



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Probing the Role of Joint Specific Environmental Cues in Guiding Stem Cell Fate



Simon F. Carroll B.Eng., M.Sc.

A thesis submitted to the University of Dublin in partial
fulfilment of the requirements for the degree of

Doctor in Philosophy

Trinity College Dublin

March 2016

Supervisors

Dr Daniel J. Kelly

Dr Conor T. Buckley



PMS
ENGINEERING



Thesis 11150

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.



Summary

Due to their relative ease of isolation, ability to proliferate extensively *in vitro* and to differentiate along multiple lineage pathways, mesenchymal stem cells / multipotent stromal cells (MSCs) are a promising cell type for tissue regeneration strategies. However, unlocking the potential of these cells requires an understanding of how the local cellular environment influences cell metabolism, phenotype and matrix synthesis and will be crucial to engineering functional cartilage tissue grafts of a clinically relevant scale.

The objective of this thesis was to investigate the influence of joint-specific environmental factors on chondrogenesis of MSCs encapsulated within 3D hydrogels and subsequent tissue formation within these constructs. Various novel bioreactor systems and numerical models were developed to monitor and predict oxygen gradients within engineered grafts and to apply specific mechanical loading regimes to MSC laden constructs. It was demonstrated that the fate of MSCs may be directed by both varying oxygen gradients in cell laden hydrogels and through the application of physiological levels of various modes of mechanical loading. In the first part of the thesis, MSCs were encapsulated in agarose hydrogels and maintained in altered external oxygen conditions while simultaneously measuring oxygen levels within the construct. MSCs were found to possess a high initial oxygen consumption rate, but to adopt a less oxidative, more chondrocyte-like metabolic profile following the onset of chondrogenic differentiation. Cartilage matrix deposition was generally maximal in regions of low oxygen tension, but collagen synthesis was inhibited in very low oxygen ($pO_2 < 2\%$) regions.

The next phase of the thesis explored how the two different mechanical stimuli integral to the joint environment, namely cyclic hydrostatic pressure (HP) and cyclic tensile strain (CTS), regulated chondrogenesis of MSCs and their progression along the endochondral pathway. The application of physiological levels of HP was found to enhance chondrogenesis of MSCs, leading to the development of more mechanically functional engineered cartilage whilst simultaneously suppressing calcification of the grafts. In contrast, the application of CTS to MSCs encapsulated into 3D hydrogels was found to enhance cellular organization, inhibit adipogenesis and promote either direct intramembranous or indirect endochondral ossification of MSCs, depending on the

context within which loading was applied and on the magnitude and frequency of the applied strain. The final phase of the thesis explored the role of CTS in regulating the differentiation of MSCs along either a chondrogenic or myogenic pathway. Tensile stimulation was found to enhance myogenesis and suppress chondrogenesis in MSC seeded collagen-alginate 3D matrices, however this response was demonstrated to be dependent on both matrix stiffness and MSC morphology.

Development of next generation tissue engineering strategies will require a deeper knowledge of how environmental stimuli influence stem cell lineage commitment *in vitro*. In particular, a more complete understanding of stem cell mechanobiology and metabolism may ultimately allow us to manipulate the *in vivo* mechanical environment to ensure such cells terminally differentiate towards a desired phenotype upon implantation into the body. The insights provided in this dissertation suggest that promoting a metabolic profile similar to that of chondrocytes might be considered to be a key determinant of the success of stem cell based cartilage tissue engineering strategies, and demonstrate that the biophysical and biochemical environment in 3D grafts can be tailored to modulate MSC lineage commitment and subsequent tissue development.

Acknowledgements

It has been a privilege to work with so many talented and committed friends and colleagues at Trinity Centre for Bioengineering, and I would like to thank Dr Daniel Kelly for the opportunity to do so. Both Dr Kelly's and Dr Buckley's enthusiasm and advice have been invaluable over the years; they were a pleasure to work with and I am sincerely grateful for their hard work and support.

This dissertation would not have been possible without the support of many people. I would like to thank, in particular, my good friends Henrique, Grainne and all the DOPs for all the fun times and good memories. Special thanks to Melanie and June for keeping me grounded during coffee breaks and for the many interventions in Probus. Many thanks to all my former and current colleagues - Alejandro, Eamonn, Jill, Adam, Lu, Masooma, Binu, Dave, Andy, Pedro, Amy, Alanna, Steve, Tariq, Thomas, Darren, Jake, Yurong and all the researchers of TCBE, who have helped make my time at Trinity such a rewarding experience. I am also extremely grateful to my family for their support over the years. Finally, a special thank you to Jane-Ann for all your encouragement, support and understanding; you have the patience of a saint.

This work was funded by Science Foundation Ireland (07-RFP-ENMF142, and the President of Ireland Young Researcher Award: 08/YI5/B1336), and the European Research Council (StemRepair – Project number 258463).

Publications

1. Carroll, S.F., Buckley, C.T., and Kelly, D.J., *Cyclic hydrostatic pressure promotes a stable cartilage phenotype and enhances the functional development of cartilaginous grafts engineered using multipotent stromal cells isolated from bone marrow and infrapatellar fat pad*. Journal of Biomechanics, 2014. 47(9): p. 2115-21.
2. Thorpe, S.D., Nagel, T., Carroll, S.F., and Kelly, D.J., *Modulating gradients in regulatory signals within mesenchymal stem cell seeded hydrogels: a novel strategy to engineer zonal articular cartilage*. PLoS One, 2013. 8(4): p. e60764.
3. Carroll, S., Buckley, C., and Kelly, D., *Oxygen consumption during chondrogenesis in hydrogels seeded with chondrocytes and stem cells isolated from different tissues*. Journal of Tissue Engineering and Regenerative Medicine, 2012. 6: p. 60-60.

Conferences

1. Carroll, S.F.; Sathy, B.N.; Buckley, C.T.; Kelly, D.J. *MSC fate in 3D fibrin hydrogels is regulated by the magnitude and frequency of tensile strain*. 21st Congress of the European Society of Biomechanics, July 5 – 8, 2015, Prague, Czech Republic.
2. Carroll, S.F.; Buckley, C.T.; Kelly, D.J. *Probing the role of joint specific environmental cues in guiding stem cell fate*. 21st Bioengineering in Ireland Conference, January 16-17th, 2015, Maynooth, Ireland.
3. Carroll, S.F.; Buckley, C.T.; Kelly, D.J. *Cellular Oxygen Consumption in Engineered Cartilage Grafts*. 20th Annual Conference of the Bioengineering Section of the Royal Academy of Medicine in Ireland, January 24th -25th, 2014, Limerick, Ireland.
4. Carroll, S.F.; Buckley, C.T.; Kelly, D.J. *The influence of hydrostatic pressure on the functional development & phenotypic stability of cartilaginous grafts engineered using mesenchymal stem cells isolated from bone marrow & infrapatellar fat pad*. Annual Meeting of the Orthopaedic Research Society, January 26–29, 2013, Henry B. Gonzalez Convention Centre, San Antonio, Texas, USA.
5. Carroll, S.F.; Buckley, C.T.; Kelly, D.J. *The influence of hydrostatic pressure on the functional development & phenotypic stability of cartilaginous grafts engineered using mesenchymal stem cells isolated from bone marrow & infrapatellar fat pad*. 19th Annual Conference of the Bioengineering Section of the Royal Academy of Medicine in Ireland, January 18th-19th, 2013, Johnstown House Hotel, Co. Meath, Ireland.
6. Carroll, S.F.; Buckley, C.T.; Kelly, D.J. *Oxygen consumption during chondrogenesis in hydrogels seeded with chondrocytes and stem cells isolated from different tissues*. 3rd TERMIS World Congress 2012, Tissue Engineering and Regenerative Medicine, September 5th – 8th, 2012, Hofburg Congress Centre, Vienna, Austria.
7. Carroll, S.F.; Buckley, C.T.; Kelly, D.J. *A comparison of oxygen consumption rates of chondrocytes and different sources of stem cells during chondrogenesis in agarose hydrogels*. 18th Annual Conference of the Bioengineering Section of the Royal Academy of Medicine in Ireland, January 27 - 28, 2012. Hilton Templepatrick Hotel, Belfast, Ireland.

Nomenclature

%ww	Percentage of wet weight
∂	Partial derivative
°C	Degrees Celsius
μM	Micromolar
μm	Micrometer
2D	2-dimensional
3D	3-dimensional
ACI	Autologous chondrocyte implantation
AGC	Aggrecan
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BM	Bone marrow
BMP	Bone morphogenic protein
BMSC	Bone marrow-derived MSC
BSA	Bovine serum albumin
CaCl₂	Calcium chloride
CC	Chondrocyte
cDNA	Complementary DNA
CFU-F	Colony forming unit-fibroblast
Chondro	Chondrogenesis
CO₂	Carbon dioxide
CTS	Cyclic tensile strain
D	Diffusion coefficient
DAB	Diaminobenzide
D_{ag}	Diffusion coefficient for agarose
DAPI	4',6-diamidino-2-phenylindole
DC	Dynamic compression
D_{H2O}	Diffusion coefficient for water
DM	Differentiation medium
D_{media}	Diffusion coefficient for culture media
DMEM	Dulbecco's Modified Eagle's Medium
DMEM-	Phenol red-free DMEM
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetraacetic acid
EM	Expansion medium
ETOH	Ethanol
FBS	Foetal bovine serum

FE	Finite element
FEA	Finite element analysis
FGF	Fibroblast growth factor
Fibro	Fibrogenesis
FPSC	Infrapatellar fat pad-derived MSC
FS	Free-swelling (unloaded)
GAG	Sulphated glycosaminoglycan
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
H₂O	Water
H₂O₂	Hydrogen peroxide
HCl	Hydrochloric acid
HP	Intermittent hydrostatic pressure
Hz	Hertz
IFP	Infrapatellar fat pad
IGF	Insulin growth factor
IgG	Immunoglobulin G
IHP	Intermittent hydrostatic pressure
ITS	Insulin transferrin selenium
K_m	Concentration at half the maximum consumption rate
kPa	Kilo Pascal
M	Molar
MACI	Matrix-induced autologous chondrocyte implantation
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MMP	Matrix metalloproteinase
MPa	Mega Pascal
mRNA	Messenger ribosomal nucleic acid
ms	Millisecond
MSC	Mesenchymal stem cell / multipotent stromal cell
Myo	Myogenesis
n	Number of observations
N	Newton
N₂	Nitrogen
ng	Nanogram
nM	Nanomolar
nM	Nanometre
Ø	Diameter
O₂	Oxygen
OA	Osteoarthritis
Osteo	Osteogenesis

P	Threshold significance level
PBS	Phosphate buffered saline
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
PG	Proteoglycan
PGA	Polyglycolic acid
PLGA	Poly(lactic-co-glycolic acid)
pO₂	Oxygen tension
pO_{2c}	Oxygen tension measured at the centre of the construct
Q_m	Maximum oxygen consumption rate
qRT-PCR	quantitative real-time reverse transcriptase PCR
RGD	Arginine-Glycine-Aspartic acid
RhoA	Ras homolog gene family, member A
RNA	Ribosomal nucleic acid
ROCK	Rho-associated protein kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Seconds
SD	Cell density
SD	Standard deviation
SEM	Standard error from the mean
sGAG	Sulphated glycosaminoglycan
sGAG	Sulphated glycosaminoglycan
SMC	Smooth muscle cell
t	Time
TGF-β	Transforming growth factor-beta
TIMP-1	Tissue inhibitors of metalloproteinases -1
U	Units
ww	Wet weight
α-SMA	α-smooth muscle actin
μL	Microlitre
μM	Micromolar
p	Cell density
φ_f	Fluid phase volume fraction
PCR	Polymerase chain reaction

