.7 cell line, American Type Culture Collection (ATCC)) were seeded per well in 6-well tissue culture plates (Iwaki, Japan). On the following day, the LhCG constructs were transferred into the wells seeded with macrophages. A cell strainer (BD Biosciences, Singapore) was used to segregate the LhCG constructs from the macrophages in each well. Hereafter, the co-culture system was established as illustrated in **Figure 5-1**.

Experimental Groups	Timepoint	Assays
(a) LhCG + MΦ++	Day 7, 14	Cell Proliferation: WST-1, Hoechst 33528
(b) LhCG + MΦ--	Day 7, 14	Apoptosis: Annexin V/PI
		Synthetic Activity: qPCR
(c) LhCG	Day 0, 7, 14	Degradation: qPCR, DMMB, hydroxyproline

Figure 5-1 Modelling the inflammatory environment of OA joints via co-culturing of LhCGs with (a) LPS-activated macrophages (LhCG + MΦ++); (b) non-activated macrophages (LhCG + MΦ--); (c) monoculture control group with only LhCGs.

5.2.3 Establishment of an interactive tri-culture platform for in vitro RA modelling

To set up the interactive platform, LhCG constructs were fabricated (as described in section 5.3.1) and seeded with SW982 cells (Cell Lines Service (CLS), Germany) one day before to the start of tri-culture, denoted by Day (-0). For cell seeding, LhCG constructs were placed into the wells of a 24-well plate (one construct/well, wells were pre-coated with 300 μ l of 1.2 wt% of agarose gel). SW982 cells were then trypsinized and re-suspended at a concentration of 1×10^6 cells/ml. Subsequently, 200 μ l of this cell suspension will then be added to each LhCG construct and allowed to settle for an hour in the incubator at 37 °C before adding another 500 μ l of culture

media. Concurrently, differentiation of THP-1 monocytes (ATCC) into macrophages (0.7×10^6 cells/well, 24-well plate) was performed using phorbol-12-myristate-13-acetate (PMA) at a concentration of 100ng/mL. On the following day (Day 0), the THP-1 derived macrophages were activated by LPS (1 μ g/mL in serum free DMEM) stimulation. After 6-hour of activation, the SF-laden LhCG constructs were transferred into the culture wells containing activated macrophages. A cell strainer was used to segregate the SF-laden LhCG constructs from the macrophages in each well. Henceforth, a tri-culture system modelling inflammatory RA joint environment was established

5.2.4 Cell viability and apoptosis assay

The viability of the chondrocytes was evaluated both qualitatively and quantitatively[282]. For qualitative Live/Dead fluorescent staining (Molecular probes, Invitrogen), calcein (0.5 μ l/ml) and ethidium homodimer (2 μ l/ml) were added to CC medium containing the LhCG construct. After 30 min of incubation at 37 °C, the construct was examined under fluorescence microscope. The live cells were stained green while the dead cells were stained red. For the quantitative WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, Roche Diagnostics, Switzerland) colorimetric assay, each LhCG construct was incubated in 10% v/v WST-1 reagent in CC medium for 2 h at 37 °C in a CO₂ incubator. After that, the absorbance was read immediately at 450 nm with a reference reading at 690nm using a microplate spectrum reader (Multiskan spectrum, Thermo).

The apoptotic chondrocytes were stained using Annexin-V/PI-based Vybrant Apoptosis Kit (Invitrogen). Cells in the early-stage of apoptosis were stained green by Annexin-V-FITC based on the externalization of phosphatidylserine while cells in the late-stage of apoptosis or dead cells were co-stained or stained red by PI.

5.2.5 Gene/protein expression analysis

Gene expression analysis was done by using quantitative real-time polymerase chain reaction (qPCR). Briefly, RNA was extracted from LhCG constructs and macrophages respectively, using TRIzol® reagent (Invitrogen) and converted to cDNA via reverse transcription. Subsequently, qPCR was performed using iQ SYBR Green Supermix (Bio-Rad) and the iQ™ qPCR system (Bio-Rad). For analysis, gene expression values relative to the respective housekeeping gene (TATA-binding protein (TBP) for chondrocytes and β -actin (ACTB) for macrophages) were calculated using the comparative $2^{-\Delta\Delta C_T}$ method. **Table 5-1** contains the list of primers (AIT Biotech, Singapore) used in this experiment. All reverse transcription and PCR reagents were purchased from Promega (Madison, MI), unless otherwise stated.

Table 5-1 qRT-PCR primer sequences for porcine, murine and human gene markers: forward (F) and reverse (R).

Gene	Accession number/ reference	Primer sequence (both 5'-3')	Annealing temperature (°C)	Product size (bp)
Porcine collagen type I (Col I)	[283]	F: CCTGCGTGTACCCCACTCA R: ACCAGACATGCCTCTTGTCCTT	58	84
Porcine collagen type II (Col II)	[284]	F: GCTATGGAGATGACAACCTGGCTC R: CACTTACCGGTGTGTTTCGTGCAG	58	256
Porcine collagen type X (Col X)	[285]	F: CAGGTACCAGAGGTCCCATC R: CATTGAGGCCCTTAGTTGCT	58	117
Porcine SOX-9	NM_000346.3	F: GCTGGCGGATCAGTACCC R: CGCGGCTGGTACTTGTA	58	165

Porcine aggrecan (Agg)	[284]	F: CGAGGAGCAGGAGTTTGTCAAC R: ATCATCACCACGCAGTCCTCTC	58	177
Porcine cartilage oligomeric matrix protein (COMP)	NM_007112.3	F: GGCACATTCCACGTGAACA R: GGTTTGCCTGCCAGTATGTC	58	127
Porcine matrix metalloproteinase-1 (MMP-1)	NM_001166229.1	F: ATCCACAGATCCTTTGGCTTCCT R: TCATACCTCCAGCATTCGTGAGCA	58	113
Porcine matrix metalloproteinase-3 (MMP-3)	NM_001166308.1	F: AGAAGTTCCTTGGGTTGGAGGTGA R:AGGCCAGGAAAGGTGCTGAAGTAA	58	115
Porcine matrix metalloproteinase 13 (MMP-13)	XM_003129808.2	F: ACCTGGACAAGTAGTTCCAAAC R:AGTGGTCAAGACCTAAGGAATGGC	58	82

Porcine inducible nitric oxide synthase (iNOS)	EU274294	F: CCAGGCAATGGAGAGAACT R: CCGAACACAGCATACCTGAA	58	80
Porcine cyclooxygenase-2 (COX-2)	AF207824	F: AGAGCAGAGAGATGAGATACCA R: GCCATTTCTTCTCTCCTGTAA	58	103
Porcine interleukin-1 beta (IL1 β)	NM_214055.1	F:AGAGCCCATCATCCTTGAAAC R:CCAGCCAGCACTAGAGATTTG	58	111
Porcine interleukin-6 (IL-6)	NM_214399.1	F: GGAGACCTGCTTGATGAGAA R: GCCTCGACATTTCCCTTATTG	58	102
Porcine A Disintegrin And Metalloproteinase with Thrombospondin Motifs-4 (ADAMTS-4)	XM_003481414.2	F: TGTGTGTGTGAGGTTTCATCC R: CTACCCTTGACTCTTCCTTTCC	58	77

Porcine ADAMTS-5	XM_003132747.4	F: CCACCACCCTCAAGAACTTT R: CCACATAAATCCTCCCGAGTAAA	58	112
Porcine inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKBKB)	NM_001099935.1	F: CTTCCCTGACAACCCAAACA R: TCGGATGACGTTCCCAAATC	58	81
Porcine TATA-binding protein 1 (TBP1)	NM_001172085.1	F: ACAGTTCAGTAGTTATGAGCCAGA R: AGATGTTCTCAAACGCTTCG	58	152
Porcine Ribosomal protein L4 (RPL4)	XM_005659862.1	F: CAAGAGTAACTACAACCTTC R: GAACTCTAC GATGAATCTTC	58	122
Murine tumor necrosis factor-alpha (TNF- α)	[282]	F: CCACGCTCTTCTGTCTACTG R: GCTACGGGCTTGTCACCTC	58	145

Murine interleukin 1 beta (IL-1 β)	NM_008361.3	F: GAGCTTTGTACAAGGAGAACCA R: GGGTGTGCCGTCTTTCATTA	58	103
Murine interleukin 6 (IL-6)	NM_031168.1	F: TTCCTCTGGTCTTCTGGAGTA R: CTCTGAAGGACTCTCGCTTTG	58	94
Murine interleukin 8 (IL-8)	NM_011339.2	F: GTCCAAAGAGGACTGTGTGTAG R: GACTAAGCAGGAAATGGAGAGG	58	94
Murine β -actin (ACTB)	[282]	F: TGTCCACCTTCCAGCAGATGT R: AGCTCAGTAACAGTCCGCCTAGA	58	101
Human tumor necrosis factor-alpha (TNF- α)	NM_000594.3	F: CCCATGTTGTAGCAAACCCTC R: TATCTCTCAGCTCCACGCCA	58	96

Human interleukin 1 beta (IL-1 β)	NM_000576.2	F: ATTTGAGTCTGCCCAGTTCC R: GTTATATCCTGGCCGCCTTT	58	99
Human interleukin-6 (IL-6)	NM_000600.3	F: GGAGACTTGCCTGGTGAAA R: CTGGCTTGTTTCCTCACTACTC	58	99
Human interleukin 8 (IL-8)	NM_000584.3	F: CTTGGCAGCCTTCCTGATTT R: GGGTGGAAAGGTTTGGAGTATG	58	111
Human matrix metalloproteinase -1 (MMP-1)	NM_002421.3	F: CTCTGACATTCACCAAGGTCTC R: GATTCCTCCAGGTCCATCAAA	58	105
Human matrix metalloproteinase-3 (MMP-3)	NM_002422.3	F: GCAAGACAGCAAGGCATAGA R: CAGCAACAGTAGGATTGGAAGA	58	81

Protein expression, more specifically, the expression of TNF- α from LPS-activated macrophages was quantified using enzyme-linked immunosorbent assay (ELISA). Briefly, co-culture supernatants were collected at different time points, and the TNF- α concentration was measured by using the mouse TNF- α ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol.

5.2.6 Biochemical analysis

LhCG constructs were collected at selected time points and were frozen at -20°C before freeze-drying for 24 h. Each of the constructs was then digested overnight in 1 ml digestion solution consisting of 0.3 mg/ml papain dissolved in 0.2 mM dithiothreitol and 0.1 mM disodium ethylene diamine tetraacetic acid. Chondrocyte density was extrapolated from DNA content measured by the fluorimetric Hoechst 33258 assay (7.7pg DNA per cell). Glycosaminoglycan (GAG) content was measured by dimethylmethylene blue (DMB) assay, whereas total collagen content was quantified by using proline/hydroxyproline assay from acid hydrolyzed samples.

5.2.7 Histology and immunohistochemistry staining

LhCG constructs collected from each time point were fixed in 4% (w/v) paraformaldehyde for 24 h prior to paraffin embedment. The embedded samples were cut into 10 μ m-thick sections using a microtome. The sections from all the groups were later stained with Safranin-O. Anti-IgG immunohistochemistry staining was used to stain for collagen type I, II and X. For collagen type I staining, the sections were first incubated with 10% horse serum (w/v, in PBS) for 20 min to prevent non-specific binding of IgG. After that, they were incubated with collagen type I primary antibody (2 μ g/ml in 1 x PBS, goat polyclonal IgG, Santa Cruz Biotechnology), followed by anti-IgG (5 μ g/ml in 1 x PBS, AlexaFluor 488, Invitrogen). For collagen II and X staining, the sections were first blocked with 10% goat

serum (w/v, in PBS), and then incubated with either collagen type II (2 µg/ml in 1 x PBS, MAB8887, Chemicon) or collagen type X primary antibody (2 µg/ml, mouse monoclonal IgG, Abcam) at 4 °C overnight. Subsequently, the sections were washed with 1 x PBS and incubated with Anti-IgG (5 µg/ml in 1 x PBS, Invitrogen Alexa Fluor, 488) at room temperature for 1 h in the dark. Lastly, 4', 6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei for all the samples.

5.2.8 Drug testing on the established disease model

Celecoxib (Santa Cruz Biotechnology) was dissolved in DMSO and diluted with cell culture medium to obtain the required final concentrations. Experimental groups were divided as follows: untreated OA disease model (LhCG (D)), OA disease model treated with celecoxib at a concentration of 10 µg/ml (LhCG (T10)), 20 µg/ml (LhCG (T20)), and 40 µg/ml (LhCG (T40)). Drug-supplemented culture medium was replaced every 3-4 days. The tissue samples were collected on day 0, 3 and 7 for cell viability quantification and gene expression analysis while cell culture supernatants were collected on day 1, 3 and 7 for nitrite determination assay and prostaglandin E2 immunoassay. The procedures for cell viability assay and gene expression analysis are the same as those described in Section 2.3 and 2.4. A Griess Reagent System (Promega) was used to measure the production of nitric oxide while a commercial prostaglandin E2 immunoassay (Abcam) was used to quantify the secretion levels of PGE2, both according to manufacturer's instructions.

5.2.9 Statistical analysis

All results are presented as mean $\pm$ SD with 3 samples in each group. Unless otherwise stated, one-way ANOVA was used for comparisons assuming equal variances. $p < 0.05$ was considered statistically significant.

5.3 A three-dimensionally engineered biomimetic cartilaginous tissue model for osteoarthritic drug evaluation [286]

OA is a highly prevalent degenerative joint disease that is characterized by the progressive degradation of extracellular matrix (ECM) components, chondrocyte destruction, remodeling of subchondral bone, and inflammation of synovium[112]. Although the risk factors for OA have been identified, the exact pathogenesis of OA remains unclear[287]. Currently, there is still a lack of an effective disease-modifying treatment for OA[288, 289], leading to increased efforts in discovering potential therapeutic agents, consequently fuelling an upsurge in the number therapeutic agents in the drug development pipeline. In order to validate the efficacy of these therapeutic agents, animal models and monolayer-cultured cells are commonly used. For animal OA models, they include spontaneous models in aging animals, genetically modified mice, as well as surgically, enzymatically or chemically induced models[122]. These models however are limited by factors such as high-cost, long experimental cycle time, ethical issues, and low-throughput[123]. As for monolayer-cultured cells, chondrocytes isolated from animal joints and cartilage tissue of OA patients are commonly used for the screening of potential anti-arthritic drugs agents[104, 131, 141, 142, 290, 291]. However, increasing studies have shown that monolayer expansion of chondrocytes behave differently from their *in vivo* counterparts due to the lack of 3D cell-cell and cell-ECM interactions[49, 50, 150, 151]. The inherent limitations associated with these conventional models thus necessitate the need for the development of an accurate and relevant 3D *in vitro* model for drug screening.[286]

Synovial inflammation is identified as one of the important contributing factors to OA pathology[110, 111]. It is now a well-documented clinical phenomenon that contributes to OA pathology, with many clinical studies verifying the presence of macrophages in the synovial membrane of OA patients[292]. Therefore, besides chondrocytes, macrophages are

believed to be actively involved in the development of OA[114, 115, 293], based on the observation that macrophages produce a wide range of cytokines and immune factors responsible for OA development and progression, comprising of interleukin-1 (IL-1), tumor necrosis factor (TNF), interleukin-6 (IL-6), prostaglandin-E2 (PGE2), nitric oxide (NO), interferon-alpha (IFN- α) and transforming growth factor-beta (TGF- β). Among these cytokines and chemokines produced, IL-1 β and TNF- α have been identified as key players in OA pathogenesis, and they are believed to be the initiators of the disease process.[120] The release of these two proinflammatory cytokines by macrophages stimulates chondrocytes and synoviocytes to likewise produce and release inflammatory mediators. This cascading mechanism is the main driver of cartilage metabolism.

Hence, in this section, we aim to establish an accurate and physiologically relevant *in vitro* 3D model, created based on the previously established LhCG construct. We hypothesized that the pathological features of OA can be induced in LhCG by co-culturing it with LPS-activated macrophages, simulating the inflammatory environment in an osteoarthritic joint. Subsequently, the fidelity of this model in mimicking OA cartilage was assessed in terms of chondrocyte responses and changes in cartilage matrix composition. In order for the established disease model to be useful as an *in vitro* drug-test model, it has to accurately predict *in vivo* therapeutic responses to various drugs. Therefore, we also investigated the predictive ability of this *in vitro* model by treating it with celecoxib, which is one of the most widely used NSAIDs for OA[294].

5.3.1 Results

5.3.1.1 Establishment of an inflammatory environment

Upon stimulation with LPS, the macrophages showed significant upregulation of TNF- α , IL-1 β and IL-6 (**Figure 5-2 (a)**). Among the genes investigated, IL-8 was the only gene that was not affected by the presence of LPS. Within the next two weeks post-activation, there was a

general decreasing trend in the expression levels of TNF- α , IL-1 β and IL-6 with only IL-8 showing otherwise. Interestingly, the gene expression of TNF- α , IL-6 and IL-8 showed an increasing trend even without LPS stimulation, as seen in the non-activated group. To further validate successful establishment of an inflammatory environment, the secretion levels of TNF- α were quantified (**Figure 5-2(b)**). For the LhCG + M Φ ++ group, a marked increase in the concentration of TNF- α was observed after LPS stimulation. The TNF- α secretion remained high for another day, followed by a significant decrease (by about 60%) after 3 days. As for the LhCG + M Φ -- group, it is noteworthy to mention that secretion of TNF- α was also detected and was found to follow a similar trend to the LhCG + M Φ ++ group even though the concentration is consistently 20-40 % lower.

Figure 5-2 Release of pro-inflammatory cytokines by macrophages. (a) qPCR analysis of four cytokines produced by LPS-activated macrophages as compared to non-activated macrophages; (b) ELISA determination of TNF- α secretion from all experimental groups. Time 0-- refers to TNF- α concentrations measured before LPS activation of macrophages while time 0 represents the concentrations measured post 6-hr LPS-activation for the LhCG+Mphage++ group. *Indicates $p < 0.05$, statistically significance differences between groups were observed.

5.3.1.2 Development of a 3D osteoarthritis cartilaginous tissue model

Cell Proliferation

By referring to the WST-1 results in **Figure 5-3 (a)**, chondrocytes in all three experimental groups remained viable and proliferated well throughout the 2 weeks of co-culture, as

indicated by the increase in the absorbance values. However, cellular proliferation among the groups increased to different levels. The difference between the groups was most obvious after two weeks, where all groups displayed significantly higher cellular proliferation as compared to LhCG day 0-control group. The most evident increase was observed in the LhCG + MΦ⁺⁺ group, with a 53% increase in the absorbance value. Furthermore, this group also showed an absorbance value that was 15% higher than the LhCG day 14-control group. A similar trend was also observed when cell density was quantified for all the groups as shown in **Figure 5-3 (b)**. After two weeks of co-culture, cell density of the LhCG+ MΦ⁺⁺ group achieved an increase of more than two-fold as compared to the LhCG day 0-control group. This is significant because the LhCG day 14-control group only showed a 38% increase in their absorbance value within the same time frame.

Figure 5-3 Assessment of increased chondrocyte proliferation. (a) WST-1 cell proliferation assay; (b) Cell density normalized to dry weight of LhCG construct, obtained from DNA quantification using Hoechst 33258 assay. *Significantly different from LhCG control group at day 7 and day 14, respectively ($p < 0.05$). #Significantly different from day 0 LhCG prior to co-culture with macrophages ($p < 0.05$).

Programmed Cell Death

After two weeks of co-culture, ‘Live’ and Annexin-V/PI staining were used to determine chondrocyte viability and apoptosis respectively. The ‘Live’ specific staining results indicated that the chondrocytes remained viable in all three groups and there was no obvious difference observed among the groups during the two-week co-culture period (**Figure 5-4**).

However, the Annexin-V/PI staining results showed that a higher number of chondrocytes in their early apoptotic stage (stained green) was found predominantly in the co-culture groups after a week. Following two weeks of study, it was evident that the co-culture groups contained the most amounts of dead (stained red) or late stage apoptotic cells (co-stained).

Figure 5-4 Side-by-side comparison of ‘Live’ specific staining of chondrocyte and Annexin-V/PI staining for the various groups at Day 7 and Day 14.

Changes in chondrocyte anabolic and catabolic activity

To investigate the occurrence increased matrix synthesis in the newly established diseased model, gene expression studies were carried out through qPCR to examine the expression levels of four important anabolic chondrocyte markers namely Col II, Aggrecan, COMP and SOX-9. For each of the genes tested, all groups were normalized to that of LhCG control group at day 0, which was designated a fold value of 1 (**Figure 5-5**).

Figure 5-5 Gene expression levels of important anabolic genes quantified by qPCR. Fold values for each gene were calculated based on their respective expression value in day0-LhCG construct. *Significantly different from LhCG control group at day 7 and day 14, respectively ($p < 0.05$). #Significantly different from day 0 LhCG prior to co-culture with macrophages ($p < 0.05$).

For LhCG control group, the expression levels for all the anabolic genes tested were shown to decrease over time, except for the expression of SOX-9 that remained stable throughout the experimental period. However, chondrocytes in both co-culture groups showed a very different gene expression profile as compared to the control group. At day 7, the LhCG + MΦ++ group showed significantly higher expression for all the anabolic genes when compared to the control group. This was especially prominent for aggrecan and SOX-9 where the expression for aggrecan was 2-3 fold higher while SOX-9 expression showed a 4 to 5-fold increase. Interestingly, the same burst of synthetic activity was also observed in LhCG + MΦ-- group; increased expression was detected for collagen type II and COMP, but this increase in anabolic gene expression was only observed at day 14.

One of the more important responses of the chondrocytes to inflammation that will eventually lead to matrix degeneration in OA is the synthesis and secretion of various degradative enzymes. Therefore, the gene expression of three most important MMPs (MMP1, 3 and 13) was analyzed. From the qPCR results in **Figure 5-6**, both the co-culture groups showed significant increase in the expression of MMP-1 and MMP-3 after one week. This was however followed by a drastic drop in both the gene expression levels by day 14. As for MMP-13, chondrocytes in both LhCG + MΦ++ and LhCG + MΦ-- group showed a continuous decrease in the expression of this enzyme.

Figure 5-6 Gene expression levels of important catabolic genes quantified by qPCR. Fold values for each gene were calculated based on their respective expression value in day0-LhCG construct. *Significantly different from LhCG control group at day 7 and day 14, respectively ($p < 0.05$). #Significantly different from day 0 LhCG prior to co-culture with macrophages ($p < 0.05$).

Apart from evaluating the transcript levels for the selected anabolic genes, the actual amount of the two main cartilaginous ECM components—GAG and collagen—synthesized

by the chondrocytes in the newly established diseased model were also assessed via biochemical assays (**Figure 5-7**). The results were normalized to cell content for instantaneous indication of GAG and collagen homeostasis, indicating their synthesis, deposition, and degradation in the LhCG samples [295]. All three groups showed increasing amounts of GAG throughout the study period. It is however important to note that among the three groups, the LhCG + MΦ++ group consistently measured significantly lower amounts of GAG (**Figure 5-7 (a)**) despite the fact that it had the highest cell density among the three groups (**Figure 5-3(b)**). The degradation of proteoglycan is more obviously shown by the Safranin-O staining results in **Figure 5-8 (c)**. The intensity of Safranin-O staining was consistently lower for the LhCG + MΦ++ group as compared to the control LhCG group, indicating a depletion of proteoglycan content.

Figure 5-7 Biochemical analyses for GAG and collagen content. (a) GAG produced per cell; (b) Total collagen produced per cell. *Significantly different from LhCG control group at day 7 and day 14, respectively ($p < 0.05$). #Significantly different from day 0 LhCG prior to co-culture with macrophages ($p < 0.05$).

The total amount of collagen secreted by chondrocytes in all three groups was quantified using hydroxyproline assay. The results are shown in **Figure 5-7 (b)**. At the end of the two-week experimental period, both the LhCG group and the LhCG + MΦ-- group showed an increased production of collagens as compared to day 0. Contrastingly, the total collagen

content of the LhCG + MΦ⁺⁺ group remained more or less the same at day 7, and decreased slightly at day 14. With immunohistochemistry, the extent of collagen degradation, specifically collagen type II can be observed more clearly. As shown in **Figure 5-8 (b)**, there are obvious qualitative differences in collagen type II levels between the co-culture groups and the LhCG control group. Unlike the brightly stained control LhCG group, the presence of collagen type II was found to be negligible in both the co-culture groups. Collagen type I levels remained insignificant in all three groups (**Figure 5-8 (a)**).

Figure 5-8 Histochemical and immunohistochemical staining comparing differences in ECM composition of LhCG in the three different groups. (a) immunohistochemical staining for collagen type I; (b) immunohistochemical staining for collagen type II; and (c) Safranin-O staining for GAG.

Phenotypic alterations of the chondrocytes

Articular chondrocytes in osteoarthritic cartilage are known to lose their differentiated phenotype; they undergo hypertrophic differentiation as evidenced by the expression and secretion of hypertrophic markers such as collagen type X[296]. Therefore in this study, the levels of expression and secretion of this hypertrophic chondrocyte-specific collagen in all the three experimental groups were investigated. By referring to the gene expression levels of this gene (**Figure 5-9(b)**), it can be seen that collagen type X was greatly expressed by the

LhCG + MΦ⁺⁺ group at the end of the experiment while its expression remained low in the other two groups. The qPCR data corresponded to the immunohistochemical staining results. The presence of this collagen type was particularly obvious in the LhCG + MΦ⁺⁺ group at day 14 as indicated by the relatively higher intensity of green fluorescent staining (**Figure 5-9(a)**).

Figure 5-9 Assessment of hypertrophic differentiation of chondrocytes in the three experimental groups via (a) immunohistochemical staining for collagen type X; and (b) qPCR analysis of the gene expression level of collagen type X. *Significantly different from LhCG control group at day 7 and day 14, respectively ($p < 0.05$). #Significantly different from day 0-LhCG prior to co-culture with macrophages ($p < 0.05$).

5.3.1.3 Validation of the established *in vitro* OA model as an accurate drug-screening model

The effect of celecoxib on cell viability is shown in **Figure 5-10(a)**. After one week, celecoxib treated at three different concentrations significantly increased cell viability as compared to the non-treated group. In this study, NO production was significantly increased by the co-culture system with LPS-stimulated macrophages. However, it was shown that after co-incubation with celecoxib at different concentrations, the secretion levels of NO were

attenuated after one week. The attenuation of NO production is especially obvious when celecoxib was used at higher concentrations ($>20\mu\text{g/ml}$), with about 35-37% of reduction as compared to the non-treated group on day 7 (**Figure 5-10 (b)**). The total PGE2 concentration (pg/ml) in the culture supernatant over 7 days in the presence or absence of celecoxib, is shown in **Figure 5-10(c)**. Celecoxib was shown to have statistically significant inhibitory effect on PGE2 release at a higher concentration (a decrease of 21% at $40\mu\text{g/ml}$ as compared to the day 7 non-treated group) coupled with a longer treatment period (after one week). As for the gene expression study on MMPs, the results showed that celecoxib at the optimal concentration of $40\mu\text{g/ml}$ significantly inhibited the induction of MMP (both 1 and 3) expression in the disease model while the non-treated group (LhCG (D)) showed statistically significant MMP upregulation on day 3 (**Figure 5-10 (d)**).

Figure 5-10 The effect of celecoxib on (a) cell viability; (b) NO production; (c) PGE2 release; (d) gene expression of (i) MMP-1 and (ii) MMP-3. *Significantly different from the untreated group (LhCG (D)) at day 3 and day 7, respectively ($p < 0.05$). #Significantly different from the day 0/day 1 untreated group ($p < 0.05$).

5.3.2 Discussion

Osteoarthritis (OA) is a prevalent degenerative joint disease resulting from a disparity between the rate of degeneration and repair of the joint tissues. Current treatment options are only focused on symptomatic relief, thus creating a large unmet need for curative therapeutic interventions[297]. The development of a relevant *in vitro* cartilage model of OA may provide an advantageous alternative for cost-effective and high throughput preliminary testing of therapeutics. Our previous work had led to the successful establishment of a 3D engineered cartilage graft (LhCG) intended for cartilage repair. Due to the excellent quality of the grafts, which are highly similar to native cartilaginous tissue, we were encouraged to explore the possibility of using this platform to develop an accurate *in vitro* osteoarthritic model.

Previous research had demonstrated that synovial inflammation (inclusive of inflamed synovium and activated synovial macrophages), plays an important role in promoting OA pathology[111]. Hence, in this study, LhCG constructs were co-cultured with LPS-activated macrophages (LhCG+MΦ++) and comparisons were made with those co-cultured with non-activated macrophages (LhCG+MΦ--). This co-culture system was designed to mimic the inflammatory environment in the OA joints since LPS is known to stimulate the release of proinflammatory cytokines such as TNF-α and IL-1β from macrophages[298, 299]. To confirm the establishment of an inflammatory environment, we examined the gene expression of macrophages by selecting four important genes for quantitative real-time PCR analysis (**Figure 5-2 (a)**). Among these were TNF-α and IL-1β, both of which have been suggested to be key for the initiation of the disease process. Furthermore, two other macrophage-derived cytokines selected were IL-6 and IL-8, as they are known to effect the subsequent structural changes in the joint tissues.[110, 120, 300] As expected, there was a burst release of TNF-α, IL-1β and IL-6 by the macrophages stimulated with LPS. Besides

inflammation, it has been shown recently that angiogenesis also plays a part in OA progression and pain[112]. IL-8, being a chemotactic factor for monocytes as well as a powerful promoter of angiogenesis, was thus only expressed later since angiogenesis is stimulated by inflammation[293]. Interestingly, a similar gene expression profile was also seen in the non-activated group. This may be due to the paracrine interactions of the chondrocytes and the macrophages demonstrated in a previous study. It was shown that the co-culture of these two cell types displayed mutually induced alterations in their respective gene expression profiles, resulting in cartilage degradation[301]. After examining the transcript levels of these cytokines, ELISA was also conducted to quantify the secretion levels of inflammatory cytokines. We focused on TNF- α because of its well-established role in OA pathogenesis. From the results (**Figure 5-2(b)**), it could be seen that the activated group showed significantly high levels of TNF- α secretion after LPS stimulation. This is in agreement with the qPCR data, and the overall trend of TNF- α secretion is in line with previous studies[282]. At this point in time, both gene expression and protein expression analyses suggested that the macrophages were fully activated in the presence of LPS whilst incomplete activation was also observed in the control group.

In order to prove that the established OA *in vitro* model is able to mimic the disease with high fidelity, the replication of chondrocyte responses seen in OA cartilage needs to be validated in the model itself. While chondrocyte reaction pattern during OA progression may seem heterogeneous, their responses may be summarized into the following categories: increased cell proliferation and apoptosis, increased anabolic activity, synthesis of degradative enzymes, and changes in chondrocytic phenotype[128]. Therefore, different tests were used in this study to determine the different cellular responses in each of these categories. Furthermore, degradation of the ECM components in the *in vitro* cartilage models was also investigated in this study.

During the initial stages of OA, increased chondrocyte proliferation is often observed and it is considered to be a reflex reaction of the chondrocytes *in vivo* as an attempt to counteract cartilage degradation. Thus, the replication of this phenomenon within the newly established *in vitro* diseased model was investigated using the WST-1 cell proliferation assay and also DNA quantification via the Hoechst 33258 assay (**Figure 5-3**). The results from both assays concurrently showed that chondrocytes in the LhCG + MΦ++ group had abnormally high cellular proliferative activities as compared to the other experimental groups. This suggested that hyperplasia has occurred in this group and that the otherwise quiescent chondrocytes were activated due to the presence of various inflammatory cytokines. Many studies also reported this increase in proliferation in osteoarthritic chondrocytes, in contrast to healthy articular chondrocytes, which essentially lack such activity [127, 296, 302-304]. Apoptotic cell death is commonly reported in OA joints and many studies have suggested that this is an important feature associated with osteoarthritic cartilage degeneration[305-307]. Currently, the scientific community remains divided in opinion on the purported effects of pro-inflammatory cytokines on chondrocyte apoptosis[127, 128]. Although our results indicated that the cytokines released by macrophages have indeed induced apoptosis of chondrocytes, the extent of apoptosis was observed to be insignificant (**Figure 5-4**).

In addition to cell proliferation and apoptosis, many *in vitro* studies have also reported that osteoarthritic chondrocytes display inappropriate activation of anabolic and catabolic activities[144, 146, 308-310]. Based on this, we determined the expression of important anabolic and catabolic genes. From the analysis of anabolic gene expression (**Figure 5-5**), we detected hyperactivity of matrix synthesis in our co-culture models. The anabolic genes tested, particularly the two main ECM components (collagen type II and aggrecan) were significantly upregulated. This phenomenon corresponds to the biosynthetic phase observed during the degeneration of cartilage in OA. This phase is seen as an attempt by the

chondrocytes to repair the damaged cartilage ECM[128]. Furthermore, we also detected marked increased expression of catabolic genes, namely MMP-1 and MMP-3, exclusively in our disease model (**Figure 5-6**). This suggests that our model mimic the degradation process seen in OA cartilage since MMP-3 is known to be strongly expressed in normal and early degenerative cartilage and it activates MMP-1[311]. Among the MMPs, MMP-13 is thought to be the main MMP responsible for the degradation of collagen type II. However, chondrocytes in both LhCG + MΦ++ and LhCG + MΦ-- groups showed a continuous decrease in the expression of this enzyme. One possible explanation for this observed phenomenon is that MMP-13 is known to be heavily involved in the terminal breakdown of cartilage at the late-stage of OA, thus its presence is neither significant nor detectable during onset of OA[312, 313]. Hence, a longer co-culture period may be needed for the up-regulation of this gene to be detected.

The final abnormal cellular response of osteoarthritic chondrocytes that we looked for in our disease model is an altered chondrocyte phenotype. Chondrocytes in OA cartilage are reported to lose their differentiated phenotype. They undergo hypertrophic differentiation and express hypertrophic markers such as collagen type X. This represents another possible reason for the failure of the compensatory synthesis pathways required to restore the integrity of the degraded ECM.[296, 314] The results from both gene expression study and immunohistochemical staining strongly suggest that only the chondrocytes in our disease model have undergone hypertrophic differentiation (**Figure 5-9**).

To further validate that our model is reliable in recapitulating the key features of osteoarthritis, we also investigated changes in the ECM content of our model since it is the main target of osteoarthritic cartilage degradation. The most prominent change in the biochemical composition of osteoarthritic cartilage is the loss of large proteoglycans[315]. The quantitative data of the total amount of GAG produced per cell in our model suggests

that proteoglycans were being degraded but the chondrocytes were able to compensate the loss of this molecule by increasing their anabolic activity, thus resulted in the overall increased amount of GAG (**Figure 5-7(a)**). This is in line with literature where the cartilage has been known to have a capacity to replace GAG; at least until advanced degradation occurs[316]. Unlike GAG, the synthesis of collagens by the chondrocytes was not fast enough to compensate for the degraded molecules as shown by a decrease in the total amount of collagens at day 14 in our model (**Figure 5-7(b)**). A possible explanation for this is that it is more difficult for the cells to recapitulate the more complex and extensive collagen network in the ECM as compared to GAG.

In order for the newly established OA model to be useful as a drug-screening tool, it has to accurately predict therapeutic responses to various drugs. To investigate the predictive ability of this *in vitro* model, we tested one of the most established OA drugs, celecoxib, on this model. The results of celecoxib treatment on our model showed reduced production of PGE2 and NO, as well as inhibition of MMP-1 and MMP-3 gene expression at the optimal drug concentration of 40µg/ml (**Figure 5-10**). All these responses are in line with some of the documented effects listed in **Table 5-2**.

Table 5-2 The direct effects of celecoxib on OA cartilage/chondrocytes.