Table of Contents

Declaration..... i

Summaryiii

Acknowledgements..... v

Publications..... vii

Conferences..... vii

Nomenclature.....viii

Table of Contents xi

List of Figures..... xv

List of Tables..... xix

Chapter 1 Introduction..... 1

1.1 General overview 1

1.2 Regulation of MSC differentiation.....2

1.3 Aims of this research.....3

Chapter 2 Literature Review 4

2.1 Articular cartilage.....4

2.1.1 Composition and structure of articular cartilage..... 4

2.1.2 Chondrocytes..... 8

2.1.3 In vivo loading of articular cartilage 10

2.2 Cartilage tissue engineering 12

2.2.1 Overview of cartilage tissue engineering 12

2.2.2 Biomaterials for cartilage tissue engineering 12

2.3 Mesenchymal stem cells 13

2.3.1 Biochemical regulation of MSC differentiation 14

2.3.2 Substrate stiffness and cell morphology 15

2.3.3 Extrinsic mechanical signals as regulators of MSC fate..... 16

2.3.4 Oxygen tension and MSC metabolism 23

2.4 Summary 25

Chapter 3	A comparison of the oxygen consumption rate of chondrocytes and MSCs of different tissue origins undergoing chondrogenesis in 3D hydrogels.....	27
3.1	Abstract	28
3.2	Introduction	29
3.3	Methods	30
3.3.1	Cell Isolation and Expansion.....	30
3.3.2	Agarose gel constructs fabrication and culture.....	30
3.3.3	Experimental design	31
3.3.4	Measurement of localised oxygen concentration	32
3.3.5	Prediction of oxygen concentration within agarose constructs	33
3.3.6	Biochemical analysis	34
3.3.7	Histology and immunohistochemistry	35
3.3.8	Statistical Analysis	35
3.4	Results	36
3.4.1	MSCs adopt a chondrocyte-like oxygen consumption rate following agarose hydrogel encapsulation and stimulation with TGF- β 3	36
3.4.2	Decreasing the external oxygen tension reduces the rate at which MSCs consume oxygen and leads to increased matrix accumulation.	38
3.4.3	Increasing the cell seeding density lowers oxygen levels in tissue engineered cartilage and increases extracellular matrix synthesis.	41
3.5	Discussion	43
3.6	Conclusion.....	46
3.7	Supplementary figures.....	47
Chapter 4	Cyclic hydrostatic pressure promotes a stable cartilage phenotype and enhances the functional development of cartilaginous grafts engineered using MSCs of different tissue origins	49
4.1	Abstract	50
4.2	Introduction	51
4.3	Methods	53
4.3.1	Agarose gel constructs fabrication and culture.....	53
4.3.2	Application of hydrostatic pressure.....	53
4.3.3	Mechanical, histological and biochemical assessment of tissues	54
4.3.4	Experimental design	54
4.3.5	Statistical Analysis	55
4.4	Results	56
4.4.1	Dynamic hydrostatic pressure enhances the functional development of cartilage tissue engineered using both BMSCs and FPSCs.....	56
4.4.2	Dynamic hydrostatic pressure suppresses calcification of cartilage grafts engineered using BMSCs	58
4.4.3	Dynamic hydrostatic pressure acts to maintain a chondrogenic phenotype in cartilaginous grafts engineered using FPSCs	60
4.5	Discussion	61

Chapter 5	The influence of cyclic tensile strain on MSC differentiation in 3D hydrogels is dependent on cell morphology and substrate stiffness.....	65
5.1	Abstract	66
5.2	Introduction	67
5.3	Methods.....	69
5.3.1	Cell Isolation and expansion	69
5.3.2	Collagen - alginate hydrogel fabrication and culture.....	69
5.3.3	Mechanical stimulation of hydrogel encapsulated MSCs.....	70
5.3.4	Characterization of mechanical properties of collagen-alginate hydrogels	73
5.3.5	RNA isolation and real-time reverse transcriptase polymerase chain reaction.....	73
5.3.6	Cell viability and F-actin cytoskeleton fluorescent imaging MSC	74
5.3.7	Statistical analysis	75
5.4	Results	75
5.4.1	Bioreactor Validation	75
5.4.2	Stiffer substrates promote chondrogenesis and progression along the endochondral pathway.....	77
5.4.3	Cyclic tensile strain enhances myogenesis but inhibits chondrogenesis of MSCs ...	77
5.4.4	A spread MSC morphology is correlated with suppression of chondrogenesis.....	80
5.4.5	Cyclic tensile strain synergizes with a spread cellular morphology to enhance markers of myogenesis.....	80
5.5	Discussion	83
Chapter 6	Cyclic tensile strain can play a role in directing both intramembranous and endochondral ossification of MSCs in 3D hydrogels.....	87
6.1	Abstract	88
6.2	Introduction	89
6.3	Methods.....	91
6.3.1	Cell Isolation and Expansion	91
6.3.2	Fibrin hydrogel fabrication and culture	91
6.3.3	Mechanical stimulation of hydrogel encapsulated MSCs.....	92
6.3.4	Biochemical analysis	92
6.3.5	RNA isolation and real-time reverse transcriptase polymerase chain reaction.....	93
6.3.6	Fluorescent staining and quantification of cellular anisotropy of MSC filamentous actin	95
6.3.7	Statistical analysis	95
6.4	Results	96
6.4.1	Cyclic tensile strain in the absence of soluble differentiation factors promotes the expression of tenogenic and osteogenic markers while inhibiting adipogenesis	96
6.4.2	Cyclic tensile strain promotes endochondral ossification of chondrogenically primed MSCs in a magnitude dependent manner	98
6.4.3	Cyclic tensile strain promotes cellular alignment of MSCs in 3D fibrin hydrogels	100
6.5	Discussion	101
Chapter 7	Discussion.....	105
7.1	Overview of research findings	105
7.2	Significance of research findings	107

7.3	Limitations and future research directions	109
Chapter 8	Conclusion.....	111
Appendix A.....		113
	Technical Drawings of Tension Bioreactor.	113
Bibliography		129

List of Figures

Figure 2-1: Illustration of a collagen fibril's mechanical properties. The fibril is stiff and strong in tension (A), but is weak and buckles easily in compression (B). A schematic representation of the collagen network interacting with the proteoglycan network, forming a porous, fibre-reinforced, composite solid matrix in articular cartilage (C) (Mow et al. 1989, Lu and Mow 2008).	6
Figure 2-2: Chondrocyte and collagen fibre organization in articular cartilage (Buckwalter et al. 1994)	7
Figure 2-3: Schematic overview of the role of growth factors at different stages of chondrogenesis. BMP (bone morphogenetic protein), TGF- β (transforming growth factor beta), PDGF (platelet-derived growth factor), FGF (fibroblast growth factor), CTGF (connective tissue growth factor), and VEGF (vascular endothelial growth factor) (Demoor et al. 2014).....	8
Figure 2-4: Diagram of autologous chondrocyte implantation (ACI) technique (Demoor et al. 2014).	9
Figure 2-5 : Schematic representation of the <i>in vivo</i> mechanical environment of articular cartilage under intermittent joint loading and motion (Wong and Carter 2003).	11
Figure 2-6: The mesengenic process (Caplan and Bruder 2001).....	14
Figure 2-7: Matrix compliance alters MSC fate in 3D matrix culture. In this case, MSC seeded in softer matrices (left) stained positive for adipogenic markers, whereas stiffer substrates (right) promoted osteogenic markers (Huebsch et al. 2010).	15
Figure 2-8: Cell morphology alters MSC fate in 2D culture. The spreading of MSCs (cultured in combined osteo/adipo-inductive medium) was limited by creating islands of ECM protein coatings of varying area on non-adhesive glass via microcontact printing techniques. Cells were stained for lipids (red) and alkaline phosphatase (blue) (A). Increased cell spreading was correlated with enhanced osteogenesis and reduced adipogenesis (B) (McBeath et al. 2004, Guilak et al. 2009).....	16
Figure 2-9: Cyclic hydrostatic pressure promotes chondrogenic gene expression in both the presence and absence of TGF- β 3 (CTR - free swelling control; IHP - intermittent hydrostatic pressure; TGF - transforming growth factor β 3; AGC – aggrecan) (Miyanishi et al. 2006).....	18
Figure 3-1: The local oxygen tension in the centre of <i>in vitro</i> engineered cartilage tissue grafts cultured in chondrogenic media was measured using a custom designed oxygen testing rig, test dish and fibre optic sensor (a). Samples were separated into cores and annuli for spatial biochemical analysis (b). Schematic of the finite element model developed to predict oxygen concentration gradients throughout the rest of the graft (c).....	32

- Figure 3-2: Cartilage tissue grafts engineered using various cell sources were cultured for 24 days. Oxygen concentration at centre of cartilage tissue grafts engineered using cells derived from various tissue sources (a). Cellular oxygen consumption rate extrapolated from FE model. (b) FE contour plots showing predicted oxygen concentration throughout the constructs (c). (a, b and c denote statistical difference vs. same cell type at day 24, FPSC constructs at same time-point and CC constructs at same time-point respectively; $P < 0.05$; $n = 3$ * indicates the location of oxygen sensor measurement within the constructs).....36
- Figure 3-3: Cartilage tissue grafts engineered using various cell sources were cultured for 24 days, separated into cores and annuli, then analysed for DNA, sGAG and collagen content (a-d). Histological and immune-histochemical staining (e) was performed on representative sections for sGAG, collagen and collagen type II (top, middle, bottom respectively). (a, b and c denote statistical difference vs. core of construct using the same cell type, FPSC constructs in the same region and CC constructs in the same region respectively; $P < 0.05$; $n = 3$).38
- Figure 3-4: Cartilage tissue grafts engineered using bone marrow MSCs were cultured in either a normoxic (20%) or low (5%) external oxygen environment for 24 days. Oxygen concentration measured at centre of the constructs (a). Cellular oxygen consumption rate extrapolated from FE model (b). FE contour plots showing predicted oxygen concentration throughout the constructs (c). (a, b denote statistical difference vs. same culture condition group at day 24 and 5% oxygen environment group at the same time point respectively; $P < 0.05$; $n = 3$. * indicates the location of oxygen measurement within the constructs).40
- Figure 3-5: Cartilage tissue grafts engineered using various cell sources were cultured for 24 days, separated into cores and annuli, then analysed for DNA, sGAG and collagen content (a-d). Histological and immuno-histochemical staining (e) was performed on representative sections for sGAG, collagen and collagen type II (top, middle, bottom respectively). (a, b and c denote statistical difference vs. core of construct using the same cell type, FPSC constructs in the same region and CC constructs in the same region respectively; $P < 0.05$; $n = 3$).40
- Figure 3-6: Cartilage tissue grafts engineered using bone marrow stem cells were seeded initially at a density of either 20 or 40 million cells/mL and cultured for 24 days. Oxygen concentration measured at the centre of constructs (a). Cellular oxygen consumption rate extrapolated from FE model (b). FE contour plots showing predicted oxygen concentration throughout the constructs (c). (a, b denote statistical difference vs. same initial seeding density group at day 24 and 40 million cell group at the same time point; $P < 0.05$; $n = 3$. * indicates the location of oxygen measurement within the constructs).41
- Figure 3-7: Cartilage tissue grafts engineered using bone marrow MSCs were seeded initially at a density of either 20 or 40 million cells/mL for 24 days, then separated into cores and annuli and analysed for DNA, sGAG and collagen content (a-d). Histological and immuno-histochemical staining (e) was performed on representative sections for sGAG, collagen and collagen type

II (top, middle, bottom respectively). (a, b denote statistical difference vs. core of construct cultured in a similar environment and same region of construct seeded at 40 million cells/mL respectively; $P < 0.05$; $n = 3$).	42
Figure 3-8: A replicate study was performed using cells from a separate set of donors and oxygen tension in the centre of each construct was measured. Data is arranged (a) by cell source, (b) by external oxygen environment and (c) by initial cell seeding density.....	47
Figure 3-9: The percentage change in cell density in sample cores and annuli after 24 days of culture. Cell density at day 24 was estimated by normalizing day 24 DNA values to the average day 0 values, which were assumed to be representative of the initial cell seeding density, for each group. Data is arranged (a) by cell source, (b) by external oxygen environment and (c) by initial cell seeding density (a, b denote a statistically significant different DNA content vs. day 0 samples, and vs the DNA content in the core region of the same samples respectively, $P < 0.05$; $n = 3$).	47
Figure 3-10: FEA models were analysed to determine the percentage volume of each sample that lay within the specified oxygen ranges. Data is arranged (a) by cell source, (b) by external oxygen environment and (c) by initial cell seeding density ($n = 3$).	48
Figure 4-1: Dynamic hydrostatic pressure enhances the accumulation of cartilage specific ECM in both BMSC and FPSC constructs when cultured in chondrogenic medium for 5 weeks, as shown by biochemical analyses (a-d) and histological/immune-histological staining (e.) *, ^ denote statistical significance vs. free swelling group of similar cell type and vs. FPSCs under similar loading conditions respectively ($P < 0.002$; $n = 4$).	57
Figure 4-2: Dynamic hydrostatic pressure enhances the functional properties of both BMSC and FPSC constructs when cultured in chondrogenic medium for 5 weeks. *, ^ denote statistical significance vs. free swelling group of similar cell type and vs. FPSCs under similar loading conditions respectively ($P < 0.002$; $n = 4$).	58
Figure 4-3: Dynamic hydrostatic pressure suppresses calcification of BMSC constructs when cultured in chondrogenic medium for 5 weeks, as shown by biochemical analysis of calcium content of BMSC constructs (a) and Alizarin Red staining of BMSC constructs for calcium deposition (b). FS and HP indicate free swelling and loaded respectively. * denotes statistical significance vs. free swelling group of similar cell type ($P < 0.002$; $n = 4$).	59
Figure 4-4: Dynamic hydrostatic pressure has no significant effect on calcification of BMSC constructs when cultured in chondrogenic medium for 3 weeks, followed by 2 weeks culture in hypertrophic medium, as shown by biochemical analysis ($n = 4$) of calcium content of BMSC constructs (a) and Alizarin Red staining of BMSC constructs for calcium deposition (b). FS and HP indicate free swelling and loaded respectively. Original image magnification is shown inset.....	59
Figure 4-5: Dynamic hydrostatic pressure acts to maintain a chondrogenic phenotype in FPSC constructs when cultured for 3 weeks in chondrogenic medium	

containing TGF- β 3, followed by 2 weeks in the presence or absence of TGF- β 3, as shown by biochemical analyses (a) and histological staining (b) of constructs. FS, FS- and HP- indicate free swelling with continuous TGF- β 3 supplementation, free swelling without TGF- β 3 and loaded without TGF- β 3 respectively. * and ^ denote statistical significance vs. FS and FS- groups respectively (P<0.001; n = 4).	60
Figure 5-1: Front panel of bioreactor control software developed in Matlab. Multiple instances of the script could be run simultaneously by utilising partitioned virtual environments, thereby allowing multiple bioreactors to run simultaneously from the same PC. The graphical user interface allows control of applied strain magnitude, frequency and duration of loading. Automated loading is facilitated via a script loop that continuously monitored the current time on the system clock, and system information such as current status and cycles remaining is displayed.	72
Figure 5-2: A novel bioreactor system for application of uniaxial cyclic tensile strain to soft 3D hydrogels was developed (a). Dog-bone shaped cell-seeded hydrogels constructs were moulded in recesses within flexible silicone strips (i). These strips were mounted on strain assemblies (ii), which were then sealed into the bioreactor chamber (iii) under sterile conditions. The displacement of external actuators (iv) was aseptically transferred to accurately stretch the strips in the bioreactor using magnets, both outside of and within the chamber, connected around a flexible membrane separator. MSCs encapsulated in collagen-alginate blended hydrogels and cultured in the bioreactor remained viable over 7 days (b). Tensile testing image analyses were performed to correlate actuator displacement with hydrogel strain (c). Representative images and data from hydrogel strain validation image analyses are shown.	76
Figure 5-3: Mechanical characterisation of collagen-alginate blended hydrogels created using 0.6% (soft) and 2.25% (stiff) final alginate concentration (a). Relative gene expression in MSCs encapsulated in stiff blended hydrogels (normalized to MSCs in soft hydrogels) after 4 days of culture (b). * indicates a significant difference vs. soft group (P<0.05; n = 5).	78
Figure 5-4: F-actin staining of MSCs encapsulated in a soft or stiff hydrogel and cultured in free swelling or loaded conditions for 4 days (a). Cell nuclei are counter-stained with Dapi (blue). Chondrogenic and myogenic relative gene expression at day 4 normalized to the soft/free-swelling control group (b). Significant differences vs. non-loaded control group are indicated (P<0.05; n = 5).	79
Figure 5-5: Live/dead staining 24 hours after MSCs were encapsulated in an immediately (round) or delayed (spread) cross-linked hydrogel (a). Relative gene expression in spread MSCs (normalized to round MSCs) after 4 days of culture. * indicates a significant difference vs. round group (P<0.05; n = 5).	81
Figure 5-6: F-actin staining of MSCs encapsulated in an immediately (round) or delayed (spread) cross-linked hydrogel and cultured in free swelling (control) or loaded conditions for 4 days (a). Cell nuclei are counter-stained with Dapi (blue). Chondrogenic and myogenic relative gene expression at day 4	

normalized to the round/free-swelling group (b). Significant differences vs. non-loaded control group are indicated ($P<0.05$; $n = 5$).	82
Figure 6-1: Relative gene expression of MSCs encapsulated in fibrin hydrogels and cultured in tensile strain bioreactor in the absence of growth factors for 7 days. The application of 10% cyclic tensile strain at 0.5 Hz was found to promote intramembranous ossification and inhibit adipogenic differentiation of MSCs. Data are presented as fold-changes, relative to the non-loaded (control) group. * indicates a significant difference vs. free swelling control group ($P<0.05$; $n = 5$). See Table 1 for details of gene symbols.....	97
Figure 6-2: Biochemical analysis of MSC encapsulated fibrin hydrogels cultured in tensile strain bioreactors in media supplemented with chondrogenic growth factor TGF- β 3 for 21 days. The application of 10% cyclic tensile strain at 0.5 Hz was found to promote cellular proliferation, which led to an increase in total cartilage matrix deposition. Significant differences between groups are indicated where $P<0.05$ ($n = 5$).	98
Figure 6-3: Chondrogenic and osteogenic gene expression of MSCs encapsulated in fibrin hydrogels and cultured in media supplemented with chondrogenic growth factor TGF- β 3 for 21 days. Cyclic tensile strain was found to promote endochondral ossification of chondrogenically primed MSCs in a magnitude and frequency dependent manner. Data are presented as fold-changes, relative to the non-loaded (control) group. RUNX-2/SOX9 ratio is presented as fold change/fold change. Significant differences between groups are indicated where $P<0.05$ ($n = 5$). See Table 1 for explanation of gene symbols.....	99
Figure 6-4: F-actin staining (a) and quantification of filament anisotropy (b) of MSCs encapsulated in fibrin hydrogels and cultured in tensile strain bioreactors in media supplemented with chondrogenic growth factor TGF- β 3 for 21 days. The application of cyclic tensile strain was found to increase cellular organisation. Significant differences between groups are indicated where $P<0.05$ ($n = 5$).	100

List of Tables

Table 2-1: Review of studies examining the influence of cyclic tensile strain on MSC differentiation on both 2D and 3D substrates	22
Table 5-1: Table 1: Primers used for Real-Time Reverse Transcription Polymerase Chain Reaction.....	74
Table 6-1: Primers used for Real-Time Reverse Transcription Polymerase Chain Reactions.....	94

Chapter 1 Introduction

1.1 General overview

Articular cartilage is the smooth, white connective tissue covering the ends of long bones. It is an avascular, aneural, but highly specialized tissue, the principal function of which is to provide a smooth, low friction, load-bearing surface in diarthrodial joints. Due to its avascular nature, articular cartilage has a limited capacity for self-repair and cartilage degeneration due to high impacts loads or pathological conditions, such as osteoarthritis, can lead to the ablation of the cartilage layer and development of debilitating lesions. Depending on the severity of the condition, surgical interventions, such as microfracture, autologous chondrocyte implantation or autologous osteochondral transplantation may be required. These treatments are not without drawbacks however, as transplantation of tissue risks donor site morbidity, chondrocytes are known to dedifferentiate during monolayer expansion, and tissue regenerated following such procedures often displays characteristics of fibrocartilage and is susceptible to degradation (Jackson and Simon 1999, Redman et al. 2005). Repaired articular cartilage often degenerates over time, leading to the debilitating condition of osteoarthritis. The most common treatment of advanced osteoarthritis is joint replacement, which is estimated to reach two million knees and hips per year by 2015 in the United States (Benders et al. 2013). This procedure involves surgical removal of the joint and implantation of a prosthesis. However, such implants have a tendency to loosen with time, thus requiring revision surgeries in younger patients (Creamer 2000).

A promising alternative approach to cartilage and joint regeneration is tissue engineering: the use of autologous or allogenic cells encapsulated in three-dimensional (3D) matrices to create functional grafts *in vitro* prior to implantation. Mesenchymal stem cells/multipotent stromal cells (MSCs) are a promising cell source for tissue engineering, have been demonstrated to possess a high capacity for proliferation *in vitro*, and can be induced to differentiate towards multiple lineages (Caplan 1991, Pittenger et al. 1999, Tuan et al. 2003). MSCs can be isolated from various tissues, such as bone marrow (BM), infrapatellar fat pad (IFP) and subcutaneous fat, via minimally invasive surgeries.

1.2 Regulation of MSC differentiation

MSCs are a multipotent progenitor cell type that give rise to skeletal tissues such as cartilage, bone and other connective tissues (Caplan 1991). However, harnessing the potential of these cells in a regenerative context will require a deep understanding of how local environmental signals can guide stem cell differentiation towards a desired phenotype. A further challenge is to define the metrics by which successful differentiation of MSCs into specialised cell types is measured. Common approaches involve investigation of genetic markers associated with specific lineages and analyses of the biochemical constituents and mechanical properties of synthesized tissues, but MSCs should also adopt a metabolic profile that mirrors that of the desired phenotype.

For the purposes of *in vitro* differentiation of MSCs, it would seem reasonable to employ a culture environment that recapitulates key aspects of the environment of the desired tissue, both biochemically and mechanically. Articular cartilage exists in a low oxygen environment, in which the resident cells (chondrocytes) are subjected to gradients in various biochemical factors and multiple modes of biophysical stimuli. Successful cartilage tissue engineering strategies must aim to produce mechanically functional grafts occupied by cells that have adapted to, and can thrive in, this environment. It is well established that biochemical stimuli, provided by growth factors such as members of the transforming growth factor- β (TGF- β) or bone morphogenic protein (BMP) super-families, can be potent promoters of chondrogenic and osteogenic phenotypes respectively, and that altering the local oxygen environment can influence MSC phenotype and subsequent tissue formation (as reviewed by Malda et al. 2003, Puetzer et al. 2010, and James 2013). However, it has also been demonstrated that, through the use of various bioreactor systems, the application of physiologically appropriate mechanical signals, such fluid shear stresses, hydrostatic pressure, cyclic compressive strains and cyclic tensile strains can drive MSC differentiation (Angele et al. 2003, Mouw et al. 2007, Kreke et al. 2008, Connelly et al. 2010). Indeed, it has been reported that mechanical loading is critical for homeostasis of articular cartilage and there is evidence to suggest that abnormal joint loading plays a key role in the onset of osteoarthritis (Paukkinen et al. 1983, Lane Smith et al. 1992, Arokoski et al. 2000, Felson et al. 2000).

Although MSCs have been reported to undergo chondrogenesis, cartilage matrix content and functional properties of the grafts remain inferior to those engineered using

chondrocytes (Mauck et al. 2006, Erickson et al. 2009). Furthermore, it has been shown that following *in vivo* subcutaneous implantation, IFP derived MSCs tend to undergo fibrous dedifferentiation or resorption, whereas bone marrow derived MSCs tend to follow an endochondral pathway (Pelttari et al. 2006, Vinardell et al. 2012), suggesting that further optimisation of 3D culture environments will be required before MSC seeded grafts can realize their full potential.

1.3 Aims of this research

The overall objective of this thesis is to investigate the influence of joint-specific environmental factors of MSC differentiation and subsequent neo-tissue formation. The specific objectives are:

1. To characterize the local oxygen gradients that develop within cartilaginous tissues engineered using chondrocytes and MSCs derived from various tissue sources, and examine the influence of this environment on cellular metabolism and cartilage tissue deposition.
2. To investigate the influence of cyclic hydrostatic pressure in directing the fate of MSCs encapsulated in physiologically relevant 3D grafts. It is hypothesised that the application of such mechanical stimuli will play a role in the maintenance of a chondrogenic phenotype and in suppressing hypertrophy and endochondral ossification.
3. To investigate the influence of cyclic tensile strain in directing the fate MSCs encapsulated in physiologically relevant 3D grafts. It is hypothesised that the application of such mechanical stimuli to chondrogenically primed MSCs will enhance progression along the endochondral pathway.

Chapter 2 Literature Review

This chapter will begin by describing the structure, composition and *in vivo* mechanical loading environment of articular cartilage. The application of cartilage tissue engineering strategies for treatment of articular cartilage defects will then be discussed, with a focus on biomaterial selection and potential cell types. Finally, mesenchymal stem cells and their potential for use in tissue engineering applications will be reviewed.

2.1 Articular cartilage

2.1.1 *Composition and structure of articular cartilage*

Articular cartilage is a highly organized hyaline cartilage composed of a dense extracellular matrix (ECM) arranged in a hierarchical, zonal configuration and sparsely populated with highly specialized cells called chondrocytes. Chondrocytes, the cells responsible for secretion and maintenance of the organic part of the ECM, account for approximately 1% of the tissue volume (Buckwalter and Mankin 1997). The low cellular density of chondrocytes within cartilage, coupled with fact that chondrocytes reside within lacunae which limit the ability of the cell to migrate, results in a poor capacity for tissue regeneration. Furthermore, the poor vascularisation of the tissue inhibits the migration of progenitor cells to the site of cartilage injuries, whilst simultaneously limiting the supply of nutrients required for cartilage matrix synthesis.