Drugs	Effects on OA cartilage/chondrocytes	Literature
NSAID (eg. Celecoxib)	Downregulation of COX-2 expression and inhibition of PGE2 production	[317-319]
	Inhibition of glycosaminoglycan release and stimulation of proteoglycan synthesis in OA cartilage explants	[320-322]
	Suppression of MMP expression in OA cartilage.	[323-325]

	Inhibition of NO production via inactivation of JNK and NF-κB	[324-326]
		[324]
	Induction of apoptosis in a dose-dependent manner in chondrocytes	[327]
	Reduction of apoptosis via COX inhibition	[319, 328]
	Downregulation of IL-1 and IL-6 expression	

5.4 Establishment of a 3D *in vitro* model to investigate the effects of crosstalk between chondrocytes, synovial fibroblasts, and macrophages on the destructive processes in RA.

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects about 1% to 2% of the world population, making it the most common form of inflammatory arthritis. It involves chronic inflammation of the synovial joints caused by massive infiltration of immunomodulatory cells into joint capsules[161]. This results in drastic thickening of the synovial membranes, followed by the formation of a proliferative synovial tissue (pannus) containing predominantly macrophages and fibroblast-like synoviocytes[301]. The formation of pannus tissue is unique to RA and this feature differentiates it from other arthritic conditions. The pannus tissue consists of both inflammatory and resident mesenchymal cells and is responsible for the invasion and destruction of the underlying cartilage and bone[36]. As a result, the role of macrophages[178], T- and B-cells[329, 330], and synovial fibroblasts (SF) [331], as well as their multilateral interactions [332, 333] in the pathophysiology of RA have been extensively studied. Among these cells, rheumatoid arthritis SFs (RASFs) and macrophages of the synovial lining are considered as the key mediators of joint destruction in

RA [334, 335]. In a disease state, these two cell types are the major source of pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α) and interleukin-1 (IL-1), which are the chief cytokines involved in the destruction of cartilage in RA [336-338]. Besides their proinflammatory role, RASFs and macrophages are also known to effect cartilage matrix destruction through excessive production of matrix-degrading enzymes like MMPs and cathepsins[165, 178].

Despite the extensive research conducted over many years, the etiology of RA is still not completely understood and current therapeutic approaches for RA are only able to alleviate clinical symptoms or delay disease progression[339]. These two factors have made RA a research focus for the development of new drug-screening models. Traditional 2D cell-culture models offer cost-efficient and easily reproducible conditions for drug testing, but they are oversimplified and consequently are unable to recapitulate the complex 3D cell organization as those of native tissues[40, 41, 194, 340]. Furthermore, monolayer cultures often lead to morphological changes that would result in the loss of tissue-specific gene expression and dedifferentiation[49]. This is especially true for cartilage cells (chondrocytes) where they show fibroblast-like morphology once they are removed from their native 3D environment in the cartilage tissue[341]. Therefore, RA research relies heavily on animal models that mimic some, or all, of the clinical symptoms observed. So far, studies conducted in several animal models have provided important insights into the mechanisms of RA-related cartilage destruction [193, 194]. These animal studies, however, are unable to reveal the underlying molecular pathways that regulate the development and progression of the disease[205]. Moreover, animal studies are often expensive, time-consuming, and ethically problematic.

Tissue engineering offers a perfect solution for addressing the limitations of current drug screening models. It offers an opportunity to develop complex physiologically relevant

in vitro models that are able to recapitulate some of the crucial features of the native tissues in an *in vitro* setting.[342] There are several studies that have worked on the development of *in vitro* models that mimic the human joint microenvironment[161, 205-207]. Besides differences in the cell types and purity used for coculture (eg. whole synovial tissue, isolated synovial fibroblasts, macrophages, T- or B-cells), these studies also differ widely in the type of cartilage matrix they employed[207]. Some used cartilage explants obtained either from human patients[177, 208] or other animal species[207] while others used *in vitro* generated, cell-containing matrices, such as high-density chondrocyte pellets[161, 209], chondrocyte-laden alginate beads[210], and chondrocyte/RA synovial cell encapsulated in fibrin matrices[206].

Based on previous experience with the development of three-dimensional (3D) chondrocyte culture, efforts will be made to study the process of RA-related joint erosion *in vitro*[103]. In this chapter, there will be a focus on the development of a reliable 3D *in vitro* model that mimics the *in vivo* cartilage destructive process mediated by activated RASFs and macrophages. It is hypothesized that a 3D cartilaginous component interacting with activated SFs and macrophages in a well-controlled environment allows extensive autocrine and paracrine communications between all cell types, thereby resulting in a more accurate representation of the *in vivo* process of RA cartilage destruction. If successfully established, the *in vitro* disease model would fulfil the need for a standardized and reproducible study model for *in vitro* experiments and will also help bridge the gap between the oversimplified cell-based models and the highly complex animal models in the current drug discovery and development pipeline.

5.4.1 Results

5.4.1.1 Establishment of a 3D interactive platform to study cartilage destruction in RA

To set up the interactive platform, LhCG constructs were fabricated and overlaid with SW982 cells (a prototype of activated SFs) a day prior to the start of tri-culture, denoted by Day (-0). Concurrently, differentiation of THP-1 monocytes into macrophages was performed using PMA. On the following day (Day 0), the THP-1 derived macrophages were activated by LPS stimulation. Upon 6-hour activation, the SF-laden LhCG constructs were transferred into the culture wells containing activated macrophages. The SF-laden LhCG constructs were segregated from the macrophages in each well using a cell strainer. Henceforth, a tri-culture system modelling inflammatory RA joint environment was established (as shown in **Figure 5-11(A)**). In addition to the tri-culture model (LF++), two co-culture models, namely L++ and LF and also a monoculture cartilage tissue model were also established and used as the study control groups. The details of the four experimental groups employed in this study are given in **Figure 5-11(B)**.

(B)

Experimental group	Abbreviation	Set-up
LhCG	L	LhCG
Synovial fibroblast-laden LhCG	LF	Synovial fibroblasts (SW982 cells)
LhCG coculture with macrophages	L++	Activated Macrophages
Synovial fibroblast-laden LhCG coculture with macrophages	LF++	Activated Macrophages

Figure 5-11 Experimental setup for the 3D *in vitro* RA model. (A) The process flow of setting up the *in vitro* model, starting from a day before tri-culture, Day (-0). (B) The details of the four experimental groups.

5.4.1.2 Characterization of the *in vitro* models

Cell proliferation and viability

(A)

Figure 5-12 Assessment of cell viability and proliferation. (A) Absorbance values measured from WST-1 cell proliferation assay; (B) Live/Dead staining of cells. * indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.

For cell proliferation, comparisons were made between the L++ group and the L group while the LF++ group were compared with the LF group, since they have an equal initial cell number. From the results shown in **Figure 5-12 (A)**, it was observed that chondrocytes in the healthy control group (L) and the L++ group remained viable and proliferated well during the 2-week experimental period. However, L++ group recorded a significantly higher absorbance value, indicating a higher cell proliferative activity as compared to the L group ($p < 0.05$). As for the fibroblast-seeded constructs, both the LF group and LF++ group showed increased proliferation as compared to the LF group on day 0. However, the LF group showed a significantly higher cell proliferative activity than the LF++ group on day 14. Interestingly, the LF++ group showed a lower absorbance value as compared to L++ on day 14 despite higher initial cell numbers. When the constructs were evaluated qualitatively for cell viability

via Live/Dead staining (**Figure 5-12 (B)**), results showed that the cells remained viable (stained green) in all the experimental groups with no observable difference among them.

Analysis of anabolic activity in chondrocytes

Collagen I, II and aggrecan synthesis by chondrocytes were analyzed in terms of the mRNA expression levels in all the experimental groups. The genes tested in all groups (LF, L++ and LF++) were normalized to that of the L group at each particular time point. The L group was designated a fold value of 1. As seen in **Figure 5-13**, it was observed that the seeding of SFs elicited change in the anabolic gene expression profile of chondrocytes almost immediately (as early as day 0). The expression of collagen I and II in the LF group was downregulated significantly on day 0 ($p < 0.05$), which was effectively a day after SF seeding. As for aggrecan, its expression level was upregulated significantly in the LF group as compared to the L group ($p < 0.05$). At day 7 and day 14, all the anabolic genes tested were significantly downregulated in the co-culture and tri-culture groups as compared to the L control group. Notably, LF++ group showed the lowest expression for all the anabolic genes tested on day 7.

Figure 5-13 Evaluation of chondrocyte response in terms of their matrix synthesis ability. * indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.

Analysis of catabolic activity in chondrocytes

Figure 5-14 Evaluation of chondrocyte response in terms of their production of matrix-degrading enzymes. * indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.

Cartilage breakdown in RA is mainly mediated by the increased production of MMPs and ADAMTS. MMP-1 and MMP-13 represent genes that are responsible for native collagen degradation while MMP-3, ADAMTS-4 and -5 represent proteoglycan degrading genes. Upon seeding of SFs for one day (day 0), chondrocytes responded by increasing the gene expression levels of the matrix degrading enzymes tested. All except for MMP-1 showed significant up-regulation as compared to the L group ($p < 0.05$, **Figure 5-14**). This is especially obvious for MMP-3, where it was expressed at 30-fold higher than the L group on day 0. In terms of collagen degrading genes, the expressions of MMP-1 and -13 were not significantly affected by the presence of SFs at later time points, as evidenced by their relatively constant expression in the LF group. The most noticeable response was from the LF++ group on day 14, where it showed a 5-fold increase for MMP-1 and a 3-fold increase for MMP-13 as compared to L group. A similar trend was also observed in the LF++ group

for proteoglycan degrading enzymes. When compared to the L group on day 14, this group showed significant upregulation for the expression of MMP-3, ADAMTS-4 and -5 ($p<0.05$).

Inflammatory response of chondrocytes

Besides actively participating in degrading their own matrix, chondrocytes were also reported to be the source of pro-inflammatory cytokines. As shown in **Figure 5-15**, chondrocytes were indeed induced to produce IL-1 β , which is an important pro-inflammatory cytokine responsible for sustaining both inflammation and cartilage erosion[343]. At day 7, only the LF group showed a significant increase in the expression of this gene. However, the expression levels of this gene were also seen to increase significantly in the LF++ group by day 14. Interestingly, the upregulation of IL-1 β was only seen in the SF-seeded groups but not in the co-culture group with macrophages (L++). As for IL-6, both the co-culture and tri-culture groups showed significant increase in the expression of this gene after two weeks. This was especially prominent for LF++, which was 20-fold higher than the L control group. In addition to the pro-inflammatory cytokine, the expression of IKBKB was also investigated. IKBKB is a key molecule in signalling to the transcription factor NF-kB, which in turn is a 'master switch' of the inflammatory cascade[344]. From the results, it was observed that the seeding of SF upregulated this gene but its expression continued to decrease over time, as shown by the decreasing trend in the LF group. However, the tri-culture LF++ group showed a significant upregulation of this signalling molecule by day 14 ($p<0.05$). No significant difference was seen between the L++ group and the L group.

Figure 5-15 Evaluation of the inflammatory response of chondrocytes. * indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.

Apoptotic activity of chondrocytes

The apoptotic activity of chondrocytes was examined in terms of the expression of two important apoptosis-regulating genes, namely Caspase-3 and -8. From **Figure 5-16**, it can be observed that SFs induced upregulation of these two genes, and the effect was amplified by the presence of macrophages. One day post-SF seeding, both Caspase-3 and -8 were upregulated markedly in the LF group as compared to the L group on the same day. However, the expression of these two genes in the LF group showed a decreasing trend thereafter. In contract, the LF++ group showed downregulation of these genes on day 7, followed by an upregulation on day 14. After two weeks, the LF++ group showed the highest

expression levels for both caspase-3 and -8 as compared to the rest of the groups. By day 14, L++ group also showed significantly higher expression of these apoptotic genes.

Figure 5-16 qPCR analysis of apoptosis-related genes expressed by chondrocytes. * indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.

Characterization of SF in the interactive culture system

In a diseased tissue, activated SFs are recognized as the effector cells in driving inflammation. Hence, the expressions of TNF- α , IL-1 β , IL-6 and IL-8 were analysed. For each gene tested in **Figure 5-17 (A)**, all the groups (LF, L++ and LF++) were normalized to that of the LF group on day 0. From the results, it was observed that SFs in the LF++ group had significantly higher expression levels for all the inflammatory cytokines tested than their counterparts in the LF group. Specifically, the LF++ group showed significantly higher expression of TNF- α than the LF group on day 14. As for IL-1 β , the LF++ group showed a 12-fold increase on day 7 and a 8-fold increase on day 14 as compared to the control LF group on day 0. A marked difference between LF and LF++ in the expression levels of IL-6 and IL-8 can also be seen on day 7. Besides driving inflammation, activated SFs are also known to be a major source of MMPs that cause cartilage destruction. Results revealed that the expression levels of both MMP-1 and MMP-3 (**Figure 5-17 (B)**) were consistently higher in the LF++ group as compared to the LF group throughout the entire experimental period.

Figure 5-17 qPCR analysis of synovial fibroblast response. (A) The expression of inflammation-induced genes and (B) matrix-degrading genes. * indicates $p < 0.05$ when compared to the LF group at day 0; # indicates $p < 0.05$ when compared to the LF group at each respective time point.

Characterization of macrophages in the interactive culture system

Macrophages infiltrate inflammatory tissues and secrete various pro-inflammatory cytokines. From the gene expression results (**Figure 5-18 (A)**), it can be observed that almost all the cytokines experienced a marked downregulation as compared to day 0 control group, except for IL-6. Interestingly, L++ group showed a steady decrease in the expression of IL-6 while LF++ group showed a 1.5-fold increase after one week. Importantly, the expression of this gene remained markedly higher than the L++ group until the end of the study. Another role of macrophages in RA is to cause joint destruction. Similar to the expression pattern of pro-inflammatory cytokines, both MMP-1 and MMP-3 had the highest expression on day 0, which was immediately after LPS-stimulation (**Figure 5-18 (B)**). For MMP-1, both L++ and LF++ group showed an increased expression on day 14, with the latter being significantly higher. As for MMP-3, both groups tested showed a continuous decreased expression of this gene during the two-week study. The L++ group showed a less drastic decrease than the LF++ group.

Figure 5-18 qPCR analysis of macrophage response. (A) Gene expression of important pro-inflammatory cytokines; (B) Gene expression of matrix-degrading enzymes. * indicates $p < 0.05$ when compared to the L++ group at day 0; # indicates $p < 0.05$ when compared to the L++ group at each respective time point.

Characterization of the cartilage matrix

After investigating cellular responses, tissue response of each group was also determined in terms of changes in their ECM content. Both co-culture and tri-culture groups measured significantly lower amounts of GAG after two weeks as compared to the L group (**Figure 5-19 (A)**). GAG degradation was found to be more severe in the LF and LF++ group. Similarly, degradation of GAG was also detected in all the co-culture and tri-culture groups via Safranin-O stain (**Figure 5-20 (B)**). Unlike the L group with continuous and brightly stained matrix, all the other groups showed fragmented and lightly stained matrix, indicating loss of GAG. In addition to GAG, total collagen content in each group was also quantified (**Figure 5-19 (B)**). Due to the seeding of SFs, the collagen content in the LF group was significantly higher than that of the L group on day 0. However, the collagen content of this group decreased to a level that was comparable to the L group after a week of culture before an increased production was detected again on day 14. As for LF++ group, its total collagen content was the highest among the groups on day 7. Following two weeks of culture, the collagen content in the LF++ group decreased significantly ($p < 0.05$) to the lowest among the

groups. No significant difference was observed between the L++ and the L group during the course of study. With immunohistochemistry, the changes in collagen content can be observed more clearly. As shown in **Figure 5-20 (A)**, the presence of collagen type II (stained green) was insignificant in all the co-culture and tri-culture groups as compared to the L control group on both day 7 and 14.

Figure 5-19 Evaluation of tissue response via biochemical analysis (A) GAG produced per cell; (B) Total collagen produced per cell. * indicates $p < 0.05$ when compared to the healthy control group, L at each respective time point; # indicates $p < 0.05$ when LF++ group was compared to the L++ group at each respective time point; ^ indicates $p < 0.05$ when LF++ group was compared to the LF group at each respective time point.

Figure 5-20 Evaluation of ECM degradation via (A) immunostaining of collagen type II and (B) histological staining of GAG. For immunostaining, cell nuclei were counter-stained with DAPI.

5.4.2 Discussion

As detailed in **Chapter 2**, RA is a complicated systemic disease that involves many cell populations and a host of signaling molecules[300]. Among the different cellular players in RA, RASFs and macrophages have been recognized for their important roles in mediating cartilage destruction[159, 345]. The underlying pathophysiological mechanisms of RA synovial fibroblasts (RASf) involve (i) indirect mechanism mediated by IL-1 β and TNF- α , which are produced by RASf and shift cartilage homeostasis towards catabolism [346] and (ii) direct cartilage destruction where tongues of proliferating fibroblast-like and macrophage-like cells can be seen penetrating the extracellular matrix of the cartilage at the pannus-cartilage interface [159, 347]. Therefore, these two cell types were used in the establishment of an *in vitro* RA model, to mimic the *in vivo* cartilage destruction process mediated by activated RASFs and macrophages. To establish the model, an interactive triculture platform consisting of a cartilaginous tissue model and also the two cell populations was set up as illustrated in **Figure 5-11 (A)** (the *in vitro* RA model has been abbreviated as LF++). Briefly, LhCG constructs were employed to represent the articular cartilage and used as a disease-modeling platform since they have similar biological properties as the native articular cartilage. SW982, a sarcoma-derived fibroblast cell line was used to model the activated SFs [348]. As for macrophages, monocyte-derived THP-1 macrophages were used [349]. Furthermore, the macrophages were stimulated with lipopolysaccharide (LPS) to attain the activated/pro-inflammatory phenotype of macrophages as observed in the RA pathophysiology.

The novel aspects of the newly established *in vitro* RA model described in this section are: 1) this RA model studies the crosstalk among all cellular players involved, providing important insight into how their interactions result in more accurate recapitulation of the *in vivo* cartilage destruction process. In contrast, many published *in vitro* models of cartilage

destruction typically focused on the responses of the cartilaginous component but not the other cellular components[161, 206]. While it is important to reproduce the diseased state of the cartilage through involvement of relevant cell types, the *in vitro* model may benefit more from replicating the *in vivo* responses of all involved, important cell types; 2) the use of a unique scaffold-free 3D cartilaginous component (LhCG constructs) that recapitulates important cartilage phenotype for disease modelling. Most previous studies made use of damaged or diseased cartilage matrices, which prevents the study of disease progression from an initially healthy cartilage. In addition, they suffer from significant and obvious construct variability that makes standardization difficult, thus hindering their potential use as high-throughput models for efficient and accurate drug screening [161, 177, 350].

In order to determine the accuracy of the established RA *in vitro* model, the replication of chondrocyte, activated SFs and macrophage responses seen in RA cartilage needs to be validated in the model itself. In RA cartilage, chondrocytes are known to have the following responses: increased apoptosis, suppression of matrix synthesis, increased production of matrix-degrading enzymes, and production of pro-inflammatory cytokines. In terms of apoptosis, it is one of the pathways of cartilage destruction since it deprives cartilage of the ability to replenish lost matrix components[351, 352]. In this study, apoptosis of chondrocytes was evaluated through the upregulation of apoptosis-regulating genes and also indicated by decreased cellular proliferative activity. These key phenomena were only observed in the LF++ group, as shown by the significant upregulation of Caspase-3 and -8 (**Figure 5-16**) and also the significantly lower cell proliferative activity (**Figure 5-12(A)**) as compared to the LF group after two weeks. Although L++ group also experienced upregulation for both Caspase-3 and -8, the apoptotic effect was overcome by a marked increase in cell proliferative activity. According to previous studies, macrophages are responsible for inducing apoptosis of parenchymal and stromal cells in an RA joint. Hence, it

is believed that the presence of macrophages is the reason behind increased apoptosis observed in the L++ and LF++ group. The results suggest that the inclusion of macrophage is crucial to recapitulate this feature in an in vitro RA model.

Besides increased chondrocyte death, chondrocytes in an RA cartilage also display reduced synthesis of matrix components and increased production of tissue-degrading enzymes, indicating disrupted cartilage homeostasis caused by a pro-inflammatory cascade[162]. In this study, the gene expression results clearly showed that all coculture and triculture groups downregulated their expression on important cartilage ECM components (col II and aggrecan) while upregulating their proteolytic enzyme expression (MMPs and ADAMTS), albeit to different extent. Importantly, in this RA model, which is the LF++ group, a consistent downregulation of all the anabolic genes tested was observed (**Figure 5-13**), while the converse is true to catabolic genes (**Figure 5-14**). Additionally, the chondrocytes in LF++ group also responded in a fashion that is similar to *in vivo* conditions of a RA joint, where the chondrocytes themselves produce cytokines to accelerate the switch from an anabolic to a catabolic state[162]. This is evidenced through the upregulated expression of a key regulator of inflammation, namely IKBKB and also the increased expression of pro-inflammatory cytokines such as IL-1 β , and IL-6 (**Figure 5-15**) [353]. The chondrocyte responses discussed thus far have corroborated well with those observed in animal models of arthritis[301, 354].

RASFs are well known for their pro-inflammatory and pro-destructive roles in an RA joint [165]. More importantly, their functional role is well supported by macrophages, which act as amplifiers of the pathogenic cascade[165, 345]. From the results in **Figure 5-17**, it is clear that the inclusion of the macrophages in the same microenvironment as activated SFs indeed promoted higher expression of inflammatory cytokines and tissue degrading enzymes expressed by the SFs. The upregulation of IL-1 β seen in activated SFs of the LF++

group is especially important as the expression of this cytokine contributed to the suppression of matrix synthesis and production of MMPs seen in chondrocytes (**Figure 5-14**)[180, 355]. The results resonated well with observations from animal studies, where intra-articular injection of IL-1 in caused proteoglycan loss, chondrocyte apoptosis and extensive matrix degradation[338, 356]. On the other hand, when the effect of SFs on macrophages was investigated, results indicated that IL-6 was significantly upregulated in the macrophages (**Figure 5-18**). Since levels of IL-6 are elevated in the synovial fluids of RA patients[357, 358], this phenomenon observed in the newly established model further suggests its fidelity in replicating *in vivo* disease progression. Therefore, it is clear that cartilage degradation in the model was induced by activated SFs and was further potentiated by the involvement of activated macrophages, which was manifested in the distinct cellular responses observed in the LF and the LF++ group.

Apart from examining the responses of each cell type used for the development of the RA model, the actual level of ECM degradation was also investigated. This is to determine the reliability of the newly established model in recapitulating the destructive process observed in RA cartilage[158]. Through histological and immunofluorescent staining, loss of the two main ECM components, namely proteoglycans and collagen II, was prominent in all experimental groups, except for the monoculture L group (**Figure 5-20**). When examined more closely, significant enhancement of proteoglycan loss was detected in both the LF and LF++ groups. Between these two groups, LF++ had more loss of collagen (**Figure 5-19 (B)**). Taken together, these results suggested that the LF++ model is reliable in recapitulating the destructive process in RA cartilage.

5.5 Summary

In **section 5.3**, the inflammatory environment aimed at simulating the *in vivo* conditions had successfully induced some of the key pathological features of osteoarthritis in the LhCG construct. The various cytokine-mediated cellular responses and progressive degradation of the ECM that mimic some of the important pathophysiological events in OA have been observed. Celecoxib treatment on the newly-established disease model indicated the ability to elucidate accurate therapeutic responses to the drug. Therefore, the results suggested that the newly established *in vitro* 3D OA model has the potential to be used as an accurate *in vitro* 3D model for future OA drug-testing.

In **section 5.4**, the results showed that chondrocytes in the newly established *in vitro* RA model (LF++ group) displayed aberrant behaviours similar to those seen in RA chondrocytes, whereby they contributed to their own ECM destruction by switching their metabolism to a catabolic state. Additionally, the inclusion of activated SFs and macrophages in the model influenced both cell types to more closely replicate their *in vivo* role in mediating cartilage destruction. This is evidenced by the extensive loss of ECM components observed in the model itself. Collectively, the preliminary results from this chapter showed that the LF++ group responded in a manner that appeared to recapitulate certain important features of an actual RA cartilage. If properly validated, this model may allow testing/screening of new therapeutic strategies and thereby contributing to the improvement of anti-rheumatic treatment.

6 Conclusion and Future Work

6.1 Conclusions

This thesis set out to develop the LhCG as a platform for *in vivo* cartilage repair, as well as for *in vitro* drug testing on engineered, diseased cartilage. As mentioned in **Chapter 1**, the engineering of a scaffold-free cartilage construct holds certain advantages (minimizes phenotypic alteration of cells, no hindrance of neotissue organization) over other techniques involving the use of synthetic biomaterials. The inherent biological characteristics (phenotype, purity) of this scaffold-free LhCG construct enhances its suitability as a platform for *in vitro* drug testing, as well as for *in vivo* cartilage repair. **Chapter 2** reviewed the previously established LhCG system, while also touching on relevant themes laid out in this thesis, such as: osteoarthritis, rheumatoid arthritis, and cartilage repair. Significant enhancement to chondrocyte extraction was demonstrated in **Chapter 3** through the multiple digestion method, leading to more efficient LhCG fabrication. Following the establishment of the LhCG platform using this method, **Chapters 4 and 5** illustrated the application of the LhCG in both *in vitro* and *in vivo* situations. Results presented in those chapters supported the hypotheses and specific aims of this thesis. In summary, this thesis has developed an engineered, versatile cartilage platform that may be used in cartilage regeneration and drug evaluation.

In conclusion, this thesis has demonstrated the versatility of LhCG to be used for both *in vivo* and *in vitro* applications. For the purpose of cartilage repair and regeneration, this thesis has established an efficient surgical procedure for the proposed Living hyaline cartilage graft (LhCG) approach. More importantly, the efficacy of LhCG in mediating repair of full-thickness chondral defects was confirmed. In terms of its *in vitro* applications, this thesis has demonstrated that setting up interactive cell culture system with the relevant cell

types can induce key pathological features of OA and RA in LhCG. Through the development of these *in vitro* disease models, a convenient platform may be created for extensive studies and systematic experimentations, which would help to advance current knowledge on OA and RA. Concurrently, this platform may be useful to accelerate the drug screening process with the hope of fast-tracking new treatments to commercial deployment.

6.2 Future work

This thesis has demonstrated the versatility of the LhCG platform through *in vivo* cartilage regeneration, as well as *in vitro* disease modeling. On top of these results, more work may be done in order to accentuate the effectiveness and capability of the LhCG in both scenarios.

In vivo cartilage regeneration

In vivo cartilage regeneration remains an important clinical need, which may have significant societal implications if appropriately addressed. Through the development of the LhCG, the key advantage was highlighted: an autologous source of chondrocytes wherein their phenotypes were maintained through *in vitro* culture in a 3D, biomimicking environment. As demonstrated in the preliminary *in vivo* porcine results in this thesis, restoration of a cartilage structure that is similar to native cartilage was effected through the implantation of the LhCG. However, mechanical recovery was not fully demonstrated in this study, primarily due to lack of time for healing. As such, long-term animal studies in a similar porcine model could be conducted with the aim of evaluating both the biological characteristics of the regenerated cartilage microstructure, as well as functional recovery of the knee-joint. On this note, functional recovery of the knee-joint in terms of its load bearing distribution across the entire cartilage could be evaluated through biomechanical assessment. In Wang et al.[359], a robotic system was employed to evaluate joint stability in human cadaveric knees. Through the use of a similar system, the cadaveric porcine knee joint could also be assessed for its

moment versus angular rotation behavior under varus and valgus loads at extension and flexion. A positive control cadaveric porcine knee joint will be used to evaluate the percentage functional recovery of the regenerated knee joint. With respect to the microstructure of the cartilage at the knee joint, Dang and Kuo[360] have impressed upon the field that the articular cartilage has a unique microstructure that is different from fibrocartilage. This suggests that it is important for the unique microstructural characteristics to be reinstated, as a precursor for functional recovery of the knee joint. Therefore, it appears that the regeneration of the various layers (superficial zone, tangential zone, radial zone, and tidemark) is required. By conducting a long-term in vivo porcine study, the effectiveness of the LhCG in regenerating the unique microstructural characteristics could be evaluated, and results could be corroborated with that of functional recovery (if any).

Upon evaluating and ascertaining that the LhCG is able to regenerate the biological and functional aspects of the cartilage and knee joint respectively, this work has a strong potential for clinical evaluation. The main attractiveness of the LhCG lies in the use of chondrocytes, and their expansion in a 3D microenvironment that maintains their pure hyaline phenotype. This technique avoids potential pitfalls in an altering hyaline phenotype when cultured in a non-physiological 2D environment for a substantial period of time (~30 days). In addition, the LhCG is sufficiently sound structurally for clinical handling, and does not face the issue of diffusing away from its implanted site. This is due to the ECM that holds the entire LhCG construct together in a robust fashion. Although clinical evaluation and eventual clinical acceptance may be a distant conclusion at this moment, it presents as an achievable target.

In vitro disease modeling

At the present moment, drug tests may be conducted using in vivo animal models, which are expensive and time-consuming. The difficulty in successfully translating a newly developed

drug is compounded by the fact that a significant percentage of drugs developed do not serve even their primary functions after a series of experimentations, even clinical trials. As van Vollenhoven[361] stated in a review of therapies for RA, clinical needs remained unmet despite the improvements made in clinical trial methodologies, and the introduction of at least 9 different biological agents with clear mechanisms of action. In order to streamline the drug screening process in an economical and high throughput fashion, there is a need to develop an alternative to in vivo animal testing, which may be provided in the form of in vitro platforms.

To conduct in vitro drug screening would warrant the use of a physiological in vitro model. To this end, this thesis has reviewed various platforms that claim physiological relevance to in vivo cartilage. This thesis developed in vitro cartilage disease models using the LhCG, and verified its similarity to in vivo disease progression (e.g. osteoarthritis, rheumatoid arthritis) through an investigation of its overall response. In addition, this model facilitated the investigation of the specific therapeutic effect of drugs (Celecoxib etc.). This is a significant advantage over other platforms. Having demonstrated that the LhCG was able to recapitulate certain important features of an actual disease cartilage, work could be done to improve the relevance and accuracy of this platform to human disease modeling. For example, cell source is a critical component. By employing the use of human cells, i.e. freshly harvested chondrocytes, monocytes, and synovial fibroblasts, the relevance of this platform to humans is significantly improved. Furthermore, more cell types can be integrated into this platform, including vascular cells and other immune cells, which are known to contribute to disease progression. Apart from improving on the model's fidelity in mimicking human diseases, its reliability in drug testing is also important. A more comprehensive list of commercially available drugs, targeting different aspects (reduce inflammation, delay joint damage, or biologics targeting specific substances in the body) of

the disease should be performed on the model itself. For example, etanercept specifically targets TNF, acting as an inhibitor to TNF. Considering that the multi-culture system includes activated synovial cells and immune cells, it is imaginable that suppression of TNF activity is expected upon exposure to appropriate drug concentrations. Some other widely used drugs that should be tested include methotrexate, leflunomide, hydroxychloroquine, and sulfasalazine, belonging to the group of disease-modifying antirheumatic drugs (DMARDs) and anakinra, certolizumab, golimumab, infliximab, and rituximab, categorized under biologic agents.

Moving on, as an effort to potentiate the use of the newly established OA and RA model as a high-throughput in vitro test system for antirheumatic substances, the main steps for generation of LhCG and the cultures with other cells, such as cell encapsulation in alginate, cell seeding and cultivation will be automated. For this purpose, an automatic platform such as a liquid-handling workstation as described in a study by Ibold et al. may be employed[161]. The efficiency of the automation process will be assessed in terms of reproducibility, consistency, and handling time. To ensure that automation does not affect the quality of in vitro models, all the processes, including cell encapsulation, cell seeding and cultivation will be performed automatically and manually. After which, the quality of LhCG constructs produced by automated and manual cultivation will be assessed via cell viability assays, histology, immunohistochemistry, biochemical analysis and gene expression studies.

Because of their potential to replicate the functions of native tissues and to allow a new form of personalized medicine, engineered tissue models have sparked the interest of many in the medical, pharmaceutical, chemical, and cosmetics industries. Based on the preliminary work done in this thesis and also the accomplishments achieved by other research groups detailed in a recent review [362], these in vitro models hold great promise in

advancing fundamental research into pathomechanisms, as well as in revolutionizing clinical pathology.

References

1. Kyriacos A. Athanasiou, E.M.D., Jerry C. Hu, *Articular cartilage tissue engineering*. Synthesis lectures on tissue engineering; #32010: Morgan & Claypool Publishers.
2. Huey, D.J., J.C. Hu, and K.A. Athanasiou, *Unlike bone, cartilage regeneration remains elusive*. Science, 2012. **338**(6109): p. 917-921.
3. Magnussen, R., et al., *Treatment of Focal Articular Cartilage Defects in the Knee*. Clinical Orthopaedics and Related Research, 2008. **466**(4): p. 952-962.
4. Temenoff, J.S. and A.G. Mikos, *Review: tissue engineering for regeneration of articular cartilage*. Biomaterials, 2000. **21**(5): p. 431-440.
5. Buckwalter, J.A., *Articular cartilage: injuries and potential for healing*. J Orthop Sports Phys Ther., 1998. **28**(4): p. 192-202.
6. Lipowitz, A.J. and C.D. Newton, *Degenerative joint disease and traumatic arthritis*. Textbook of small animal orthopaedics, International Veterinary Information Service, Ithaca, 1985.
7. Zhang, L., J. Hu, and K.A. Athanasiou, *The role of tissue engineering in articular cartilage repair and regeneration*. Crit Rev Biomed Eng., 2009. **37**(1-2): p. 1-57.
8. Thomas G. Koch, L.M., Younes Leysi-Derilou, Lise C. Berg, *Joint Cartilage Tissue Engineering and Pre-Clinical Safety and Efficacy Testing*, *Tissue Engineering for Tissue and Organ Regeneration* 2011.
9. Langer, R.S. and J.P. Vacanti, *Tissue engineering: the challenges ahead*. Sci Am., 1999. **280**(4): p. 86-9.
10. Chung, C. and J.A. Burdick, *Engineering cartilage tissue*. Advanced Drug Delivery Reviews, 2008. **60**(2): p. 243-262.

11. Kock, L., C.C. van Donkelaar, and K. Ito, *Tissue engineering of functional articular cartilage: the current status*. Cell and Tissue Research, 2012. **347**(3): p. 613-27. doi: 10.1007/s00441-011-1243-1. Epub 2011 Oct 27.
12. Darling, E.M. and K.A. Athanasiou, *Rapid phenotypic changes in passaged articular chondrocyte subpopulations*. Journal of Orthopaedic Research, 2005. **23**(2): p. 425-432.
13. Goessler, U.R., et al., *Human chondrocytes differentially express matrix modulators during in vitro expansion for tissue engineering*. Int J Mol Med., 2005. **16**(4): p. 509-15.
14. Mahmoudifar, N. and P.M. Doran, *Extent of cell differentiation and capacity for cartilage synthesis in human adult adipose-derived stem cells: comparison with fetal chondrocytes*. Biotechnol Bioeng., 2010. **107**(2): p. 393-401. doi: 10.1002/bit.22798.
15. Diekman, B.O., et al., *Chondrogenesis of adult stem cells from adipose tissue and bone marrow: induction by growth factors and cartilage-derived matrix*. Tissue Eng Part A, 2010. **16**(2): p. 523-33. doi: 10.1089/ten.TEA.2009.0398.
16. Fan, J., et al., *Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration*. Tissue Eng Part B Rev., 2009. **15**(1): p. 75-86. doi: 10.1089/ten.teb.2008.0586.
17. Wang, L., et al., *A comparison of human bone marrow-derived mesenchymal stem cells and human umbilical cord-derived mesenchymal stromal cells for cartilage tissue engineering*. Tissue Eng Part A, 2009. **15**(8): p. 2259-66. doi: 10.1089/ten.tea.2008.0393.
18. Keeney, M., J.H. Lai, and F. Yang, *Recent progress in cartilage tissue engineering*. Current Opinion in Biotechnology, 2011. **22**(5): p. 734-740.