The function of cartilage tissue is to provide a lubricated articulating surface, whilst resisting compression and distributing point loads, thereby reducing peak stresses on the subchondral bone (Buckwalter and Mankin 1997). The tissue is a porous, viscoelastic material which is composed predominately of a densely woven, strong, collagen fibrillar network (15–22% by wet weight) enmeshed with proteoglycan macromolecules (4–7% by wet weight), water (80% by wet weight) and many ionic species of dissolved electrolytes with positive and negative charges (<<1% by wet weight) (Lu and Mow 2008). Collagen type II represents 90% to 95% of the collagen in ECM; collagen types I, IV, V, VI, IX, and XI are also present, but contribute only a minor proportion of the total content (Sandell and

Aigner 2001). Proteoglycans (PGs) are composed of a protein core and can have numerous sulphated glycosaminoglycan (GAG) branches. Cartilage PGs are large protein-polysaccharide molecules, which are composed of a protein core, can have numerous GAG branches, and can exist either as monomers or as aggregates (Mow et al. 1989). Aggrecan, for example, consists of a single ~200 nm long protein core to which about 150 GAG chains are covalently attached, whereas keratan sulphate and chondroitin sulphate, the two sulphated glycosaminoglycans found in articular cartilage, are polymer chains of specific repeating disaccharide units. It is these structural components, together with water, that determine the biomechanical behaviour of articular cartilage. (Lai and Mow 1980).

Collagen fibrils, while possessing little resistance to compression, contribute to the tensile strength of cartilage (Figure 2-1), whilst the negatively charged, hydrophilic proteoglycans attract water molecules, thus inducing a swelling pressure that increases the tissues resistance to compression. PG aggregation promotes immobilisation of the PGs within the collagen meshwork, adding structural rigidity to the extracellular matrix (Mow et al. 1989).

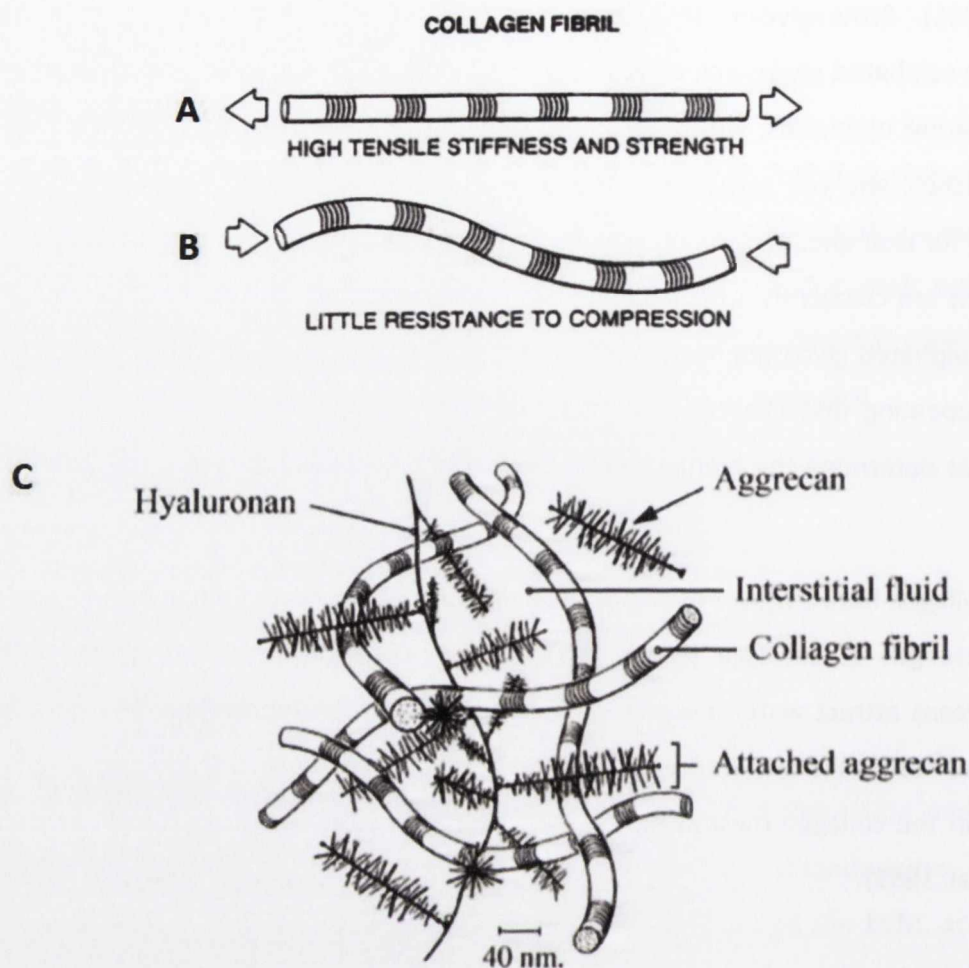


Figure 2-1: Illustration of a collagen fibril's mechanical properties. The fibril is stiff and strong in tension (A), but is weak and buckles easily in compression (B). A schematic representation of the collagen network interacting with the proteoglycan network, forming a porous, fibre-reinforced, composite solid matrix in articular cartilage (C) (Mow et al. 1989, Lu and Mow 2008).

A notable feature of articular cartilage is the depth dependent properties and structure. The tissue is 2 to 4mm deep and can be divided into several zones – the superficial tangential zone, the middle zone, the deep zone and the calcified zone (Figure 2-2) (as reviewed by Buckwalter et al. 1994). In the superficial zone, collagen fibres are densely packed and aligned parallel to the surface, thus providing resistance to shear forces at the articular surface. The middle zone contains proteoglycans and more randomly aligned collagen fibres, provides compressive strength, and acts as a transitional zone between the superficial and deep regions. In the deep zone, collagen fibrils are arranged perpendicular to the articular surface, thus providing an anchor to the subchondral bone via the calcified zone. The deep zone contains the greatest concentration of

proteoglycans and is responsible for providing the tissues highest resistance to compression. Here, the cell population is sparse and the chondrocytes are hypertrophic (Fox et al. 2009).

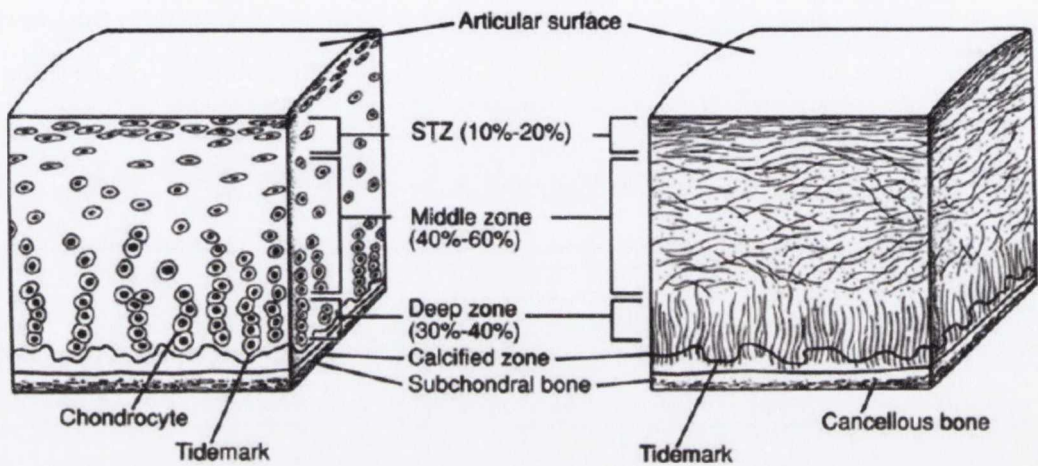


Figure 2-2: Chondrocyte and collagen fibre organization in articular cartilage (Buckwalter et al. 1994)

2.1.2 Chondrocytes

Chondrocytes develop as a result of mesenchymal cell condensation, followed by chondro-progenitor cell differentiation. Mature chondrocytes found in articular cartilage are generally spherical, non-dividing (post-mitotic) cells that, as mentioned previously, are responsible for the regulation of ECM turnover by synthesizing cartilage matrix markers such as collagen type II and aggrecan. Since cartilage is avascular, nutrients and oxygen are supplied to the cells primarily via diffusion from the synovial fluid and subchondral bone, resulting in chondrocytes occupying a low oxygen niche *in vivo*. Chondrocytes located nearer the subchondral bone mature and enter the final stage of development and become hypertrophic, no longer synthesizing type II collagen, but mainly type X collagen and alkaline phosphatase, resulting in calcification and endochondral ossification of the matrix (Figure 2-3) (Temenoff and Mikos 2000, Demoor et al. 2014).

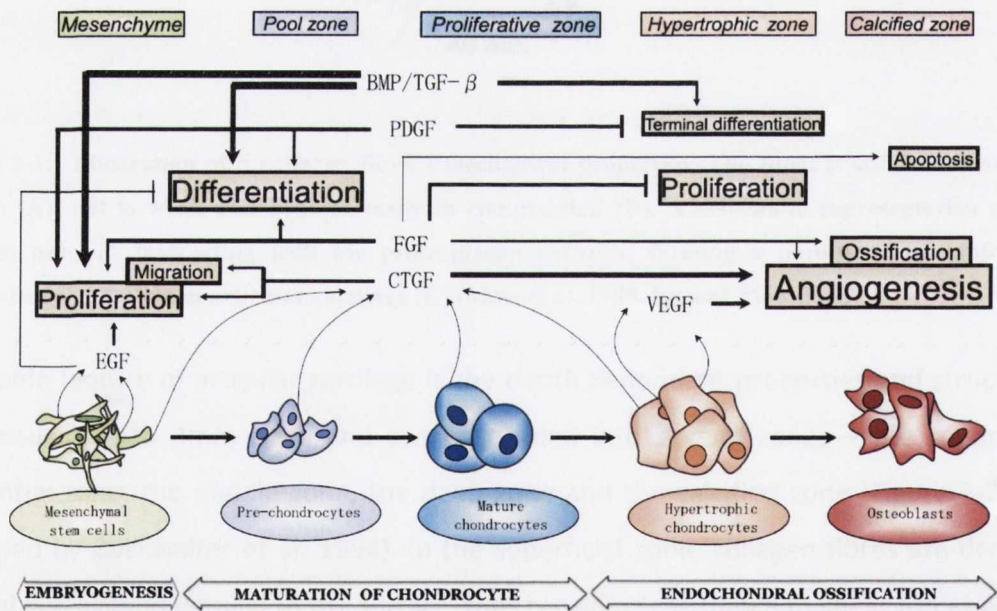


Figure 2-3: Schematic overview of the role of growth factors at different stages of chondrogenesis. BMP (bone morphogenetic protein), TGF-β (transforming growth factor beta), PDGF (platelet-derived growth factor), FGF (fibroblast growth factor), CTGF (connective tissue growth factor), and VEGF (vascular endothelial growth factor) (Demoor et al. 2014).

Growth factors such as transforming growth factor- β 3 (TGF- β 3) are expressed in pre-chondrocytes, and TGF- β 3 has been shown to guide chondrogenic differentiation, inhibit endochondral ossification and increase cartilage ECM synthesis (Grimaud et al. 2002), whereas other growth factors, such as bone morphogenic protein-2 (BMP-2) can be employed to induce hypertrophy (Enomoto-Iwamoto et al. 1998). Current techniques for treatment of cartilage defects include Autologous Chondrocyte Implantation (ACI, Figure 2-4), which has been shown to have to some success in regenerating hyaline cartilage (Peterson et al. 2002, Marlovits et al. 2006). However, while chondrocytes may appear to be the natural choice for tissue engineering applications, there are drawbacks associated with chondrocyte availability and culture. Traditionally, chondrocytes for ACI or tissue engineering approaches are harvested from non-loaded bearing cartilaginous regions. However, in both cases, donor site morbidity remains a risk, and only limited numbers of cells can be obtained via this method, so an *in vitro* proliferation step is required. Furthermore, since chondrocytes have been demonstrated to de-differentiate during expansion in monolayer culture, resulting in the secreted matrix resembling a more fibro-cartilaginous tissue, it would appear that there is still room for improvement, and that another cell source may be desirable.