19. Ahmed, T.A.E. and M.T. Hincke, *Strategies for Articular Cartilage Lesion Repair and Functional Restoration*. Tissue Engineering Part B: Reviews, 2009. **16**(3): p. 305-329.
20. Yoon, D. and J. Fisher, *Chondrocyte Signaling and Artificial Matrices for Articular Cartilage Engineering*, in *Tissue Engineering*, J. Fisher, Editor 2007, Springer US. p. 67-86.
21. Munirah, S., et al., *The use of fibrin and poly(lactic-co-glycolic acid) hybrid scaffold for articular cartilage tissue engineering: an in vivo analysis*. Eur Cell Mater., 2008. **15**: p. 41-52.
22. Chen, G., et al., *Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold*. Biochem Biophys Res Commun., 2004. **322**(1): p. 50-5.
23. Whitney, G.A., et al., *Methods for producing scaffold-free engineered cartilage sheets from auricular and articular chondrocyte cell sources and attachment to porous tantalum*. Biores Open Access., 2012. **1**(4): p. 157-65. doi: 10.1089/biores.2012.0231.
24. Marlovits, S., et al., *Chondrogenesis of aged human articular cartilage in a scaffold-free bioreactor*. Tissue Engineering, 2003. **9**(6): p. 1215-26.
25. Park, K., et al., *Scaffold-free, engineered porcine cartilage construct for cartilage defect repair--in vitro and in vivo study*. Artif Organs., 2006. **30**(8): p. 586-96.
26. Lefebvre, V. and P. Smits, *Transcriptional control of chondrocyte fate and differentiation*. Birth Defects Res C Embryo Today., 2005. **75**(3): p. 200-12.
27. Hu, J.C. and K.A. Athanasiou, *A self-assembling process in articular cartilage tissue engineering*. Tissue Engineering, 2006. **12**(4): p. 969-79.

28. Adkisson, H.D.t., et al., *The potential of human allogeneic juvenile chondrocytes for restoration of articular cartilage*. Am J Sports Med., 2010. **38**(7): p. 1324-33. doi: 10.1177/0363546510361950. Epub 2010 Apr 27.
29. Arufe, M.C., et al., *Chondrogenic potential of subpopulations of cells expressing mesenchymal stem cell markers derived from human synovial membranes*. J Cell Biochem., 2010. **111**(4): p. 834-45. doi: 10.1002/jcb.22768.
30. Hwang, N.S., et al., *In vivo commitment and functional tissue regeneration using human embryonic stem cell-derived mesenchymal cells*. Proceedings of the National Academy of Sciences, 2008. **105**(52): p. 20641-20646.
31. Toh, W.S., et al., *Cartilage repair using hyaluronan hydrogel-encapsulated human embryonic stem cell-derived chondrogenic cells*. Biomaterials., 2010. **31**(27): p. 6968-80. doi: 10.1016/j.biomaterials.2010.05.064. Epub 2010 Jun 17.
32. Medvedev, S.P., et al., *Human induced pluripotent stem cells derived from fetal neural stem cells successfully undergo directed differentiation into cartilage*. Stem Cells Dev., 2011. **20**(6): p. 1099-112. doi: 10.1089/scd.2010.0249. Epub 2010 Oct 17.
33. Yamanaka, S., *A fresh look at iPS cells*. Cell., 2009. **137**(1): p. 13-7. doi: 10.1016/j.cell.2009.03.034.
34. Lee, C.M., et al., *Development of an in vitro model of injury-induced osteoarthritis in cartilage explants from adult horses through application of single-impact compressive overload*. Am J Vet Res., 2013. **74**(1): p. 40-7. doi: 10.2460/ajvr.74.1.40.
35. Enders, J.T., et al., *A model for studying human articular cartilage integration in vitro*. J Biomed Mater Res A., 2010. **94**(2): p. 509-14. doi: 10.1002/jbm.a.32719.

36. Pretzel, D., et al., *A novel in vitro bovine cartilage punch model for assessing the regeneration of focal cartilage defects with biocompatible bacterial nanocellulose*. Arthritis Research & Therapy, 2013. **15**(3): p. R59.
37. Astashkina, A., B. Mann, and D.W. Grainger, *A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity*. Pharmacology & Therapeutics, 2012. **134**(1): p. 82-106.
38. Nyga, A., U. Cheema, and M. Loizidou, *3D tumour models: Novel in vitro approaches to cancer studies*. Journal of Cell Communication and Signaling, 2011. **5**(3): p. 239-248.
39. Mehta, G., et al., *Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy*. Journal of Controlled Release, 2012.
40. Cukierman, E., et al., *Taking cell-matrix adhesions to the third dimension*. Science., 2001. **294**(5547): p. 1708-12.
41. Griffith, L.G. and M.A. Swartz, *Capturing complex 3D tissue physiology in vitro*. Nat Rev Mol Cell Biol., 2006. **7**(3): p. 211-24.
42. Ashwell, M.S., et al., *Changes in chondrocyte gene expression following in vitro impaction of porcine articular cartilage in an impact injury model*. Journal of Orthopaedic Research, 2013. **31**(3): p. 385-391.
43. Bartlett, R.L., S. Sharma, and A. Panitch, *Cell-penetrating peptides released from thermosensitive nanoparticles suppress pro-inflammatory cytokine response by specifically targeting inflamed cartilage explants*. Nanomedicine: Nanotechnology, Biology, and Medicine, 2013. **9**(3): p. 419-427.
44. Pretzel, D., et al., *In vitro model for the analysis of synovial fibroblast-mediated degradation of intact cartilage*. Arthritis Research and Therapy, 2009. **11**(1).

45. Chen, X., et al., *Chondrogenic differentiation of umbilical cord-derived mesenchymal stem cells in type I collagen-hydrogel for cartilage engineering*. Injury, 2013. **44**(4): p. 540-549.
46. Kaupp, J.A., J.F. Weber, and S.D. Waldman, *Mechanical stimulation of chondrocyte-agarose hydrogels*. Journal of visualized experiments : JoVE, 2012(68).
47. Park, H., et al., *Injectable chitosan hyaluronic acid hydrogels for cartilage tissue engineering*. Acta Biomaterialia, 2013. **9**(1): p. 4779-4786.
48. Kundu, J., et al., *An additive manufacturing-based PCL-alginate-chondrocyte bioprinted scaffold for cartilage tissue engineering*. Journal of Tissue Engineering and Regenerative Medicine, 2013.
49. Benay, P.D. and J.D. Shaffer, *Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels*. Cell, 1982. **30**(1): p. 215-224.
50. Bonaventure, J., et al., *Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads*. Experimental Cell Research, 1994. **212**(1): p. 97-104.
51. Izadifar, Z., X. Chen, and W. Kulyk, *Strategic design and fabrication of engineered scaffolds for articular cartilage repair*. Journal of functional biomaterials, 2012. **3**(4): p. 799-838.
52. Athanasiou, K.A., E.M. Darling, and J.C. Hu, *Articular Cartilage Tissue Engineering*. Synthesis Lectures on Tissue Engineering, 2009. **1**(1): p. 1-182.
53. Vijayan, S., et al., *Cartilage repair: A review of Stanmore experience in the treatment of osteochondral defects in the knee with various surgical techniques*. Indian J Orthop., 2010. **44**(3): p. 238-45. doi: 10.4103/0019-5413.65136.

54. Williams, R., III and R. Brophy, *Decision Making in Cartilage Repair Procedures*, in *Cartilage Repair Strategies*, R. Williams, III, Editor 2007, Humana Press. p. 37-53.
55. Redman, S.N., S.F. Oldfield, and C.W. Archer, *Current strategies for articular cartilage repair*. Eur Cell Mater., 2005. **9**: p. 23-32; discussion 23-32.
56. Kalson, N.S., P.D. Gikas, and T.W.R. Briggs, *Current strategies for knee cartilage repair*. International Journal of Clinical Practice, 2010. **64**(10): p. 1444-1452.
57. *Arthroscopic lavage and debridement for osteoarthritis of the knee: an evidence-based analysis*. Ont Health Technol Assess Ser., 2005. **5**(12): p. 1-37. Epub 2005 Sep 1.
58. Bernstein, J. and T. Quach, *A perspective on the study of Moseley et al: questioning the value of arthroscopic knee surgery for osteoarthritis*. Cleve Clin J Med., 2003. **70**(5): p. 401, 405-6, 408-10.
59. Beiser, I.H. and I.O. Kanat, *Subchondral bone drilling: a treatment for cartilage defects*. J Foot Surg., 1990. **29**(6): p. 595-601.
60. Steadman, J.R., W.G. Rodkey, and J.J. Rodrigo, *Microfracture: surgical technique and rehabilitation to treat chondral defects*. Clin Orthop Relat Res., 2001(391 Suppl): p. S362-9.
61. Bekkers, J.E., M. Inklaar, and D.B. Saris, *Treatment selection in articular cartilage lesions of the knee: a systematic review*. Am J Sports Med., 2009. **37**(Suppl 1): p. 148S-55S. doi: 10.1177/0363546509351143.
62. Mithoefer, K., et al., *Clinical Efficacy of the Microfracture Technique for Articular Cartilage Repair in the Knee: An Evidence-Based Systematic Analysis*. The American Journal of Sports Medicine, 2009. **37**(10): p. 2053-2063.

63. Steadman, J.R., W.G. Rodkey, and K.K. Briggs, *Microfracture to treat full-thickness chondral defects: surgical technique, rehabilitation, and outcomes*. J Knee Surg., 2002. **15**(3): p. 170-6.
64. Mithoefer, K., et al., *The microfracture technique for the treatment of articular cartilage lesions in the knee. A prospective cohort study*. J Bone Joint Surg Am., 2005. **87**(9): p. 1911-20.
65. Miller, B.S., et al., *Patient satisfaction and outcome after microfracture of the degenerative knee*. J Knee Surg., 2004. **17**(1): p. 13-7.
66. Hunziker, E.B., *Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects*. Osteoarthritis Cartilage., 2002. **10**(6): p. 432-63.
67. Steadman, J.R., et al., *The microfracture technique in the treatment of full-thickness chondral lesions of the knee in National Football League players*. J Knee Surg., 2003. **16**(2): p. 83-6.
68. Mithoefer, K., et al., *High-impact athletics after knee articular cartilage repair: a prospective evaluation of the microfracture technique*. Am J Sports Med., 2006. **34**(9): p. 1413-8. Epub 2006 May 30.
69. Hangody, L., et al., *Autologous osteochondral mosaicplasty. Surgical technique*. J Bone Joint Surg Am., 2004. **86-A**(Suppl 1): p. 65-72.
70. Robert, H., *Chondral repair of the knee joint using mosaicplasty*. Orthopaedics & Traumatology: Surgery & Research, 2011. **97**(4): p. 418-429.
71. Hangody, L., et al., *Autologous osteochondral grafting, Technique and long-term results*. Injury, 2008. **39**(1, Supplement): p. 32-39.

72. Bentley, G., et al., *A prospective, randomised comparison of autologous chondrocyte implantation versus mosaicplasty for osteochondral defects in the knee*. J Bone Joint Surg Br., 2003. **85**(2): p. 223-30.
73. Brittberg, M., et al., *Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation*. N Engl J Med., 1994. **331**(14): p. 889-95.
74. Bartlett, W., et al., *Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation for osteochondral defects of the knee: A PROSPECTIVE, RANDOMISED STUDY*. Journal of Bone & Joint Surgery, British Volume, 2005. **87-B**(5): p. 640-645.
75. Johnstone, B., et al., *Tissue engineering for articular cartilage repair--the state of the art*. Eur Cell Mater., 2013. **25**: p. 248-67.
76. Tuan, R.S., *A second-generation autologous chondrocyte implantation approach to the treatment of focal articular cartilage defects*. Arthritis Research & Therapy, 2007. **9**(5): p. 109-109.
77. Wang, W., et al., *The restoration of full-thickness cartilage defects with BMSCs and TGF-beta 1 loaded PLGA/fibrin gel constructs*. Biomaterials, 2010. **31**(34): p. 8964-8973.
78. Bhumiratana, S., et al., *Large, stratified, and mechanically functional human cartilage grown in vitro by mesenchymal condensation*. Proceedings of the National Academy of Sciences, 2014. **111**(19): p. 6940-6945.
79. McCormick, F., et al., *Treatment of Focal Cartilage Defects With a Juvenile Allogeneic 3-Dimensional Articular Cartilage Graft*. Operative Techniques in Sports Medicine. **21**(2): p. 95-99.
80. DuRaine, G.D., et al., *Emergence of Scaffold-Free Approaches for Tissue Engineering Musculoskeletal Cartilages*. Ann Biomed Eng, 2014. **21**: p. 21.

81. Okano, T., et al., *A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide)*. J Biomed Mater Res., 1993. **27**(10): p. 1243-51.
82. Sato, M., et al., *Articular Cartilage Regeneration Using Cell Sheet Technology*. The Anatomical Record, 2014. **297**(1): p. 36-43.
83. Yang, J., et al., *Cell sheet engineering: Recreating tissues without biodegradable scaffolds*. Biomaterials, 2005. **26**(33): p. 6415-6422.
84. McCormick, F., et al., *Treatment of Focal Cartilage Defects With a Juvenile Allogeneic 3-Dimensional Articular Cartilage Graft*. Operative Techniques in Sports Medicine, 2013. **21**(2): p. 95-99.
85. Ebihara, G., et al., *Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model*. Biomaterials., 2012. **33**(15): p. 3846-51. doi: 10.1016/j.biomaterials.2012.01.056. Epub 2012 Feb 25.
86. Mitani, G., et al., *The properties of bioengineered chondrocyte sheets for cartilage regeneration*. BMC Biotechnology, 2009. **9**(1): p. 17.
87. Furukawa, K.S., et al., *Rapid and large-scale formation of chondrocyte aggregates by rotational culture*. Cell Transplant., 2003. **12**(5): p. 475-9.
88. Wolf, F., et al., *Cartilage tissue engineering using pre-aggregated human articular chondrocytes*. Eur Cell Mater., 2008. **16**: p. 92-9.
89. Imabayashi, H., et al., *Redifferentiation of dedifferentiated chondrocytes and chondrogenesis of human bone marrow stromal cells via chondrosphere formation with expression profiling by large-scale cDNA analysis*. Experimental Cell Research, 2003. **288**(1): p. 35-50.
90. Huey, D.J. and K.A. Athanasiou, *Alteration of the Fibrocartilaginous Nature of Scaffoldless Constructs Formed from Leporine Meniscus Cells and Chondrocytes*

- through Manipulation of Culture and Processing Conditions. Cells Tissues Organs*, 2013. **197**(5): p. 360-371.
91. Fickert, S., et al., *One-Year Clinical and Radiological Results of a Prospective, Investigator-Initiated Trial Examining a Novel, Purely Autologous 3-Dimensional Autologous Chondrocyte Transplantation Product in the Knee*. Cartilage, 2011.
 92. Gigout, A., M.D. Buschmann, and M. Jolicoeur, *Chondrocytes cultured in stirred suspension with serum-free medium containing pluronic-68 aggregate and proliferate while maintaining their differentiated phenotype*. Tissue Eng Part A, 2009. **15**(8): p. 2237-48. doi: 10.1089/ten.tea.2008.0256.
 93. Kolettas, E., et al., *Expression of cartilage-specific molecules is retained on long-term culture of human articular chondrocytes*. J Cell Sci., 1995. **108**(Pt 5): p. 1991-9.
 94. Athanasiou, K.A., et al., *Self-Organization and the Self-Assembling Process in Tissue Engineering*. Annual Review of Biomedical Engineering, 2013. **15**(1): p. 115-136.
 95. Detamore, M.S. and K.A. Athanasiou, *Evaluation of three growth factors for TMJ disc tissue engineering*. Ann Biomed Eng., 2005. **33**(3): p. 383-90.
 96. Huey, D.J. and K.A. Athanasiou, *Maturation growth of self-assembled, functional menisci as a result of TGF-beta1 and enzymatic chondroitinase-ABC stimulation*. Biomaterials., 2011. **32**(8): p. 2052-8. doi: 10.1016/j.biomaterials.2010.11.041. Epub 2010 Dec 10.
 97. Sanchez-Adams, J. and K.A. Athanasiou, *Dermis isolated adult stem cells for cartilage tissue engineering*. Biomaterials., 2012. **33**(1): p. 109-19. doi: 10.1016/j.biomaterials.2011.09.038. Epub 2011 Sep 28.
 98. Hoben, G.M., et al., *Self-assembly of fibrochondrocytes and chondrocytes for tissue engineering of the knee meniscus*. Tissue Engineering, 2007. **13**(5): p. 939-46.

99. MacBarb, R.F., et al., *Engineering functional anisotropy in fibrocartilage neotissues*. Biomaterials., 2013. **34**(38): p. 9980-9. doi: 10.1016/j.biomaterials.2013.09.026. Epub 2013 Sep 24.
100. Huey, D.J., J.C. Hu, and K.A. Athanasiou, *Chondrogenically-tuned expansion enhances the cartilaginous matrix forming capabilities of primary, adult, leporine chondrocytes*. Cell transplantation, 2013. **22**(2): p. 331-340.
101. Leddy, H.A., H.A. Awad, and F. Guilak, *Molecular diffusion in tissue-engineered cartilage constructs: effects of scaffold material, time, and culture conditions*. J Biomed Mater Res B Appl Biomater., 2004. **70**(2): p. 397-406.
102. Gong, Y., et al., *Microcavitary hydrogel-mediating phase transfer cell culture for cartilage tissue engineering*. Tissue Eng Part A, 2010. **16**(12): p. 3611-22. doi: 10.1089/ten.TEA.2010.0219. Epub 2010 Aug 30.
103. Su, K., et al., *Creating a living hyaline cartilage graft free from non-cartilaginous constituents: An intermediate role of a biomaterial scaffold*. Advanced Functional Materials, 2012. **22**(5): p. 972-978.
104. Kim, J.H., et al., *Effect of Phellodendron amurense in protecting human osteoarthritic cartilage and chondrocytes*. Journal of Ethnopharmacology, 2011. **134**(2): p. 234-242.
105. Conaghan, P.G., J. Dickson, and R.L. Grant, *Care and management of osteoarthritis in adults: summary of NICE guidance*. BMJ, 2008. **336**(7642): p. 502-503.
106. Clouet, J., et al., *From osteoarthritis treatments to future regenerative therapies for cartilage*. Drug Discovery Today, 2009. **14**(19-20): p. 913-925.
107. Samson, D.J., et al., *Treatment of primary and secondary osteoarthritis of the knee*. Evid Rep Technol Assess (Full Rep). 2007(157): p. 1-157.