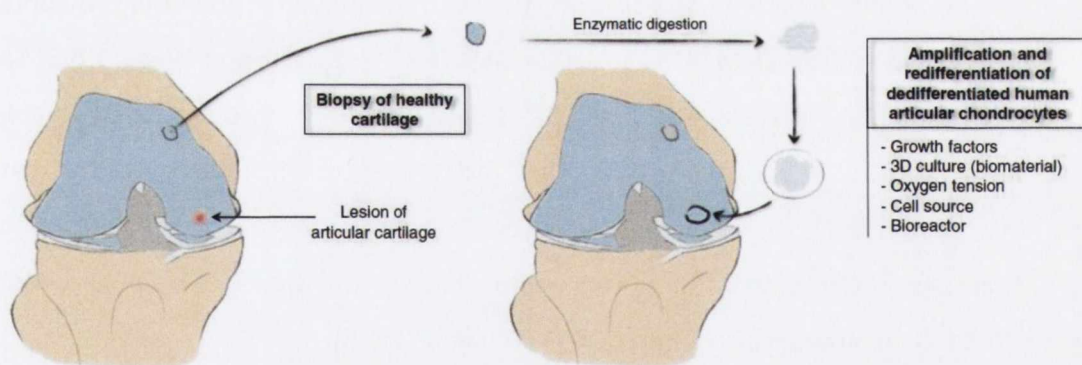


Figure 2-4: Diagram of autologous chondrocyte implantation (ACI) technique (Demoor et al. 2014).

2.1.3 *In vivo loading of articular cartilage*

Joint forces in healthy articular cartilage are transmitted across the joint by the generation of pressure over the contact area of two opposing cartilage surfaces. During gait, these contact areas, separated by a thin film of fluid, sweep swiftly across the cartilage surfaces, thereby introducing intermittent hydrostatic pressurization of the fluid phase of cartilage and generating shear and tensile stresses near the contact area surface (Figure 2-5). While compressive loading of the joint naturally leads to compressive strains normal to the articular cartilage surface, the rolling action of the joint motion induces cyclic tensile strains tangential to the surface (Askew and Mow 1978). Since the exudation of interstitial fluid from the porous cartilage is inhibited by the relatively low permeability of the matrix and confined by the impermeable subchondral bone (Maroudas et al. 1968), fluid flow is restricted to regions near the articular surface. Furthermore, as the matrix is compressed, pore size is reduced, thus resulting in a further, non-linear strain-dependent reduction in permeability (as reviewed by Mow et al. 1984). Therefore, the magnitude of total cartilage thinning during physical activities is relatively small, with peak compressive strains occurring in the superficial and transitional zones and high magnitude hydrostatic pressures in the middle and deep zones (Waterton et al. 2000, Eckstein et al. 2001). This hydrostatic pressure has been shown both theoretically and experimentally to support over 90% of the applied stress (Macirowski et al. 1994, Ateshian and Wang 1995, Soltz and Ateshian 1998, Soltz and Ateshian 2000), and thereby provide a stress-shielding mechanism for articular cartilage. Continuous loading leads to consolidation of the matrix and reduced thickness of the superficial layer. However, subsequent removal of load leads to hydrostatic tension in the porous matrix, causing the matrix to swell and imbibe water, and thus restoring cartilage thickness (Suh et al. 1995).

In Vivo Mechanical Behavior

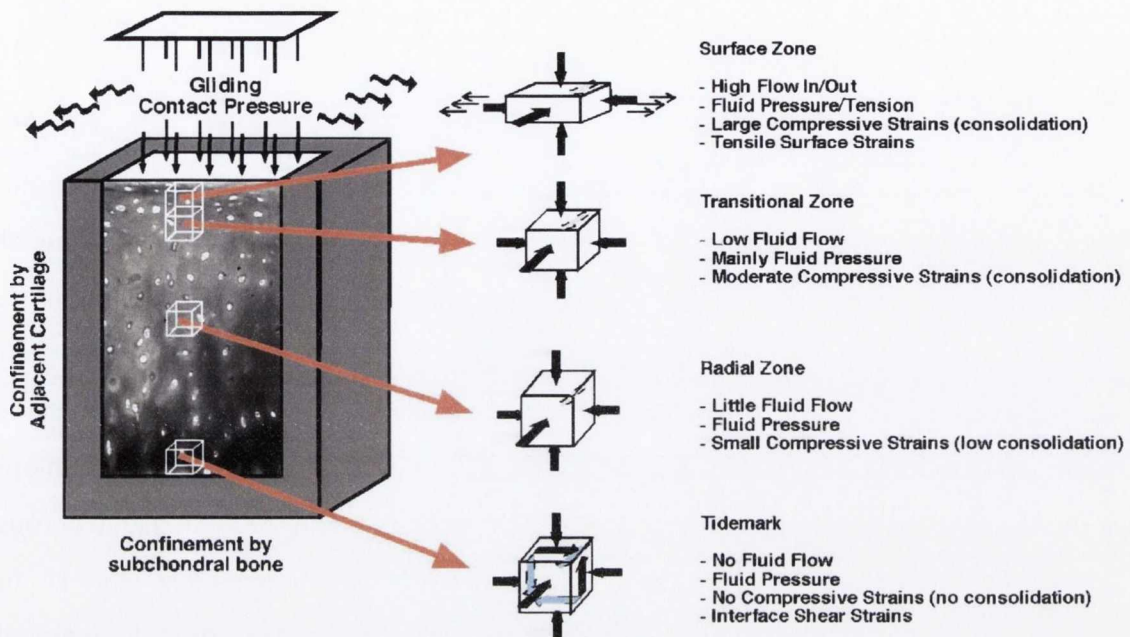


Figure 2-5 : Schematic representation of the *in vivo* mechanical environment of articular cartilage under intermittent joint loading and motion (Wong and Carter 2003).

Numerical modelling techniques have revealed that even under simple compressive loading conditions, chondrocytes are subjected to a complex local mechanical environment consisting of tension, compression, shear, and fluid pressure (Guilak and Mow 2000), and mechanical loading of the joint environment has been shown to be critical for cartilage homeostasis. Increased functional loading of joints though moderate exercise has been correlated with increased in articular cartilage thickness, proteoglycan content, and mechanical stiffness of the tissue (Jurvelin et al. 1986, Kiviranta et al. 1988, Säämänen et al. 1988). However, strenuous exercise can lead to the formation of cartilage lesions (Brama et al. 2000), whilst a lack of mechanical loading due to, for example, immobilization of the joint, can lead to a decrease in proteoglycan content, thinning and softening of the non-calcified part of articular cartilage and an increase in subchondral vascular eruptions (Paukkonen et al. 1983, Lane Smith et al. 1992, O'Connor 1997, Vanwanseele et al. 2002). Furthermore, there is evidence to suggest that an abnormal joint loading environment plays a key role in the onset of osteoarthritis (Arokoski et al. 2000, Felson et al. 2000, Griffin and Guilak 2005, Cox et al. 2013).

2.2 Cartilage tissue engineering

2.2.1 Overview of cartilage tissue engineering

The goal of cartilage tissue engineering is the *in vitro* development of implantable cartilage tissue grafts that can induce and/or maintain the desired phenotype of the encapsulated cells. Furthermore, it is desirable that engineered grafts should be of sufficient structural stability to withstand the mechanical rigors of the joint environment. Ultimately, the implanted cells should synthesize cartilage-specific ECM and the graft integrate with host tissue at the defect site, thus repairing the defect. Since chondrocytes expanded in monolayer culture displayed a tendency to de-differentiate towards a more fibrous phenotype (Benya and Shaffer 1982, Temenoff and Mikos 2000), a highly porous 3D scaffold that provides structural support and chondro-inductive stimuli to encapsulated cells is desirable. Therefore, both cell type and scaffold biomaterial selection are of key importance when engineering cartilage tissue grafts.

2.2.2 Biomaterials for cartilage tissue engineering

Features of the ideal scaffold would include:

- biocompatible - to minimize immunological responses and to integrate with the adjacent tissue
- biodegradable - gradual, controlled resorption should be simultaneous with cellular and tissue ingrowth
- porous - with interconnected pores, to allow cell migration of loaded cells or native cells as well as diffusion of nutrients, gases and waste
- adhesive - for cell attachment as well as chondro-inductivity, and for delivery of bioactive molecules
- structurally and mechanically stable - to support loading and deformation of the tissue

Many biomaterials, both naturally-derived and synthetic, have been studied for use as scaffolds. Polysaccharides are the most frequently employed natural polymer in biomedical applications and possess useful properties such as water solubility, non-toxicity, stability in variable pH and can be bio-functionalized via chemical modification.

Polysaccharide based hydrogels, such as agarose and alginate, have been shown to induce enhanced production of type II collagen and GAG in chondrocytes, thereby promoting and maintaining a chondrogenic phenotype (Rahfoth et al. 1998, Domm et al. 2002).

Fibrin is the polymerised form of fibrinogen, which is a natural component found in blood, and is commonly used as an adhesive or sealant in surgical applications (Radosevich et al. 1997). Fibrinogen exists physiologically in a liquid state within the blood stream, with activation of polymerisation via thrombin in instances of vascular lesioning or other pathological situations to form fibrin clots. Fibrin gels have been shown to maintain the chondrogenic phenotype leading to functional chondrogenesis in long term culture when seeded with chondrocytes (Eyrich et al. 2007). However, there is evidence to suggest that fibrin is not as chondro-permissive as other well established hydrogels, with bone marrow derived stem cells showing a diminished chondrogenic potential when encapsulated in fibrin (Thorpe et al. 2012).

Collagen is another commonly used natural biomaterial, and features qualities such as biodegradability, low antigenicity and the ability to facilitate cell adhesion (Lee et al. 2001). Collagen is a biological protein abundant in the extra cellular matrix of many tissues, where it provides the principal structural and mechanical support. Collagen gels have also been shown to promote a cartilage ECM synthesis in chondrocytes (Wakitani et al. 1989), but a disadvantage of using collagen as a biomaterial for tissue repair is its high degradation rate, which can lead to a rapid loss of mechanical properties (Angele et al. 2004). However, it is possible to overcome these functional limitations by blending collagen based gels with more structurally stable materials (Kim and Kim 2013).

2.3 Mesenchymal stem cells

Musculoskeletal tissues are in a constant state of flux, constantly remodelling to grow and adapt to the physiological environment. Tissues such as bone, cartilage, muscle and fat are maintained by osteocytes, chondrocytes, myocytes and adipocytes respectively, which share a common progenitor cell type – the mesenchymal stem cell / multipotent stromal cell (MSC). MSCs are multipotent, self-renewing stem cells that reside in adult mesenchymal tissues such as bone marrow, infrapatellar fat pad, synovial membrane and

adipose tissue and are responsible for directing and controlling re-modelling, repair and rejuvenation of these tissues (Figure 2-6) (Caplan and Bruder 2001). Autologous MSCs can be harvested from various tissues with relative ease and, when seeded in monolayer culture, possess the ability proliferate extensively *in vitro*. MSCs also have the ability to sense and respond to their local chemical and physical environment and can be induced to differentiate along multiple lineages *ex vivo* (Pittenger et al. 1999). These characteristics make MSCs an ideal candidate for tissue engineering applications. However, MSC differentiation is complex process requiring temporal and spatial control of the biochemical and biophysical environment.

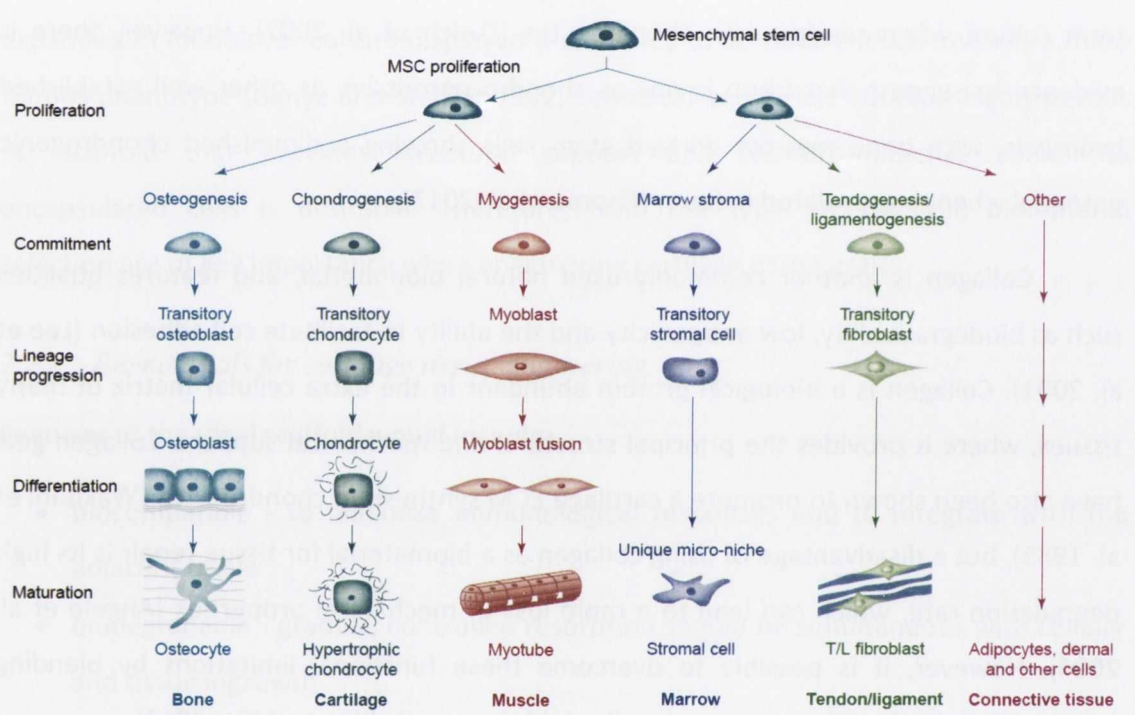


Figure 2-6: The mesengenic process (Caplan and Bruder 2001).

2.3.1 Biochemical regulation of MSC differentiation

The ability of growth factors to induce differentiation in MSCs is well documented. Chondrogenic differentiation of MSCs can be achieved by culturing MSCs in high cell-density micromass culture in a chemically defined, serum free medium supplemented with TGF- β (Mackay et al. 1998, Pittenger et al. 1999). However, it has also been shown the TGF- β induces a smooth muscle phenotype in MSCs cultured *in vitro* (Kinner et al. 2002, Jeon et al. 2006, Gao et al. 2010), suggesting that there are additional factors that

regulate the chondrogenic/myogenic lineage switch that must be considered. Furthermore, rather than adopting a stable chondrogenic phenotype following TGF- β induced chondrogenesis, bone marrow derived MSCs can continue to progress along an endochondral path, thereby becoming hypertrophic chondrocytes and resulting in mineralization of engineered grafts (Pelttari et al. 2006). While calcification may be desirable in specific regions of grafts engineered for treatment of osteochondral defects, understanding how this process occurs and how to stabilize the chondrogenic phenotype is of critical importance.

2.3.2 Substrate stiffness and cell morphology

Previous studies have demonstrated that MSCs can respond and adapt to changes in their local mechanical environment. For example, it has been shown that lineage commitment can be controlled by tuning substrate stiffness, with softer matrices promoting a chondrogenic, neurogenic or adipogenic phenotype, matrices of intermediate stiffness being more myogenic and comparatively rigid matrices promoting osteogenesis (Figure 2-7) (Engler et al. 2006, Rowlands et al. 2008, Chatterjee et al. 2010, Huebsch et al. 2010, Park et al. 2011, Bian et al. 2013). The stiffness of MSC substrates has also been shown to influence cell shape in 2D culture, with stiffer substrates being associated with a more spread cell morphology (Yeung et al. 2005, Winer et al. 2008), but there is evidence to suggest that this is not necessarily the case in more physiologically relevant 3D matrices (Huebsch et al. 2010, Parekh et al. 2011).

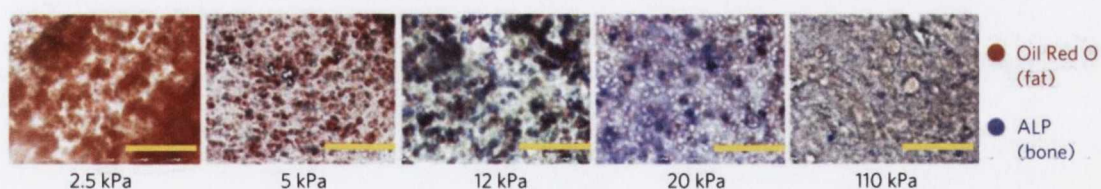


Figure 2-7: Matrix compliance alters MSC fate in 3D matrix culture. In this case, MSC seeded in softer matrices (left) stained positive for adipogenic markers, whereas stiffer substrates (right) promoted osteogenic markers (Huebsch et al. 2010).

Furthermore, cell morphology itself is known to regulate cell fate, with MSCs that are allowed to adopt a more stellate morphology undergoing osteogenesis or myogenesis,

while cells constrained in a round shape exhibit enhanced markers of chondrogenesis or adipogenesis (Figure 2-8) (McBeath et al. 2004, Gao et al. 2010).

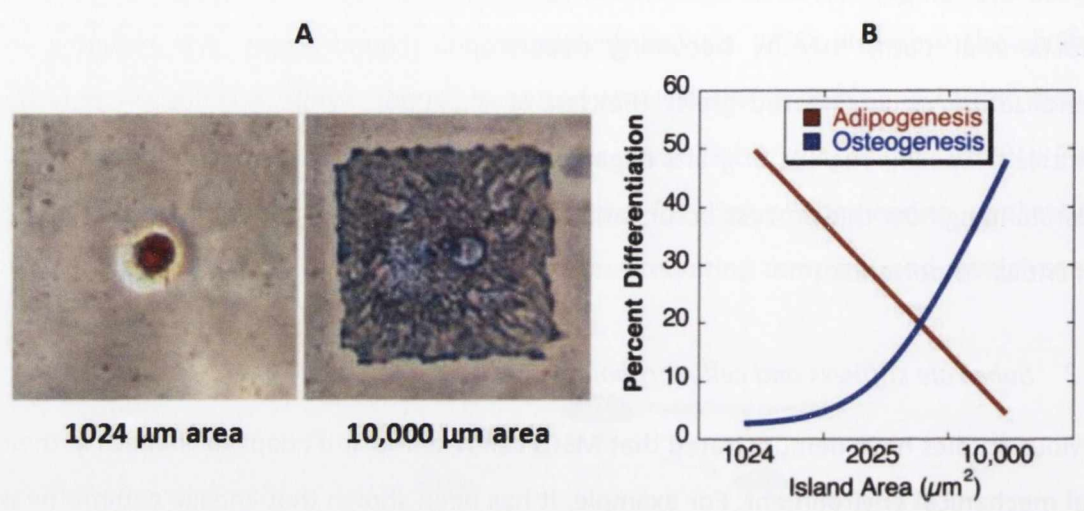


Figure 2-8: Cell morphology alters MSC fate in 2D culture. The spreading of MSCs (cultured in combined osteo/adipo-inductive medium) was limited by creating islands of ECM protein coatings of varying area on non-adhesive glass via microcontact printing techniques. Cells were stained for lipids (red) and alkaline phosphatase (blue) (A). Increased cell spreading was correlated with enhanced osteogenesis and reduced adipogenesis (B) (McBeath et al. 2004, Guilak et al. 2009)

2.3.3 Extrinsic mechanical signals as regulators of MSC fate

A considerable body of data exists demonstrating that the application of physiological levels of mechanical stimuli, such as dynamic compression (DC), hydrostatic pressure (HP) and cyclic tensile strain (CTS), can influence MSC lineage commitment.

2.3.3.1 Dynamic compression

Due, perhaps, to the relative ease of bioreactor design and the relevancy of this form of stimulation to physiological loading of cartilage tissue, dynamic compression is among the more commonly studied modes of extrinsic loading. It has been demonstrated that DC can be as effective as TGF- β 1 stimulation in inducing MSC expression of chondrogenic genes aggrecan and collagen type II in agarose hydrogels (Huang et al. 2004). Indeed, multiple studies report enhanced chondrogenic gene expression and/or cartilage-specific ECM deposition as a response to compressive loading in the absence of growth factors

(Huang et al. 2004, Huang et al. 2005, Campbell et al. 2006, Park et al. 2006, Mauck et al. 2007, Terraciano et al. 2007) and increased Sox9 and aggrecan gene expression has been observed as early as only one hour after the initiation of dynamic compressive loading in PEG hydrogels (Terraciano et al. 2007).

Studies involving compressive loading in conjunction with growth factors reveal a more complex pattern. DC has been reported to lead to enhanced synthesis of GAG and collagen and a more uniform ECM distribution in MSC seeded hyaluronic acid hydrogels cultured long term (70 days) in chondrogenic medium supplemented with TGF- β 3 (Bian et al. 2012). Interestingly, in constructs that were transferred to hypertrophic induction media after 14 days, dynamic compressive loading was also shown to significantly reduce the expression of hypertrophic markers and to suppress the degree of calcification (Bian et al. 2012). However, it has been shown that the influence of compressive loading is dependent on growth factor dosage, with much stronger effects on chondrogenic gene expression observed at lower TGF- β 1 concentrations (Li et al. 2010) and that in the presence of TGF- β 1, biosynthesis in loaded MSC agarose constructs was less than in unloaded controls (Kisiday et al. 2009). Furthermore, longer-term studies have shown that cartilage ECM deposition by MSCs encapsulated in agarose hydrogels is reduced when mechanical loading is initiated at the onset of TGF- β induced chondrogenesis (Campbell et al. 2006, Thorpe et al. 2008). It has also been reported that delayed dynamic compressive loading (applied after the discontinuation of the growth factors) significantly increased tissue mechanical properties relative to DC applied concurrently with TGF- β 3 supplementation (Lima et al. 2007). Chondrogenic gene expression can be enhanced in the presence of TGF- β 1 if DC application is delayed until day 16, whereas initiation of DC at day 6 suppresses aggrecan expression in MSCs. Taken together, these data suggest that there is a complex interplay between DC and growth factor stimulation of MSCs and that cellular response to extrinsic mechanical loading is dependent on the stage of chondrogenesis of the MSC.

2.3.3.2 Hydrostatic pressure

Application of physiological levels of cyclic hydrostatic pressure have been reported to promote a chondrogenic phenotype in MSCs cultured both with and without growth factor supplementation. HP has been demonstrated to enhance SOX9, collagen type II

and aggrecan gene expression in MSC pellets cultured without TGF- β 3 (Miyanishi et al. 2006). Furthermore, although supplementation with TGF was found to be the more potent chondrogenic stimulus than HP loading, combining HP and TGF supplementation resulted in the greatest increase in expression of the chondrogenic genes examined (Figure 2-9). This pro-chondrogenic influence of HP loading has since been demonstrated to be magnitude dependent, with greater collagen type II expression and collagen matrix deposition being found to be correlated with increasing magnitude of applied HP within the range examined (i.e. 0.1, 1 and 10 Hz) (Miyanishi et al. 2006).

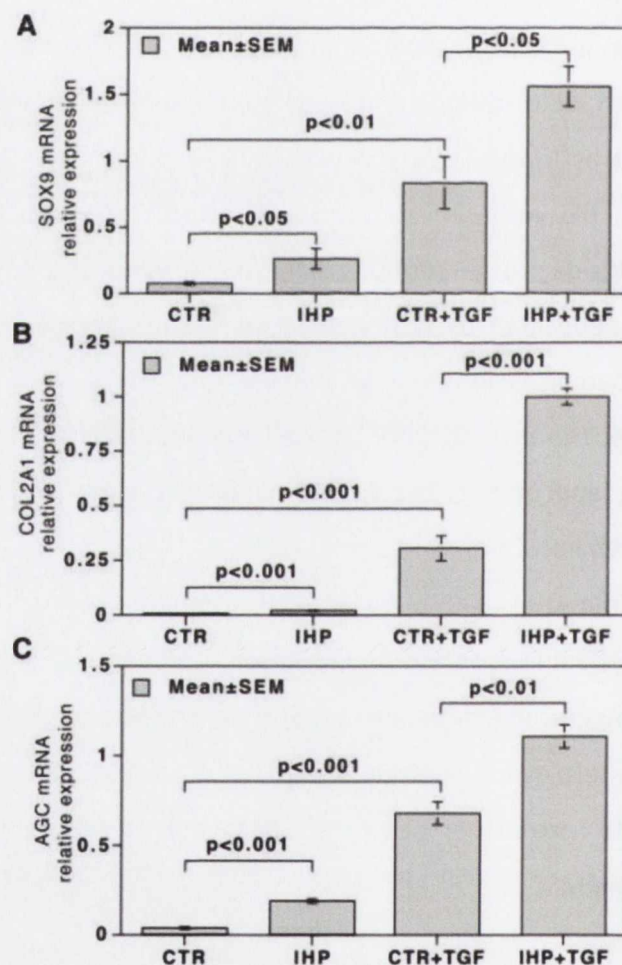


Figure 2-9: Cyclic hydrostatic pressure promotes chondrogenic gene expression in both the presence and absence of TGF- β 3 (CTR - free swelling control; IHP - intermittent hydrostatic pressure; TGF - transforming growth factor β 3; AGC – aggrecan) (Miyanishi et al. 2006).