108. Jr., W.C.S.; Available from:
<http://www.medicinenet.com/osteoarthritis/article.htm>.
109. Sokolove, J. and C.M. Lepus, *Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations*. Therapeutic Advances in Musculoskeletal Disease, 2013. **5**(2): p. 77-94.
110. de Lange-Brokaar, B.J.E., et al., *Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review*. Osteoarthritis and Cartilage, 2012. **20**(12): p. 1484-1499.
111. Scanzello, C.R. and S.R. Goldring, *The role of synovitis in osteoarthritis pathogenesis*. Bone, 2012. **51**(2): p. 249-257.
112. S.H.Hassanali, G.O.O.a., *Osteoarthritis: A look at pathophysiology and approach to new treatments: a review*. East African Orthopaedic Journal, 2011.
113. Lebre, M.C. and P.P. Tak, *Macrophage subsets in immune-mediated inflammatory disease: Lessons from rheumatoid arthritis, spondyloarthritis, behçet, Åôs disease and gout*. The Open Arthritis Journal, 2010. **3**: p. 18-23.
114. Blom, A.B., et al., *Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis*. Osteoarthritis and Cartilage, 2004. **12**(8): p. 627-635.
115. Blom, A.B., et al., *Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: Involvement of matrix metalloproteinase 3*. Arthritis and Rheumatism, 2007. **56**(1): p. 147-157.
116. Fernandes, J.C., J. Martel-Pelletier, and J.P. Pelletier, *The role of cytokines in osteoarthritis pathophysiology*. Biorheology., 2002. **39**(1-2): p. 237-46.

117. Kapoor, M., et al., *Role of proinflammatory cytokines in the pathophysiology of osteoarthritis*. Nat Rev Rheumatol., 2011. **7**(1): p. 33-42. doi: 10.1038/nrrheum.2010.196. Epub 2010 Nov 30.
118. Civinini, R., et al., *Growth factors in the treatment of early osteoarthritis*. Clinical Cases in Mineral and Bone Metabolism, 2013. **10**(1): p. 26-29.
119. Zhu, S., et al., *Alendronate protects against articular cartilage erosion by inhibiting subchondral bone loss in ovariectomized rats*. Bone, 2013. **53**(2): p. 340-349.
120. Fernandes, J.C., J. Martel-Pelletier, and J.P. Pelletier, *The role of cytokines in osteoarthritis pathophysiology*. Biorheology, 2002. **39**(1-2): p. 237-246.
121. Nagase, H., et al., *Deiodinase 2 upregulation demonstrated in osteoarthritis patients cartilage causes cartilage destruction in tissue-specific transgenic rats*. Osteoarthritis and Cartilage, 2013. **21**(3): p. 514-523.
122. Ameye, L.G. and M.F. Young, *Animal models of osteoarthritis: Lessons learned while seeking the 'Holy Grail'*. Current Opinion in Rheumatology, 2006. **18**(5): p. 537-547.
123. Little, C.B. and M.M. Smith, *Animal models of osteoarthritis*. Current Rheumatology Reviews, 2008. **4**(3): p. 175-182.
124. Fioravanti, A., et al., *In vitro effects of VA441, a new selective cyclooxygenase-2 inhibitor, on human osteoarthritic chondrocytes exposed to IL-1 α* . Journal of Pharmacological Sciences, 2012. **120**(1): p. 6-14.
125. van der Kraan, P.M., *Osteoarthritis year 2012 in review: Biology*. Osteoarthritis and Cartilage, 2012. **20**(12): p. 1447-1450.
126. Pritzker, K.P.H., et al., *Osteoarthritis cartilage histopathology: grading and staging*. Osteoarthritis and Cartilage, 2006. **14**(1): p. 13-29.

127. Aigner, T., et al., *Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritic human articular knee cartilage: A study of proliferation, programmed cell death (apoptosis), and viability of chondrocytes in normal and osteoarthritic human knee cartilage.* Arthritis & Rheumatism, 2001. **44**(6): p. 1304-1312.
128. Sandell, L.J. and T. Aigner, *Articular cartilage and changes in arthritis An introduction: Cell biology of osteoarthritis.* Arthritis Research, 2001. **3**(2): p. 107-113.
129. Johnson, E.O., et al., *Apoptosis in osteoarthritis: morphology, mechanisms, and potential means for therapeutic intervention.* J Surg Orthop Adv., 2008. **17**(3): p. 147-52.
130. Hashimoto, S., et al., *Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis.* Arthritis & Rheumatism, 1998. **41**(9): p. 1632-1638.
131. Schwager, J., et al., *Rose hip and its constituent galactolipids confer cartilage protection by modulating cytokine, and chemokine expression.* BMC Complementary and Alternative Medicine, 2011. **11**.
132. Lippiello, L., D. Hall, and H.J. Mankin, *Collagen synthesis in normal and osteoarthritic human cartilage.* Journal of Clinical Investigation, 1977. **59**(4): p. 593-600.
133. Aigner, T., et al., *Activation of collagen type II expression in osteoarthritic and rheumatoid cartilage.* Virchows Arch B Cell Pathol Incl Mol Pathol., 1992. **62**(6): p. 337-45.
134. Eyre, D.R., et al., *Biosynthesis of collagen and other matrix proteins by articular cartilage in experimental osteoarthrosis.* Biochemical Journal, 1980. **188**(3): p. 823-837.

135. Aigner, T. and J. Dudhia, *Phenotypic modulation of chondrocytes as a potential therapeutic target in osteoarthritis: a hypothesis*. Annals of the Rheumatic Diseases, 1997. **56**(5): p. 287-291.
136. Yoshihara, Y., et al., *Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis*. Annals of the Rheumatic Diseases, 2000. **59**(6): p. 455-461.
137. Dean, D.D., O.E. Muniz, and D.S. Howell, *Association of collagenase and tissue inhibitor of metalloproteinases (TIMP) with hypertrophic cell enlargement in the growth plate*. Matrix., 1989. **9**(5): p. 366-75.
138. Zhou, X., et al., *Tetrandrine inhibits the Wnt/ β -catenin signalling pathway and alleviates osteoarthritis: An in vitro and in vivo study*. Evidence-based Complementary and Alternative Medicine, 2013. **2013**.
139. Morell, M., et al., *Analgesic effect of the neuropeptide cortistatin in murine models of arthritic inflammatory pain*. Arthritis and Rheumatism, 2013. **65**(5): p. 1390-1401.
140. Jalb, B.A., et al., *Alterations in expression of cartilage-specific genes for aggrecan and collagen type II in osteoarthritis*. Romanian Journal of Morphology and Embryology, 2011. **52**(2): p. 587-591.
141. Chen, Y.J., et al., *EGb761 inhibits inflammatory responses in human chondrocytes and shows chondroprotection in osteoarthritic rat knee*. Journal of Orthopaedic Research, 2013. **31**(7): p. 1032-1038.
142. Csaki, C., A. Mobasheri, and M. Shakibaei, *Synergistic chondroprotective effects of curcumin and resveratrol in human articular chondrocytes: inhibition of IL-1 β -induced NF-kappaB-mediated inflammation and apoptosis*. Arthritis Research & Therapy, 2009. **11**(6): p. R165.

143. Von Der Mark, K., et al., *Type x collagen synthesis in human osteoarthritic cartilage. indication of chondrocyte hypertrophy*. Arthritis & Rheumatism, 1992. **35**(7): p. 806-811.
144. Bulstra, S.K., et al., *Metabolic characteristics of in vitro cultured human chondrocytes in relation to the histopathologic grade of osteoarthritis*. Clinical Orthopaedics and Related Research, 1989(242): p. 294-302.
145. Johansson, N., et al., *Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development*. Developmental Dynamics, 1997. **208**(3): p. 387-397.
146. Bolton, M.C., J. Dudhia, and M.T. Bayliss, *Age-related changes in the synthesis of link protein and aggrecan in human articular cartilage: Implications for aggregate stability*. Biochemical Journal, 1999. **337**(1): p. 77-82.
147. Cs-Szab $\sqrt{\geq}$, G., et al., *Changes in messenger RNA and protein levels of proteoglycans and link protein in human osteoarthritic cartilage samples*. Arthritis and Rheumatism, 1997. **40**(6): p. 1037-1045.
148. Cohen-Solal, M., T. Funck-Brentano, and E. Hay, *Animal models of osteoarthritis for the understanding of the bone contribution*. BoneKEy Rep, 2013. **2**.
149. Kobayashi, K., et al., *Sodium iodoacetate-induced experimental osteoarthritis and associated pain model in rats*. Journal of Veterinary Medical Science, 2003. **65**(11): p. 1195-1199.
150. Ma, B., et al., *Gene expression profiling of dedifferentiated human articular chondrocytes in monolayer culture*. Osteoarthritis and Cartilage, 2013. **21**(4): p. 599-603.

151. Takata, N., et al., *Comparison between loose fragment chondrocytes and condyle fibrochondrocytes in cellular proliferation and redifferentiation*. Journal of Orthopaedic Science, 2011. **16**(5): p. 589-597.
152. Esch, M.B., T.L. King, and M.L. Shuler, *The Role of Body-on-a-Chip Devices in Drug and Toxicity Studies*. Annual Review of Biomedical Engineering, 2011. **13**(1): p. 55-72.
153. Song, Y.W. and E.H. Kang, *Autoantibodies in rheumatoid arthritis: rheumatoid factors and anticitrullinated protein antibodies*. Vol. 103. 2010. 139-146.
154. Schellekens, G.A., et al., *The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide*. Arthritis Rheum., 2000. **43**(1): p. 155-63.
155. van der Helm-van Mil, A.H., J.Z. Wesoly, and T.W. Huizinga, *Understanding the genetic contribution to rheumatoid arthritis*. Curr Opin Rheumatol., 2005. **17**(3): p. 299-304.
156. Di Giuseppe, D., et al., *Cigarette smoking and risk of rheumatoid arthritis: a dose-response meta-analysis*. Arthritis Research & Therapy, 2014. **16**(2): p. R61.
157. Boissier, M.-C., et al., *Rheumatoid arthritis: From autoimmunity to synovitis and joint destruction*. Journal of Autoimmunity, 2012. **39**(3): p. 222-228.
158. Goldring, S.R., *Pathogenesis of bone and cartilage destruction in rheumatoid arthritis*. Rheumatology (Oxford). 2003. **42**(Suppl 2): p. ii11-6.
159. Huber, L.C., et al., *Synovial fibroblasts: key players in rheumatoid arthritis*. Rheumatology, 2006. **45**(6): p. 669-675.
160. Izquierdo, E., et al., *Synovial fibroblast hyperplasia in rheumatoid arthritis: clinicopathologic correlations and partial reversal by anti-tumor necrosis factor therapy*. Arthritis Rheum., 2011. **63**(9): p. 2575-83. doi: 10.1002/art.30433.

161. Ibold, Y., et al., *Development of a high-throughput screening assay based on the 3-dimensional pannus model for rheumatoid arthritis*. J Biomol Screen., 2007. **12**(7): p. 956-65.
162. McInnes, I.B. and G. Schett, *Cytokines in the pathogenesis of rheumatoid arthritis*. Nat Rev Immunol, 2007. **7**(6): p. 429-442.
163. Juarez, M., A. Filer, and C.D. Buckley, *Fibroblasts as therapeutic targets in rheumatoid arthritis and cancer*. Swiss Med Wkly., 2012. **142:w13529**.(doi): p. 10.4414/smw.2012.13529.
164. Ruth, J.H., et al., *CXCL16-mediated cell recruitment to rheumatoid arthritis synovial tissue and murine lymph nodes is dependent upon the MAPK pathway*. Arthritis Rheum., 2006. **54**(3): p. 765-78.
165. Müller Ladner, U., et al., *Cells of the synovium in rheumatoid arthritis. Synovial fibroblasts*. Arthritis Research & Therapy, 2007. **9**(6): p. 223-223.
166. Tolboom, T.C., et al., *Invasiveness of fibroblast-like synoviocytes is an individual patient characteristic associated with the rate of joint destruction in patients with rheumatoid arthritis*. Arthritis Rheum., 2005. **52**(7): p. 1999-2002.
167. Müller-Ladner, U., et al., *Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice*. The American Journal of Pathology, 1996. **149**(5): p. 1607-1615.
168. Brentano, F., et al., *The role of Toll-like receptor signalling in the pathogenesis of arthritis*. Cell Immunol., 2005. **233**(2): p. 90-6.
169. Brentano, F., et al., *RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3*. Arthritis Rheum., 2005. **52**(9): p. 2656-65.

170. Pierer, M., et al., *Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands*. J Immunol., 2004. **172**(2): p. 1256-65.
171. Jungel, A., et al., *Expression of interleukin-21 receptor, but not interleukin-21, in synovial fibroblasts and synovial macrophages of patients with rheumatoid arthritis*. Arthritis Rheum., 2004. **50**(5): p. 1468-76.
172. Ikeuchi, H., et al., *Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine*. Arthritis Rheum., 2005. **52**(4): p. 1037-46.
173. Mulherin, D., O. Fitzgerald, and B. Bresnihan, *Synovial tissue macrophage populations and articular damage in rheumatoid arthritis*. Arthritis Rheum., 1996. **39**(1): p. 115-24.
174. Tak, P.P., et al., *Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity*. Arthritis Rheum., 1997. **40**(2): p. 217-25.
175. Koch, A., *Angiogenesis as a target in rheumatoid arthritis*. Annals of the Rheumatic Diseases, 2003. **62**(Suppl 2): p. ii60-ii67.
176. Janusz, M.J. and M. Hare, *Cartilage degradation by cocultures of transformed macrophage and fibroblast cell lines. A model of metalloproteinase-mediated connective tissue degradation*. J Immunol., 1993. **150**(5): p. 1922-31.
177. Scott, B.B., et al., *Rheumatoid arthritis synovial fibroblast and U937 macrophage/monocyte cell line interaction in cartilage degradation*. Arthritis Rheum., 1997. **40**(3): p. 490-8.
178. Kinne, R.W., B. Stuhlmuller, and G.R. Burmester, *Cells of the synovium in rheumatoid arthritis. Macrophages*. Arthritis Res Ther., 2007. **9**(6): p. 224. doi: 10.1186/ar2333.

179. Borovecki, F., N. Pecina-Slaus, and S. Vukicevic, *Biological mechanisms of bone and cartilage remodelling,Ä genomic perspective*. International Orthopaedics, 2007. **31**(6): p. 799-805.
180. Abramson, S.B. and A. Amin, *Blocking the effects of IL-1 in rheumatoid arthritis protects bone and cartilage*. Rheumatology (Oxford). 2002. **41**(9): p. 972-80.
181. Schiff, M.H., *Role of interleukin 1 and interleukin 1 receptor antagonist in the mediation of rheumatoid arthritis*. Annals of the Rheumatic Diseases, 2000. **59**(suppl 1): p. i103-i108.
182. Matsuno, H., et al., *The role of TNF-alpha in the pathogenesis of inflammation and joint destruction in rheumatoid arthritis (RA): a study using a human RA/SCID mouse chimera*. Rheumatology (Oxford). 2002. **41**(3): p. 329-37.
183. Kobayashi, I. and M. Ziff, *Electron microscopic studies of the cartilage-pannus junction in rheumatoid arthritis*. Arthritis Rheum., 1975. **18**(5): p. 475-83.
184. Woolley, D.E., M.J. Crossley, and J.M. Evanson, *Collagenase at sites of cartilage erosion in the rheumatoid joint*. Arthritis Rheum., 1977. **20**(6): p. 1231-9.
185. Edwards, J.C., *Fibroblast biology. Development and differentiation of synovial fibroblasts in arthritis*. Arthritis Res., 2000. **2**(5): p. 344-7. Epub 2000 Jun 8.
186. Mitchell, N.S. and N. Shepard, *Changes in proteoglycan and collagen in cartilage in rheumatoid arthritis*. J Bone Joint Surg Am., 1978. **60**(3): p. 342-8.
187. Dodge, G.R., I. Pidoux, and A.R. Poole, *The Degradation of Type II Collagen in Rheumatoid Arthritis: An Immunoelectron Microscopic Study*. Matrix, 1991. **11**(5): p. 330-338.
188. Otero, M. and M.B. Goldring, *Cells of the synovium in rheumatoid arthritis. Chondrocytes*. Arthritis Research & Therapy, 2007. **9**(5): p. 220-220.

189. Tetlow, L.C. and D.E. Woolley, *Comparative immunolocalization studies of collagenase 1 and collagenase 3 production in the rheumatoid lesion, and by human chondrocytes and synoviocytes in vitro*. Br J Rheumatol., 1998. **37**(1): p. 64-70.
190. Cunnane, G., et al., *Synovial tissue protease gene expression and joint erosions in early rheumatoid arthritis*. Arthritis Rheum., 2001. **44**(8): p. 1744-53.
191. Arner, E.C., *Aggrecanase-mediated cartilage degradation*. Curr Opin Pharmacol., 2002. **2**(3): p. 322-9.
192. Plaas, A., et al., *Aggrecanolysis in human osteoarthritis: confocal localization and biochemical characterization of ADAMTS5-hyaluronan complexes in articular cartilages*. Osteoarthritis Cartilage., 2007. **15**(7): p. 719-34. Epub 2007 Mar 23.
193. Kannan, K., R.A. Ortmann, and D. Kimpel, *Animal models of rheumatoid arthritis and their relevance to human disease*. Pathophysiology., 2005. **12**(3): p. 167-81.
194. Asquith, D.L., et al., *Animal models of rheumatoid arthritis*. Eur J Immunol., 2009. **39**(8): p. 2040-4. doi: 10.1002/eji.200939578.
195. Courtenay, J.S., et al., *IMMUNIZATION AGAINST HETEROLOGOUS TYPE-II COLLAGEN INDUCES ARTHRITIS IN MICE*. Nature, 1980. **283**(5748): p. 666-668.
196. Rowley, M.J., K.S. Nandakumar, and R. Holmdahl, *The role of collagen antibodies in mediating arthritis*. Modern Rheumatology, 2008. **18**(5): p. 429-441.
197. Frasnelli, M.E., et al., *TLR2 modulates inflammation in zymosan-induced arthritis in mice*. Arthritis Research & Therapy, 2005. **7**(2): p. R370-R379.
198. Wooley, P.H., et al., *PRISTANE-INDUCED ARTHRITIS - THE IMMUNOLOGICAL AND GENETIC FEATURES OF AN EXPERIMENTAL MURINE MODEL OF AUTOIMMUNE-DISEASE*. Arthritis and Rheumatism, 1989. **32**(8): p. 1022-1030.