HP has been shown to promote chondrogenesis, but have no effect on osteogenic gene expression, on bone marrow-derived MSCs (BMSC) seeded in collagen type I matrices when cultured in media supplemented with both chondrogenic and osteogenic growth factors (Wagner et al. 2008). It has also been reported that HP failed to induce robust chondrogenic differentiation in both synovial membrane-derived stem cells and infrapatellar fat pad-derived stem cells in the absence of TGF- β 3 (Vinardell et al. 2012). However, following TGF- β 3 (1 ng/mL) induced chondrogenesis, HP promoted Sox9 expression, enhanced GAG accumulation and, interestingly, inhibited expression of hypertrophic markers collagen type X and Indian Hedgehog. Also, a study examining the influence of HP on endochondral ossification of chondrocyte seeded hydrogels found that HP downregulated MMP-13 (matrix metalloproteinase -13; an enzyme in hypertrophic cartilage responsible for hydrolysing type II collagen and aggrecan) and type I collagen, while upregulating TIMP-1 (an inhibitor of matrix metalloproteinase), leading the authors to conclude that HP may slow chondrocyte differentiation and have a chondro-protective, anti-angiogenic influence on cartilage tissue (Wong et al. 2003). Furthermore, a more recent study revealed that the application of HP can reduce alkaline phosphatase activity in BMSC seeded agarose and fibrin grafts, thus adding further weight to the hypothesis that HP plays a role in the maintenance of the chondrogenic phenotype and regulating endochondral ossification of mature chondrocytes (Steward et al. 2012).

2.3.3.3 Cyclic tensile strain

There is currently a paucity of studies examining the influence of cyclic tensile strain (CTS) on chondrocytes and MSCs encapsulated in physiologically relevant 3D matrices. Likely reasons for this include the relative difficulty associated with this mode of mechanical stimulation (i.e. firmly gripping and accurately straining soft 3D hydrogels, whilst avoiding slippage or causing damage). Also, tensile strains are typically associated with connective tissues such as tendon, ligament and muscle, as opposed to articular cartilage, where the predominant mode of extrinsic loading is compressive. However, as discussed previously intermittent compressive loading is known to induce cyclic tensile strains in articular cartilage. Additionally, endochondral ossification, the process by which all long bones are formed, requires the development of a cartilage intermediate, and there is evidence to suggest that tensile strain is a regulator of this process. It has long been suggested that

ossification during limb development occurs in the regions of maximal tensile strains (Carey 1922), while *in vivo* studies of bone healing have shown that callus distraction can produce a systemic increase in concentrations of several bone growth factors; something which is not seen after rigid osteotomy (Sato et al. 1999, Weiss et al. 2002). Furthermore, while dynamic compression has been shown to suppress myogenesis of MSCs (Thorpe et al. 2012), there is evidence to suggest CTS may induce a pro-myogenic response in MSCs (Park et al. 2004). Therefore, understanding how tensile deformation influences MSC lineage commitment may be key to understanding the phenotype switch between a chondrogenic/myogenic phenotype and stabilization of phenotype of the mature chondrocyte prior to endochondral ossification.

It has previously been reported that cyclic tensile strain (CTS) can direct MSC organization and differentiation when seeded on 2D substrates. Multiple studies demonstrate that the application of uniaxial CTS can enhance markers of osteogenesis, fibrogenesis and myogenesis, and suppress chondrogenesis and adipogenesis in MSCs in monolayer culture (Simmons et al. 2003, Hamilton et al. 2004, Park et al. 2004, Friedl et al. 2007, Chen et al. 2008, Qi et al. 2008, Ghazanfari et al. 2009, Jang et al. 2011, Maul et al. 2011, Huang et al. 2012, Li et al. 2015). The magnitude of tensile loading has also been shown to be important, with low strains inducing osteogenesis and higher strains promoting the expression of fibrous or myogenic markers (Chen et al. 2008, Jang et al. 2011). These studies also show that in 2D culture, uniaxial CTS leads to cell reorganisation and alignment (Wang and Grood 2000) and that tension induced differentiation signals are dependent on MSC orientation (Kurpinski et al. 2006, Heo et al. 2011, Subramony et al. 2013). However, although there is an abundance of data regarding CTS in 2D cultures, these 2D culture systems are generally not considered truly representative of the physiological environment of cells *in vivo*, and MSCs have been shown to respond differentially to tensile loading when seeded on 2D substrates compared to when encapsulated within 3D matrices (Rathbone et al. 2012).

Fewer studies have examined the influence of CTS on MSCs seeded in more physiologically relevant 3D matrices (see Table 2-1 for summary), and most naturally focus on the influence of CTS on tenogenic and ligamentogenic differentiation. These studies generally show an upregulation in fibroblast-related genes, increased collagen deposition and enhanced mechanical properties following the application of CTS

(Juncosa-Melvin et al. 2007, Farng et al. 2008, Kuo and Tuan 2008, Connelly et al. 2010, Doroski et al. 2010, Baker et al. 2011, Yang et al. 2012), although there is evidence to suggest that, for fibrous tissue generation, MSCs should be primed towards a fibroblast lineage prior to loading (Kreja et al. 2012).

The few studies that have explored the role of tensile loading on chondrogenesis of MSCs have generally found that this mechanical stimulus promotes the development of a more fibrocartilaginous phenotype. For example, Connelly et al. found that the application of CTS to MSCs encapsulated into fibrin hydrogels enhanced glycosaminoglycan (GAG) deposition and collagen type 1 expression (Connelly et al., 2010). Furthermore, McMahon et al. also found CTS enhanced GAG synthesis of MSCs in collagen-GAG scaffolds, and attributed this to the involvement of stretch-activated ion channels (McMahon et al. 2008).

It has been reported that CTS can induce osteogenic differentiation in MSC seeded 3D constructs. The application of CTS has been shown to promote MSC expression of BMP-2 in collagen gels in the absence of osteogenic supplements (Sumanasinghe et al. 2006). Furthermore, investigations comparing of the influence of tensile strain versus compressive strain on MSCs in alginate hydrogels also found that the application of CTS upregulated osteogenic gene expression, whereas DC upregulated chondrogenic genes (Haudenschild et al. 2009). However, the mechanism of how extrinsic CTS influences MSC lineage commitment, and the dependency of this mechanism on factors such as matrix stiffness and cell shape, has not yet been fully elucidated. While it has been show that, in 2D culture, the application of CTS can regulate the chondrogenic/myogenic lineage switch in MSCs, this has yet to be demonstrated in more physiologically relevant 3D matrices.

Table 2-1: Review of studies examining the influence of cyclic tensile strain on MSC differentiation on both 2D and 3D substrates

Author	Cell substrate	2D/3D	Main findings	Summary	As evidenced by:
(Simmons et al. 2003)	Collagen I coating	2D	CTS (equibiaxial) inhibited proliferation and enhanced mineralization	↑Osteo	↑Calcium, ERK1/2, p38
(Hamilton et al. 2004)	Collagen I coating	2D	CTS (10%, 1Hz) inhibited proliferation; promoted perpendicular alignment and enhanced smooth muscle cell markers	↑Myo	Staining: ↑α-actin, calponin.
(Park et al. 2004)	Collagen or elastin coating	2D	Uniaxial (10%, 1Hz) CTS enhanced smooth muscle cell markers and Collagen I expression, promoted perpendicular alignment, but had no effect on bone, vascular & ligament markers. Equiaxial CTS suppressed smooth muscle cell markers.	↑Myo	Uniaxial: ↑α-actin, SM-22α, CNN. No effect: ALP, OCN, COL1I, ACAN, COL1II
(Kurpinski et al. 2006)	Collagen I coating	2D	CTS with Parallel Cell Alignment Increased Contractile Marker Calponin 1 and decreased both chondro/osteogenic gene expression. Perpendicular-Oriented Microgrooves Altered Effects of Uniaxial Strain on MSCs.	↑Myo ↓Chondro, Osteo	↑CNN1, ↓BGN, COL10A1, COL11A1, COMP
(Friedl et al. 2007)	Silicone/plastic	2D	CTS significantly stimulated the expression levels of the early chondrogenic and osteogenic marker genes	↑Osteo, ↑Chondro	↑SOX9, LUM, DCN, RUNX2, SPARC, SPP1, ALPL
(Chen et al. 2008)	Collagen I coating	2D	CTS induced alignment. CTS (3%) upregulated osteogenic genes, whereas CTS (10%) upregulated tendon/ligament genes.	↑Osteo/Fibro	↑COL I & III, tenascin C (TNC), Cbfa1, ALP, MMP-3, ↓MSPC
(Qi et al. 2008)	Silicone strips	2D	CTS promoted proliferation and osteogenic differentiation	↑Osteo	↑ALP, Cbfa1, Ets-1
(Diederichs et al. 2009)	Silicone dish	2D	CTS promotes fibrous gene expression and calcification	↑Osteo/Fibro	↑Collagen types 1 & 3, calcification
(Ghazanfari et al. 2009)	Collagen I coating	2D	CTS promoted proliferation, cellular perpendicular alignment and smooth muscle cell differentiation	↑Myo	↑α-actin staining
(Heo et al. 2011)	Aligned PCL fibres	2D	Static strain regulated fibrous gene expression dependent on scaffold and cell orientation	↑Fibro	↑collagen I, tenascin C, LOX gene expression.
(Maul et al. 2011)	Collagen I coating	2D	CTS promoted perpendicular alignment, enhanced smooth muscle markers in a frequency/magnitude dependent manner.	↑Myo	Staining: ↑SMA, CNN, MHC protein expression
(Jang et al. 2011)	Fibronectin coating	2D	CTS promoted proliferation, cellular alignment. 3% CTS enhanced osteogenesis, 10% enhanced SMC markers	↑Osteo, Myo	3%: ↑α-SMA, CDM expression. 10%: ↑BMP2, OPN
(Huang et al. 2012)	Silicone membrane	2D	CTS induced cardiomyocyte markers	↑Cardiomyo	↑GATA-4, β-MHC, Nkx2.5 and MEF2c expression
(Subramony et al. 2013)	PLGA sheet	2D	Fibroblastic differentiation occurred only with BOTH alignment and strain	↑Fibro	↑collagen III, FN, TNC, SCX, ↑collagen I & III deposition
(Li et al. 2015)	Collagen IV coating	2D	CTS inhibited adipogenic differentiation through TGFβ1/Smad2 signalling	↓Adipo	↓Lipid drops (oil red O), ↓PPARγ-2, adiponectin, and C/EBPα ↑p-Smad2/7-Smad2
(Altman et al. 2002)	Collagen gel	3D	CTS up-regulated ligament fibroblast markers, including collagen types I and III and tenascin-C and promoted cell alignment. No regulation of chondro/osteogenic markers	↑Fibro	↑collagen I, collagen III, TNC expression. Not detected: Collagen II. No change: OCN, OPN
(Sumanasinghe et al. 2006)	Collagen gel	3D	CTS can induce osteogenic differentiation in the absence of osteogenic supplements	↑Osteo	↑BMP2
(Nieponcice et al. 2007)	Fibrin gel	3D	CTS promoted a smooth muscle cell-like lineage	↑Myo	↑α-actin, h1-calponin, collagen content
(Juncosa-Melvin et al. 2007)	Collagen sponge	3D	CTS enhanced the structural and material properties of repaired tendon.	↑Fibro	↑stiffness ↑collagen I, collagen III gene expression. No change: Fibronectin, decorin gene expression
(Fang et al. 2008)	Collagen coated PCL	3D	CTS upregulated tendon related genes	↑Fibro	↑collagen I, collagen III, Scleraxis. No change: Tenascin C
(Byrne et al. 2008)	Collagen/GAG	3D	CTS enhanced chondrogenic differentiation[attenuated in the presence of a stretch-activated ion channel inhibitor)	↑Chondro	↑GAG synthesis
(Byrne et al. 2008)	Collagen/GAG	3D	CTS can induce osteogenic differentiation	↑Osteo	↑OCN vs constrained control
(Kuo and Tuan 2008)	Collagen gel / 2D plastic	Both	CTS enhanced tenogenesis	↑Fibro	↓Wnt4, Fizzled-5 mRNA levels, ↑Wnt5a. No change: COL 1, 2, 12
(Haudenschild et al. 2009)	Alginate	3D	CTS enhanced osteogenesis and inhibited chondrogenesis, whereas dynamic compression (DC) up-regulated chondrogenic gene expression	↑Osteo ↓Chondro	CTS: ↑BMP1, ALP, NELL1 and ↓BMPRIa. Wnt receptor Fritzzled-7 DC: ↑Wnt3, Wnt5, COL11A2
(Connelly et al. 2010)	Fibrin gel	3D	CTS promotes fibrochondrocyte-like differentiation	↑Fibro	↑GAG & collagen content, ↑Collagen I mRNA & protein, No effect: Collagen type II, Aggrecan, osteocalcin mRNA
(Doroski et al. 2010)	PEG-based hydrogel	3D	CTS significantly upregulated all tendon/ligament fibroblastic genes examined	↑Fibro	↑collagen I, collagen III, tenascin C expression. No change: osteo/chondro/adipo
(Baker et al. 2011)	PCL nanofibres	3D	CTS enhanced fibrous gene expression, collagen deposition and tensile modulus of graft	↑Fibro	↑COL1I, Fibronectin, lysyl oxidase, collagen deposition
(Kreja et al. 2012)	PLA fibre bundle	3D	CTS upregulated tendon/ligament fibroblast-related genes in fibroblasts only. No effect found in undifferentiated MSCs	↑Fibro	↑COL I, COL III, FN1, TNC expression (in fibroblasts only) ↑collagen III, tenascin C, and decorin.
(Yang et al. 2012)	PEG-based hydrogel	3D	CTS upregulates tendon/ligament fibroblast-related genes	↑Fibro	Effects of CTS appear to be independent of cell shape
(Govoni et al. 2014)	Hyaluronan based mesh	3D	CTS enhanced markers of myogenesis (α-sarcomeric actin-1 and α-smooth muscle actin expression)	↑Myo	↑α-sr-1, α-SMA expression