199. Glant, T.T., A. Finnegan, and K. Mikecz, *Proteoglycan-induced arthritis: Immune regulation, cellular mechanisms, and genetics*. Critical Reviews in Immunology, 2003. **23**(3): p. 199-250.
200. Keffer, J., et al., *TRANSGENIC MICE EXPRESSING HUMAN TUMOR-NECROSIS-FACTOR - A PREDICTIVE GENETIC MODEL OF ARTHRITIS*. Embo Journal, 1991. **10**(13): p. 4025-4031.
201. van Gaalen, F.A., et al., *Association of autoantibodies to glucose-6-phosphate isomerase with extraarticular complications in rheumatoid arthritis*. Arthritis and Rheumatism, 2004. **50**(2): p. 395-399.
202. Joe, B., et al., *Animal models of rheumatoid arthritis and related inflammation*. Curr Rheumatol Rep., 1999. **1**(2): p. 139-48.
203. Geiler, T., et al., *A NEW MODEL FOR RHEUMATOID-ARTHRITIS GENERATED BY ENGRAFTMENT OF RHEUMATOID SYNOVIAL TISSUE AND NORMAL HUMAN CARTILAGE INTO SCID MICE*. Arthritis and Rheumatism, 1994. **37**(11): p. 1664-1671.
204. Fugger, L., et al., *EXPRESSION OF HLA-DR4 AND HUMAN CD4 TRANSGENES IN MICE DETERMINES THE VARIABLE REGION BETA-CHAIN T-CELL REPERTOIRE AND MEDIATES AN HLA-DR-RESTRICTED IMMUNE-RESPONSE*. Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(13): p. 6151-6155.
205. Andreas, K., et al., *Key regulatory molecules of cartilage destruction in rheumatoid arthritis: An in vitro study*. Arthritis Research and Therapy, 2008. **10**(1).
206. Schultz, O., et al., *Development of in vitro model systems for destructive joint diseases: novel strategies for establishing inflammatory pannus*. Arthritis Rheum., 1997. **40**(8): p. 1420-8.

207. Pretzel, D., et al., *In vitro model for the analysis of synovial fibroblast-mediated degradation of intact cartilage*. Arthritis Res Ther., 2009. **11**(1): p. R25. doi: 10.1186/ar2618. Epub 2009 Feb 18.
208. Ermis, A., et al., *Invasion of human cartilage by cultured multicellular spheroids of rheumatoid synovial cells--a novel in vitro model system for rheumatoid arthritis*. J Rheumatol., 1998. **25**(2): p. 208-13.
209. Lubke, C., et al., *Growth characterization of neo porcine cartilage pellets and their use in an interactive culture model*. Osteoarthritis Cartilage., 2005. **13**(6): p. 478-87.
210. Lemare, F., et al., *Dedifferentiated chondrocytes cultured in alginate beads: restoration of the differentiated phenotype and of the metabolic responses to interleukin-1beta*. J Cell Physiol., 1998. **176**(2): p. 303-13.
211. Peck, Y. and D.A. Wang, *Three-dimensionally engineered biomimetic tissue models for in vitro drug evaluation: delivery, efficacy and toxicity*. Expert Opin Drug Deliv., 2013. **10**(3): p. 369-83. doi: 10.1517/17425247.2013.751096. Epub 2013 Jan 6.
212. Comley, J., *3D cell culture: Easier said than done!* Drug Discovery World, 2010. **11**(3): p. 25-41.
213. Griffith, L.G. and M.A. Swartz, *Capturing complex 3D tissue physiology in vitro*. Nat Rev Mol Cell Biol, 2006. **7**(3): p. 211-224.
214. Sivaraman, A., et al., *A microscale in vitro physiological model of the liver: Predictive screens for drug metabolism and enzyme induction*. Current Drug Metabolism, 2005. **6**(6): p. 569-591.
215. Chiang, H. and C.C. Jiang, *Repair of articular cartilage defects: Review and perspectives*. Journal of the Formosan Medical Association, 2009. **108**(2): p. 87-101.

216. Luyten, F.P. and J. Vanlauwe, *Tissue engineering approaches for osteoarthritis*. Bone, 2012. **51**(2): p. 289-296.
217. Ringe, J., G.R. Burmester, and M. Sittlinger, *Regenerative medicine in rheumatic disease-progress in tissue engineering*. Nature Reviews Rheumatology, 2012. **8**(8): p. 493-498.
218. Cortial, D., et al., *Activation by IL-1 of bovine articular chondrocytes in culture within a 3D collagen-based scaffold. An in vitro model to address the effect of compounds with therapeutic potential in osteoarthritis*. Osteoarthritis and Cartilage, 2006. **14**(7): p. 631-640.
219. Sun, L., X. Wang, and D.L. Kaplan, *A 3D cartilage - Inflammatory cell culture system for the modeling of human osteoarthritis*. Biomaterials, 2011. **32**(24): p. 5581-5589.
220. Lück, C., et al., *Growth characterization of neo porcine cartilage pellets and their use in an interactive culture model*. Osteoarthritis and Cartilage, 2005. **13**(6): p. 478-487.
221. Smolian, H., et al., *Establishment of an in vitro model for rheumatoid arthritis as test system for therapeutical substances*. Etablierung eines in vitro Testsystems für die rheumatoide Arthritis zur Testung therapeutisch relevanter Wirkstoffe., 2001. **18**(4): p. 265-280.
222. Andreas, K., et al., *Antirheumatic drug response signatures in human chondrocytes: Potential molecular targets to stimulate cartilage regeneration*. Arthritis Research and Therapy, 2009. **11**(1).
223. Ibold, Y., et al., *Development of a High-Throughput Screening Assay Based on the 3-Dimensional Pannus Model for Rheumatoid Arthritis*. Journal of Biomolecular Screening, 2007. **12**(7): p. 956-965.

224. Buckwalter, J.A. and H.J. Mankin, *Articular cartilage. Part II: Degeneration and osteoarthritis, repair, regeneration, and transplantation*. Journal of Bone and Joint Surgery - Series A, 1997. **79**(4): p. 612-632.
225. Jakob, M., et al., *Enzymatic digestion of adult human articular cartilage yields a small fraction of the total available cells*. Connective Tissue Research, 2003. **44**(3-4): p. 173-180.
226. Cakmak, O., et al., *Injectable tissue-engineered cartilage using commercially available fibrin glue*. Laryngoscope, 2013. **123**(12): p. 2986-2992.
227. Erggelet, C., et al., *Autologous chondrocyte implantation versus ACI using 3D-bioresorbable graft for the treatment of large full-thickness cartilage lesions of the knee*. Archives of orthopaedic and trauma surgery, 2010. **130**(8): p. 957-964.
228. Crawford, A. and S.C. Dickinson, *Chondrocyte isolation, expansion, and culture on polymer scaffolds*. Methods in molecular biology (Clifton, N.J.), 2004. **238**: p. 147-158.
229. Yonenaga, K., et al., *The optimal conditions of chondrocyte isolation and its seeding in the preparation for cartilage tissue engineering*. Tissue Engineering - Part C: Methods, 2010. **16**(6): p. 1461-1469.
230. Lau, T.T., et al., *Optimization of chondrocyte isolation and phenotype characterization for cartilage tissue engineering*. Tissue Eng Part C Methods., 2015. **21**(2): p. 105-11. doi: 10.1089/ten.TEC.2014.0159. Epub 2014 Oct 15.
231. Hayman, D., et al., *The effects of isolation on chondrocyte gene expression*. Tissue engineering, 2006. **12**(9): p. 2573-81.
232. Oseni, A.O., P.E. Butler, and A.M. Seifalian, *Optimization of chondrocyte isolation and characterization for large-scale cartilage tissue engineering*. Journal of Surgical Research, 2013. **181**(1): p. 41-48.

233. Peng, S., et al., *Evaluation of a mPEG-polyester-based hydrogel as cell carrier for chondrocytes*. Journal of Biomedical Materials Research - Part A, 2013. **101**(11): p. 3311-3319.
234. Xia, Z., et al., *A method of isolating viable chondrocytes with proliferative capacity from cryopreserved human articular cartilage*. Cell and Tissue Banking, 2013. **14**(2): p. 267-276.
235. Yuan, T., et al., *Collagen hydrogel as an immunomodulatory scaffold in cartilage tissue engineering*. Journal of Biomedical Materials Research - Part B Applied Biomaterials, 2013.
236. Shui, W., et al., *Characterization of chondrocyte scaffold carriers for cell-based gene therapy in articular cartilage repair*. Journal of Biomedical Materials Research - Part A, 2013. **101**(12): p. 3542-3550.
237. Niemeyer, P., et al., *Influence of cell quality on clinical outcome after autologous chondrocyte implantation*. American Journal of Sports Medicine, 2012. **40**(3): p. 556-561.
238. Pietschmann, M.F., et al., *Cell quality affects clinical outcome after MACI procedure for cartilage injury of the knee*. Knee Surgery, Sports Traumatology, Arthroscopy, 2009. **17**(11): p. 1305-1311.
239. Siczkowski, M. and F.M. Watt, *Subpopulations of chondrocytes from different zones of pig articular cartilage: Isolation, growth and proteoglycan synthesis in culture*. Journal of Cell Science, 1990. **97**(2): p. 349-360.
240. Sharma, B., et al., *Designing zonal organization into tissue-engineered cartilage*. Tissue engineering, 2007. **13**(2): p. 405-414.
241. Klein, T.J., et al., *Tissue engineering of articular cartilage with biomimetic zones*. Tissue Engineering - Part B: Reviews, 2009. **15**(2): p. 143-157.

242. Hwang, N.S., et al., *Response of zonal chondrocytes to extracellular matrix-hydrogels*. FEBS Letters, 2007. **581**(22): p. 4172-4178.
243. Buckwalter, J.A. and H.J. Mankin, *Articular cartilage: tissue design and chondrocyte-matrix interactions*. Instructional course lectures, 1998. **47**: p. 477-486.
244. Martin, J. and J. Buckwalter, *Aging, articular cartilage chondrocyte senescence and osteoarthritis*. Biogerontology, 2002. **3**(5): p. 257-264.
245. Montoya, F., et al., *Clinical and experimental approaches to knee cartilage lesion repair and mesenchymal stem cell chondrocyte differentiation*. Biological Research, 2013. **46**(4): p. 441-451.
246. Saris, D.B.F., et al., *Characterized Chondrocyte Implantation Results in Better Structural Repair When Treating Symptomatic Cartilage Defects of the Knee in a Randomized Controlled Trial Versus Microfracture*. The American Journal of Sports Medicine, 2008. **36**(2): p. 235-246.
247. Gobbi, A., G. Karnatzikos, and A. Kumar, *Long-term results after microfracture treatment for full-thickness knee chondral lesions in athletes*. Knee Surg Sports Traumatol Arthrosc., 2014. **22**(9): p. 1986-96. doi: 10.1007/s00167-013-2676-8. Epub 2013 Sep 20.
248. Gross, A.E., et al., *Fresh Osteochondral Allografts for Posttraumatic Knee Defects: Long-term Followup*. Clinical Orthopaedics and Related Research, 2008. **466**(8): p. 1863-1870.
249. Harris, J.D., et al., *Failures, re-operations, and complications after autologous chondrocyte implantation - a systematic review*. Osteoarthritis and Cartilage, 2011. **19**(7): p. 779-791.

250. Filardo, G., et al., *Second-generation arthroscopic autologous chondrocyte implantation for the treatment of degenerative cartilage lesions*. Knee Surgery, Sports Traumatology, Arthroscopy, 2012. **20**(9): p. 1704-1713.
251. Caron, M.M.J., et al., *Redifferentiation of dedifferentiated human articular chondrocytes: Comparison of 2D and 3D cultures*. Osteoarthritis and Cartilage, 2012. **20**(10): p. 1170-1178.
252. Makris, E.A., et al., *Repair and tissue engineering techniques for articular cartilage*. Nat Rev Rheumatol, 2015. **11**(1): p. 21-34.
253. Ahern, B.J., et al., *Preclinical animal models in single site cartilage defect testing: a systematic review*. Osteoarthritis and Cartilage, 2009. **17**(6): p. 705-713.
254. Hurtig, M.B., et al., *Preclinical studies for cartilage repair: Recommendations from the international cartilage repair society*. Cartilage, 2011. **2**(2): p. 137-152.
255. Marlovits, S., et al., *Definition of pertinent parameters for the evaluation of articular cartilage repair tissue with high-resolution magnetic resonance imaging*. Eur J Radiol., 2004. **52**(3): p. 310-9.
256. Marlovits, S., et al., *Magnetic resonance observation of cartilage repair tissue (MOCART) for the evaluation of autologous chondrocyte transplantation: determination of interobserver variability and correlation to clinical outcome after 2 years*. Eur J Radiol., 2006. **57**(1): p. 16-23. Epub 2005 Oct 3.
257. Peterson, L., et al., *Two- to 9-year outcome after autologous chondrocyte transplantation of the knee*. Clin Orthop Relat Res., 2000(374): p. 212-34.
258. Wakitani, S., et al., *Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage*. J Bone Joint Surg Am., 1994. **76**(4): p. 579-92.
259. Harman, B.D., et al., *Osteochondral autograft transplantation in the porcine knee*. Am J Sports Med., 2006. **34**(6): p. 913-8.

260. Ng, S.S., et al., *Biomechanical study of the edge outgrowth phenomenon of encapsulated chondrocytic isogenous groups in the surface layer of hydrogel scaffolds for cartilage tissue engineering*. Acta Biomater., 2012. **8**(1): p. 244-52. doi: 10.1016/j.actbio.2011.08.018. Epub 2011 Aug 25.
261. Hirshberg, A., et al., *Collagen fibres in the wall of odontogenic keratocysts: a study with picrosirius red and polarizing microscopy*. J Oral Pathol Med., 1999. **28**(9): p. 410-2.
262. Montes, G.S. and L.C. Junqueira, *The use of the Picrosirius-polarization method for the study of the biopathology of collagen*. Mem Inst Oswaldo Cruz., 1991. **86**(Suppl 3): p. 1-11.
263. Villar-Suárez, V., et al., *Effects of Extracellular Matrix on the Morphology and Behaviour of Rabbit Auricular Chondrocytes in Culture*. Journal of Biomedicine and Biotechnology, 2005. **2005**(4): p. 364-373.
264. Tare, R.S., et al., *Tissue engineering strategies for cartilage generation, Micromass and three dimensional cultures using human chondrocytes and a continuous cell line*. Biochemical and Biophysical Research Communications, 2005. **333**(2): p. 609-621.
265. Vinatier, C., et al., *A silanized hydroxypropyl methylcellulose hydrogel for the three-dimensional culture of chondrocytes*. Biomaterials, 2005. **26**(33): p. 6643-6651.
266. Wang, C., R.R. Varshney, and D.A. Wang, *Therapeutic cell delivery and fate control in hydrogels and hydrogel hybrids*. Adv Drug Deliv Rev., 2010. **62**(7-8): p. 699-710. doi: 10.1016/j.addr.2010.02.001. Epub 2010 Feb 6.
267. Chen, F.H., K.T. Rousche, and R.S. Tuan, *Technology Insight: adult stem cells in cartilage regeneration and tissue engineering*. Nat Clin Pract Rheum, 2006. **2**(7): p. 373-382.

268. Murphy, M.K., et al., *Enhancing post-expansion chondrogenic potential of costochondral cells in self-assembled neocartilage*. PLoS One., 2013. **8**(2): p. e56983. doi: 10.1371/journal.pone.0056983. Epub 2013 Feb 21.
269. Chang, C.H., et al., *Tissue engineering-based cartilage repair with allogeneous chondrocytes and gelatin-chondroitin-hyaluronan tri-copolymer scaffold: a porcine model assessed at 18, 24, and 36 weeks*. Biomaterials., 2006. **27**(9): p. 1876-88. Epub 2005 Nov 8.
270. Li, W.-J., et al., *Evaluation of articular cartilage repair using biodegradable nanofibrous scaffolds in a swine model: a pilot study*. Journal of Tissue Engineering and Regenerative Medicine, 2009. **3**(1): p. 1-10.
271. Pabbruwe, M.B., et al., *Induction of cartilage integration by a chondrocyte/collagen-scaffold implant*. Biomaterials, 2009. **30**(26): p. 4277-4286.
272. Garnero, P., J.C. Rousseau, and P.D. Delmas, *Molecular basis and clinical use of biochemical markers of bone, cartilage, and synovium in joint diseases*. Arthritis & Rheumatism, 2000. **43**(5): p. 953-968.
273. Hosseini, S., et al., *The importance of superficial collagen fibrils for the function of articular cartilage*. Biomechanics and Modeling in Mechanobiology, 2014. **13**(1): p. 41-51.
274. Kääb, M., I. Ap Gwynn, and H. Nötzli, *Collagen fibre arrangement in the tibial plateau articular cartilage of man and other mammalian species*. Journal of Anatomy, 1998. **193**(01): p. 23-34.
275. Hurtig, M.B., et al., *Preclinical Studies for Cartilage Repair Recommendations from the International Cartilage Repair Society*. Cartilage, 2011. **2**(2): p. 137-152.
276. Hu, J.C. and K.A. Athanasiou, *A self-assembling process in articular cartilage tissue engineering*. Tissue Eng., 2006. **12**(4): p. 969-79.

277. Schubert, T., et al., *Long-term effects of chondrospheres on cartilage lesions in an autologous chondrocyte implantation model as investigated in the SCID mouse model*. Int J Mol Med., 2009. **23**(4): p. 455-60.
278. O'Driscoll, S.W., F.W. Keeley, and R.B. Salter, *The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion. An experimental investigation in the rabbit*. J Bone Joint Surg Am., 1986. **68**(7): p. 1017-35.
279. O'Driscoll, S.W., F.W. Keeley, and R.B. Salter, *Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous passive motion. A follow-up report at one year*. J Bone Joint Surg Am., 1988. **70**(4): p. 595-606.
280. Lau, T.T., et al., *Inducing ossification in an engineered 3D scaffold-free living cartilage template*. Biomaterials, 2012. **33**(33): p. 8406-8417.
281. Gong, Y., et al., *Microcavitary hydrogel-mediating phase transfer cell culture for cartilage tissue engineering*. Tissue Engineering - Part A, 2010. **16**(12): p. 3611-3622.
282. Wang, C., et al., *In vitro performance of an injectable hydrogel/microsphere based immunocyte delivery system for localised anti-tumour activity*. Biomaterials, 2009. **30**(36): p. 6986-6995.
283. Wynn, R.F., et al., *A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow*. Blood, 2004. **104**(9): p. 2643-2645.

284. Jefferies, D., et al., *Differences in metabolic parameters and gene expression related to osteochondrosis/osteoarthritis in pigs fed 25-hydroxyvitamin D-3*. Veterinary Research, 2002. **33**(4): p. 383-396.
285. Zhang, F., et al., *Redifferentiation of dedifferentiated chondrocytes by adenoviral vector-mediated TGF- β 3 and collagen-1 silencing shRNA in 3D culture*. Annals of Biomedical Engineering, 2011. **39**(12): p. 3042-3054.
286. Peck, Y., et al., *A three-dimensionally engineered biomimetic cartilaginous tissue model for osteoarthritic drug evaluation*. Mol Pharm., 2014. **11**(7): p. 1997-2008. doi: 10.1021/mp500026x. Epub 2014 Mar 10.
287. van den Berg, W.B., *Osteoarthritis year 2010 in review: Pathomechanisms*. Osteoarthritis and Cartilage, 2011. **19**(4): p. 338-341.
288. Berenbaum, F., *Osteoarthritis year 2010 in review: pharmacological therapies*. Osteoarthritis and Cartilage, 2011. **19**(4).
289. Hawker, G.A., et al., *Osteoarthritis year 2010 in review: Non-pharmacologic therapy*. Osteoarthritis and Cartilage, 2011. **19**(4): p. 366-374.
290. Fioravanti, A., et al., *In vitro effects of VA441, a new selective cyclooxygenase-2 inhibitor, on human osteoarthritic chondrocytes exposed to IL-1 β* . Journal of Pharmacological Sciences, 2012. **120**(1): p. 6-14.
291. Zhou, X., et al., *Tetrandrine inhibits the Wnt/ β -catenin signalling pathway and alleviates osteoarthritis: An in vitro and in vivo study*. Evidence-based Complementary and Alternative Medicine, 2013. **2013**.
292. Benito, M.J., et al., *Synovial tissue inflammation in early and late osteoarthritis*. Annals of the Rheumatic Diseases, 2005. **64**(9): p. 1263-1267.
293. Kinne, R.W., et al., *Macrophages in rheumatoid arthritis*. Arthritis Research, 2000. **2**(3): p. 189-202.

294. Zweers, M.C., et al., *Celecoxib: Considerations regarding its potential disease-modifying properties in osteoarthritis*. Arthritis Research and Therapy, 2011. **13**(5).
295. Hoemann, C., *Molecular and Biochemical Assays of Cartilage Components*, in *Cartilage and Osteoarthritis*, F.d.r. Ceuninck, M. Sabatini, and P. Pastoureau, Editors. 2004, Humana Press. p. 127-156.
296. Dreier, R., *Hypertrophic differentiation of chondrocytes in osteoarthritis: The developmental aspect of degenerative joint disorders*. Arthritis Research and Therapy, 2010. **12**(5).
297. Fajardo, M. and P.E. Di Cesare, *Disease-modifying therapies for osteoarthritis: Current status*. Drugs and Aging, 2005. **22**(2): p. 141-161.
298. Cowan, D.B., et al., *CD14-independent activation of cardiomyocyte signal transduction by bacterial endotoxin*. American Journal of Physiology - Heart and Circulatory Physiology, 2000. **279**(2 48-2): p. H619-H629.
299. Dranoff, G., *Cytokines in cancer pathogenesis and cancer therapy*. Nature Reviews Cancer, 2004. **4**(1): p. x11-22.
300. McInnes, I.B. and G. Schett, *Cytokines in the pathogenesis of rheumatoid arthritis*. Nature Reviews Immunology, 2007. **7**(6): p. 429-442.
301. Dreier, R., et al., *Paracrine interactions of chondrocytes and macrophages in cartilage degradation: articular chondrocytes provide factors that activate macrophage-derived pro-gelatinase B (pro-MMP-9)*. Journal of Cell Science, 2001. **114**(21): p. 3813-3822.
302. Rothwell, A.G. and G. Bentley, *Chondrocyte multiplication in osteoarthritic articular cartilage*. Journal of Bone and Joint Surgery - Series B, 1973. **55**(3): p. 588-594.

303. Tallheden, T., et al., *Proliferation and differentiation potential of chondrocytes from osteoarthritic patients*. Arthritis research & therapy., 2005. **7**(3): p. R560-568.
304. Lafeber, F.P.J.G., et al., *Local changes in proteoglycan synthesis during culture are different for normal and osteoarthritic cartilage*. American Journal of Pathology, 1992. **140**(6): p. 1421-1429.
305. Aigner, T., H.A. Kim, and H.I. Roach, *Apoptosis in osteoarthritis*. Rheumatic Disease Clinics of North America, 2004. **30**(3): p. 639-653.
306. Aigner, T. and H.A. Kim, *Apoptosis and cellular vitality: Issues in osteoarthritic cartilage degeneration*. Arthritis and Rheumatism, 2002. **46**(8): p. 1986-1996.
307. Blanco, F.J., et al., *Osteoarthritis chondrocytes die by apoptosis: A possible pathway for osteoarthritis pathology*. Arthritis & Rheumatism, 1998. **41**(2): p. 284-289.
308. Cs-Szabó , G., et al., *Changes in messenger RNA and protein levels of proteoglycans and link protein in human osteoarthritic cartilage samples*. Arthritis and Rheumatism, 1997. **40**(6): p. 1037-1045.
309. Aigner, T., et al., *Activation of collagen type II expression in osteoarthritic and rheumatoid cartilage*. Virchows Archiv - B Cell Pathology Including Molecular Pathology, 1992. **62**(6): p. 337-345.
310. Aigner, T., et al., *Mechanisms of Disease: Role of chondrocytes in the pathogenesis of osteoarthritis - Structure, chaos and senescence*. Nature Clinical Practice Rheumatology, 2007. **3**(7): p. 391-399.
311. Aigner, T., et al., *Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using complementary DNA-array technology*. Arthritis and Rheumatism, 2001. **44**(12): p. 2777-2789.

312. Shlopov, B.V., et al., *Osteoarthritic lesions: Involvement of three different collagenases*. Arthritis and Rheumatism, 1997. **40**(11): p. 2065-2074.
313. Billingham, R.C., et al., *Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage*. Journal of Clinical Investigation, 1997. **99**(7): p. 1534-1545.
314. Studer, D., et al., *Molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and mesenchymal stem cells*. European Cells and Materials, 2012. **24**: p. 118-135.
315. Pearle, A.D., R.F. Warren, and S.A. Rodeo, *Basic Science of Articular Cartilage and Osteoarthritis*. Clinics in sports medicine, 2005. **24**(1): p. 1-12.
316. Hosseininia, S., L.R. Lindberg, and L.E. Dahlberg, *Cartilage collagen damage in hip osteoarthritis similar to that seen in knee osteoarthritis; A case-control study of relationship between collagen, glycosaminoglycan and cartilage swelling*. BMC Musculoskeletal Disorders, 2013. **14**.
317. Kawashima, M., et al., *The anti-inflammatory effect of cyclooxygenase inhibitors in fibroblast-like synoviocytes from the human temporomandibular joint results from the suppression of PGE2 production*. Journal of Oral Pathology and Medicine, 2013. **42**(6): p. 499-506.
318. Dave, M. and A.R. Amin, *Yin-Yang regulation of prostaglandins and nitric oxide by PGD2 in human arthritis: Reversal by celecoxib*. Immunology Letters, 2013. **152**(1): p. 47-54.
319. Alvarez-Soria, M.A., et al., *Long-term NSAID treatment directly decreases COX-2 and mPGES-1 production in the articular cartilage of patients with osteoarthritis*. Osteoarthritis and Cartilage, 2008. **16**(12): p. 1484-1493.

320. El Hajjaji, H., et al., *Celecoxib Has a Positive Effect on the Overall Metabolism of Hyaluronan and Proteoglycans in Human Osteoarthritic Cartilage*. Journal of Rheumatology, 2003. **30**(11): p. 2444-2451.
321. Mastbergen, S.C., et al., *Differential direct effects of cyclo-oxygenase-1/2 inhibition on proteoglycan turnover of human osteoarthritic cartilage: an in vitro study*. Arthritis Research & Therapy, 2006. **8**(1).
322. Mastbergen, S.C., J.W. Bijlsma, and F.P. Lafeber, *Selective COX-2 inhibition is favorable to human early and late-stage osteoarthritic cartilage: A human in vitro study*. Osteoarthritis and Cartilage, 2005. **13**(6): p. 519-526.
323. Alvarez-Soria, M.A., et al., *Diacerein has a weak effect on the catabolic pathway of human osteoarthritis synovial fibroblast - Comparison to its effects on osteoarthritic chondrocytes*. Rheumatology, 2008. **47**(5): p. 627-633.
324. Nakamura, H., et al., *Effects of celecoxib on human chondrocytes - enhanced production of chemokines*. Clinical and Experimental Rheumatology, 2007. **25**(1): p. 11-16.
325. Tsutsumi, R., et al., *Celecoxib inhibits production of MMP and NO via down-regulation of NF- κ B and JNK in a PGE2 independent manner in human articular chondrocytes*. Rheumatology International, 2008. **28**(8): p. 727-736.
326. Matsuda, K., S. Nakamura, and T. Matsushita, *Celecoxib inhibits nitric oxide production in chondrocytes of ligament-damaged osteoarthritic rat joints*. Rheumatology International, 2006. **26**(11): p. 991-995.
327. Jeffrey, J.E. and R.M. Aspden, *Cyclooxygenase inhibition lowers prostaglandin E2 release from articular cartilage and reduces apoptosis but not proteoglycan degradation following an impact load in vitro*. Arthritis Research and Therapy, 2007. **9**(6).

328. Sanchez, C., et al., *Metabolism of human articular chondrocytes cultured in alginate beads. Longterm effects of interleukin 1 α and nonsteroidal antiinflammatory drugs*. Journal of Rheumatology, 2002. **29**(4): p. 772-782.
329. Cope, A.P., H. Schulze-Koops, and M. Aringer, *The central role of T cells in rheumatoid arthritis*. Clin Exp Rheumatol., 2007. **25**(5 Suppl 46): p. S4-11.
330. Panayi, G.S., *B cells: a fundamental role in the pathogenesis of rheumatoid arthritis?* Rheumatology (Oxford). 2005. **44**(Suppl 2): p. ii3-ii7.
331. Kontoyiannis, D. and G. Kollias, *Fibroblast biology: Synovial fibroblasts in rheumatoid arthritis - leading role or chorus line?* Arthritis Research, 2000. **2**(5): p. 342-343.
332. Tran, C.N., S.K. Lundy, and D.A. Fox, *Synovial biology and T cells in rheumatoid arthritis*. Pathophysiology : the official journal of the International Society for Pathophysiology / ISP, 2005. **12**(3): p. 183-189.
333. Fox, D.A., et al., *Cell-cell Interactions in Rheumatoid Arthritis Synovium*. Rheumatic diseases clinics of North America, 2010. **36**(2): p. 311-323.
334. Szekanecz, Z. and A.E. Koch, *Macrophages and their products in rheumatoid arthritis*. Current Opinion in Rheumatology, 2007. **19**(3): p. 289-295.
335. Juarez, M., A. Filer, and C.D. Buckley, *Fibroblasts as therapeutic targets in rheumatoid arthritis and cancer*. Swiss Medical Weekly, 2012. **142**(FEBRUARY).
336. Schultz, O., et al., *Development of in vitro model systems for destructive joint diseases. Novel strategies for establishing inflammatory pannus*. Arthritis & Rheumatism, 1997. **40**(8): p. 1420-1428.
337. Dayer, J.M., *The process of identifying and understanding cytokines: from basic studies to treating rheumatic diseases*. Best Pract Res Clin Rheumatol., 2004. **18**(1): p. 31-45.

338. van den Berg, W.B., *Animal models of arthritis. What have we learned?* J Rheumatol Suppl., 2005. **72**: p. 7-9.
339. Smolen, J. and E.C. Keystone, *Rheumatoid arthritis: where are we now?* Rheumatology, 2012. **51**(suppl 5): p. v1-v2.
340. Nelson, C.M., et al., *Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures.* Science., 2006. **314**(5797): p. 298-300.
341. Ma, B., et al., *Gene expression profiling of dedifferentiated human articular chondrocytes in τ -monolayer culture.* Osteoarthritis and Cartilage. **21**(4): p. 599-603.
342. Ringe, J. and M. Sittinger, *Tissue engineering in the rheumatic diseases.* Arthritis Research & Therapy, 2009. **11**(1): p. 211-211.
343. van den Berg, W.B., *Animal models of arthritis. What have we learned?* The Journal of Rheumatology, 2005. **72**: p. 7-9.
344. Firestein, G.S., *NF-kappaB: Holy Grail for rheumatoid arthritis?* Arthritis Rheum., 2004. **50**(8): p. 2381-6.
345. Kinne, R.W., et al., *Macrophages in rheumatoid arthritis.* Arthritis Res, 2000. **2**(3): p. 189 - 202.
346. Goldring, S.R., *Pathogenesis of bone and cartilage destruction in rheumatoid arthritis.* Rheumatology, 2003. **42**(suppl 2): p. ii11-ii16.
347. Karouzakis, E., et al., *Molecular and cellular basis of rheumatoid joint destruction.* Immunology Letters, 2006. **106**(1): p. 8-13.
348. Chang, J.H., et al., *Validity of SW982 synovial cell line for studying the drugs against rheumatoid arthritis in fluvastatin-induced apoptosis signaling model.* Indian J Med Res., 2014. **139**(1): p. 117-24.

349. Schmutz, C., et al., *Monocytes/macrophages express chemokine receptor CCR9 in rheumatoid arthritis and CCL25 stimulates their differentiation*. Arthritis Research & Therapy, 2010. **12**(4): p. R161.
350. Neidhart, M., R.E. Gay, and S. Gay, *Anti-interleukin-1 and anti-CD44 interventions producing significant inhibition of cartilage destruction in an in vitro model of cartilage invasion by rheumatoid arthritis synovial fibroblasts*. Arthritis Rheum., 2000. **43**(8): p. 1719-28.
351. Yatsugi, N., et al., *Apoptosis of articular chondrocytes in rheumatoid arthritis and osteoarthritis: correlation of apoptosis with degree of cartilage destruction and expression of apoptosis-related proteins of p53 and c-myc*. J Orthop Sci., 2000. **5**(2): p. 150-6.
352. Kim, H.A. and Y.W. Song, *Apoptotic chondrocyte death in rheumatoid arthritis*. Arthritis Rheum., 1999. **42**(7): p. 1528-37.
353. Chomarat, P., et al., *Contribution of IL-1, CD14, and CD13 in the increased IL-6 production induced by in vitro monocyte-synoviocyte interactions*. J Immunol., 1995. **155**(7): p. 3645-52.
354. van de Loo, F.A., et al., *Role of interleukin-1, tumor necrosis factor alpha, and interleukin-6 in cartilage proteoglycan metabolism and destruction. Effect of in situ blocking in murine antigen- and zymosan-induced arthritis*. Arthritis Rheum., 1995. **38**(2): p. 164-72.
355. Jacques, C., et al., *The role of IL-1 and IL-1Ra in joint inflammation and cartilage degradation*. Vitam Horm., 2006. **74**: p. 371-403.
356. Pettipher, E.R., G.A. Higgs, and B. Henderson, *Interleukin 1 induces leukocyte infiltration and cartilage proteoglycan degradation in the synovial joint*. Proc Natl Acad Sci U S A., 1986. **83**(22): p. 8749-53.

357. Srirangan, S. and E.H. Choy, *The Role of Interleukin 6 in the Pathophysiology of Rheumatoid Arthritis*. Therapeutic Advances in Musculoskeletal Disease, 2010. **2**(5): p. 247-256.
358. Md Yusof, M.Y. and P. Emery, *Targeting interleukin-6 in rheumatoid arthritis*. Drugs., 2013. **73**(4): p. 341-56. doi: 10.1007/s40265-013-0018-2.
359. Wang, X., et al., *Asymmetric Varus and Valgus Stability of the Anatomic Cadaver Knee and the Load Sharing Between Collateral Ligaments and Bearing Surfaces*. Journal of Biomechanical Engineering, 2014. **136**(8): p. 081005-081005.
360. Dang, A.C. and A.C. Kuo, *Cartilage Biomechanics and Implications for Treatment of Cartilage Injuries*. Operative Techniques in Orthopaedics, 2014. **24**(4): p. 288-292.
361. van Vollenhoven, R.F., *Treatment of rheumatoid arthritis: state of the art 2009*. Nat Rev Rheumatol, 2009. **5**(10): p. 531-541.
362. Benam, K.H., et al., *Engineered in vitro disease models*. Annu Rev Pathol., 2015. **10:195-262**.(doi): p. 10.1146/annurev-pathol-012414-040418.