2.3.4 Oxygen tension and MSC metabolism

The efficacy of stem cell based cartilage tissue engineering strategies is typically evaluated based on changes in the expression of chondrogenic matrix related genes, the biochemical composition and biomechanical function of the graft (Kafienah and Sims 2004, Cernanec et al. 2007, Fehrer et al. 2007, Buckley et al. 2010, Meyer et al. 2010, Farrell et al. 2012, Thorpe et al. 2012, Farrell et al. 2014). However other changes in phenotype, such as cellular metabolic activity, may play an equally important role in determining whether a stem cell can differentiate to fulfil a tissue specific function. In the case of cartilage tissue engineering strategies, it would seem reasonable to assume that chondrogenically primed MSCs would need to adopt oxygen and nutrient consumption rates approaching that of chondrocytes in order to thrive following implantation into an articular cartilage defect. Chondrocytes are known to reside in an avascular, low oxygen *in vivo* environment, which ranges from around 6% in the superficial zone to as low as 1% in the deep zone (Zhou et al. 2004, Fermor et al. 2007). MSCs, however, typically reside in tissues such as bone marrow, which has been shown to possess oxygen levels of around 4% - 7% *in vivo* (Grant and Smith 1963, Kofoed et al. 1985, Harrison et al. 2002), and due to their perivascular niche (as reviewed by Caplan 2007, da Silva Meirelles et al. 2008), MSCs occupying such a tissue are likely to experience an oxygen tension at the higher end of this range. This would suggest that undifferentiated MSCs possess a different oxygen consumption rate to that of chondrocytes. Therefore, determining if MSCs used to engineer cartilage grafts adopt a metabolic profile similar to chondrocytes might be considered a key determinant of the success of stem cell based cartilage tissue engineering strategies. Furthermore, given that MSC phenotype and its biosynthetic activity is influenced by the local oxygen tension (Malladi et al. 2006, Fehrer et al. 2007, Buckley et al. 2010, Meyer et al. 2010, Sheehy et al. 2012, Pattappa et al. 2013), a detailed knowledge of how MSCs consume factors such as oxygen will play a key role in successfully engineering tissues of clinically relevant scales and modulating gradients in such regulatory signals will be critical to generating spatial complexity into tissue engineered grafts (Thorpe et al. 2013).

Chondrocytes generate energy in the form of adenosine triphosphate (ATP) via glycolysis and oxidative phosphorylation, and the rate at which chondrocytes consume oxygen is dependent on both the local oxygen and glucose environment. Studies have shown that chondrocytes are well adapted to low oxygen environments, deriving over 95% of their energy from glycolysis and demonstrating minimal oxygen consumption (Rajpurohit et al. 1996, Lee and Urban 1997, Heywood and Lee 2008). However, in the absence of sufficient glucose, a relative upregulation of oxygen consumption occurs (Heywood et al. 2006, Heywood et al. 2010), a phenomenon known as the Crabtree effect. Also, consistent with the Warburg effect, chondrocytes retain high levels of glycolysis even in an aerobic environment (Bywaters and MacKinnon 1937, Rosenthal et al. 1941), but culture in an anoxic environment (<1% oxygen) leads to a suppression of glycolysis (Lee and Urban 1997). It has been shown that chondrocyte ATP generation is optimal when chondrocytes are cultured in a low oxygen (5% oxygen) environment, compared to anoxia (1% oxygen) or normoxia (20% oxygen) (Fermor et al. 2010), and anoxia has been shown to severely compromise cartilage matrix production (Grimshaw and Mason 2000).

Undifferentiated MSCs also demonstrate a mixed metabolism, but have a higher oxygen consumption rate than chondrocytes and derive a significantly larger portion of ATP generation via oxidative phosphorylation (Pattappa et al. 2011). Both osteogenic and chondrogenic differentiation of MSCs have been shown to alter the balance of oxidative phosphorylation and glycolysis, with culture of MSC pellets in chondrogenic media shown to result in a reduction in the oxygen consumption rate, and MSCs undergoing osteogenesis increasing their glucose consumption rate (Pattappa et al. 2011). It has also been shown that environmental oxygen tension influences the differentiation capacity of MSCs and their metabolic behaviour. MSC populations expanded under normoxic conditions demonstrate premature senescence, enhanced capacity for osteogenic differentiation and an increase in oxygen consumption when compared to MSCs expanded under hypoxia, where a greater proportion of ATP production is derived from glycolysis (Pattappa et al. 2013). Furthermore, chondrogenic differentiation of MSCs under hypoxia leads to enhanced matrix deposition and the development of cartilage grafts with superior mechanical properties (Buckley et al. 2010). However, while the external culture environment can be controlled with relative ease, depending on construct geometry and scale, large gradients in oxygen availability (and thus gradients in

cell viability and matrix deposition) can arise between the periphery and centre of *in vitro* engineered cartilage grafts (Martin et al. 1999, Malda et al. 2004, Meyer et al. 2010, Buckley et al. 2011), potentially resulting in a necrotic core and graft failure. Understanding how parameters such as cell source, cell seeding density, oxygen consumption rate and culture environment influence oxygen gradients, and thus cellular metabolism and matrix synthesis, within engineered tissues will be critical to developing functional cartilage grafts.

2.4 Summary

The articulating joint is a complex, low oxygen environment, featuring gradients in nutrient availability and extracellular matrix composition and architecture, and subjected to multiple modes of extrinsic mechanical stimuli. Hence, chondrocytes occupying the diverse cartilage zones exhibit varying morphologies, experience gradients in oxygen and glucose availability and are subjected to a variety of modes of mechanical loading. While MSCs offer an attractive alternative to chondrocytes for tissue engineering based regenerative strategies, differentiation of MSCs into the required phenotype, and the subsequent stabilizing and maintaining of that phenotype, will require temporal and spatial control of the biochemical and biophysical environment. The studies presented in this dissertation aim to isolate and decouple these various stimuli with a view to identifying the conditions necessary for engineering of functional cartilage tissue grafts.

Chapter 3 A comparison of the oxygen consumption rate of chondrocytes and MSCs of different tissue origins undergoing chondrogenesis in 3D hydrogels

3.1 Abstract

Understanding how the local cellular environment influences cell metabolism, phenotype and matrix synthesis will be crucial to engineering functional cartilage tissue grafts of a clinically relevant scale. The objective of this study was to investigate how factors such as cell source, environmental oxygen tension and cell seeding density influence the local oxygen environment within *in vitro* engineered cartilage grafts. Furthermore, the subsequent impact of such factors on the cellular oxygen consumption rate and cartilage matrix synthesis was also examined. Bone marrow derived MSCs, infrapatellar fat pad derived MSCs and chondrocytes were seeded into agarose hydrogels and cultured in chondrogenic medium supplemented with transforming growth factor- β 3 (TGF- β 3). The local oxygen tension (pO_2) was measured in the centre of the constructs, and numerical modelling was employed to predict oxygen gradients and the average oxygen consumption rate within the engineered tissues. MSCs were found to possess a high initial oxygen consumption rate, but to adopt a less oxidative, more chondrocyte-like metabolic profile with the onset of chondrogenic differentiation, resulting in net increases in tissue oxygenation. Cartilage matrix deposition was generally maximal in regions of low oxygen tension, but collagen synthesis was inhibited in very low oxygen ($pO_2 < 2\%$) regions. These findings suggest that promoting a metabolic profile similar to that of chondrocytes might be considered a key determinant of the success of stem cell based cartilage tissue engineering strategies.

3.2 Introduction

The efficacy of stem cell based cartilage tissue engineering strategies is typically evaluated based on changes in the expression of chondrogenic matrix related genes, the biochemical composition and biomechanical function of the graft (Kafienah and Sims 2004, Cernanec et al. 2007, Fehrer et al. 2007, Buckley et al. 2010, Meyer et al. 2010, Farrell et al. 2012, Thorpe et al. 2012, Carroll et al. 2014, Farrell et al. 2014). As discussed in Chapter 2, it would seem reasonable to assume that chondrogenically primed MSCs would need to adopt oxygen and nutrient consumption rates approaching that of chondrocytes (CCs) in order to thrive following implantation into an articular cartilage defect. Therefore, determining if MSCs used to engineer cartilage grafts adopt a metabolic profile similar to chondrocytes might also be considered a key determinant of the success of stem cell based cartilage tissue engineering strategies.

While the external culture environment can be controlled with relative ease, depending on construct geometry and scale, large gradients in oxygen availability (and thus gradients in cell viability and matrix deposition) can arise between the periphery and centre of *in vitro* engineered cartilage grafts (Martin et al. 1999, Malda et al. 2004, Meyer et al. 2010, Buckley et al. 2011), potentially resulting in a necrotic core and graft failure. Understanding how parameters such as cell source, cell seeding density, oxygen consumption rate and culture environment influence oxygen gradients, and thus cellular metabolism and matrix synthesis, within engineered tissues will be critical to developing functional cartilage grafts.

The first objective of this study was to compare the oxygen consumption rate of chondrocytes and progenitor cells isolated from bone marrow (BMSCs) and infrapatellar fat pad (FPSCs) following encapsulation into three dimensional hydrogels. Cell seeded constructs were maintained in chondrogenic medium supplemented with TGF- β 3 for 24 days, with the oxygen levels measured within the engineered tissues at day 0 and day 24. The hypothesis was that the oxygen levels within tissue grafts engineered using MSCs would initially be lower than those in grafts engineered using chondrocytes (due to the more oxidative phenotype of MSCs), but, as the MSCs adopt a more chondrogenic phenotype, the consumption rate would reduce towards that observed in chondrocytes.

The second objective of this study was to explore how altering the external oxygen tension would affect local levels of oxygen availability and gradients within the engineered tissue. The final objective was to measure how oxygen levels and subsequent levels of matrix synthesis within engineered tissues depended on the initial cell seeding density.

3.3 Methods

3.3.1 Cell Isolation and Expansion

Bone marrow MSCs, infrapatellar fat pad MSCs and chondrocytes were isolated aseptically from the femoral diaphysis, the knee joint capsule and the femoral condyle, respectively, of porcine donors as described previously (Buckley et al. 2010, Buckley et al. 2011). Isolated BMSCs, FPSCs and CCs were plated separately to T-75 cm² flasks (Sarstedt, Wexford, Ireland) at a density of 5×10^3 cells/cm² and expanded in a normoxic (20% oxygen tension) environment in a media consisting of high glucose DMEM containing 10% foetal bovine serum and 1% penicillin (100 U/ml) - streptomycin (100 mg/ml) (all from GIBCO, Biosciences, Dublin, Ireland) and supplemented with fibroblastic growth factor-2 (FGF-2, 5 ng/ml; ProSpec-Tany TechnoGene Ltd, Israel). Each group consisted of cells from a single donor (i.e. donors were not pooled). Following colony formation, the cells were trypsinised, counted and expanded to passage 3 at a seeding density of 5×10^3 cells/cm² at each passage. Complete medium exchanges were performed twice weekly.

3.3.2 Agarose gel constructs fabrication and culture

BMSC, FPSC and CC constructs were fabricated separately. Cell suspensions were mixed with 4% agarose (Type VII; Sigma-Aldrich) at a ratio of 1:1 at ~40°C to yield a final gel concentration of 2% at a density of either 20 or 40 million cells/mL. The agarose/cell suspension was cast in stainless steel moulds and allowed to set for 20min to produce a solid slab, which was then cored with sterile biopsy punches to produce cylindrical constructs (Ø5×3 mm). Constructs were maintained for 24 days in a chemically defined chondrogenic medium consisting of high glucose DMEM supplemented with 100 U/mL

penicillin/streptomycin (both from Gibco), 100 mg/mL sodium pyruvate, 40 mg/mL L-proline, 50 mg/mL L-ascorbic acid-2-phosphate, 1.5 mg/mL bovine serum albumin, 1 × insulin–transferrin–selenium, 100 nM dexamethasone (all Sigma-Aldrich), and 10ng/mL of transforming growth factor-β3 (TGF-β3, ProSpec-Tany TechnoGene, Ltd.). Constructs were cultured in either a normoxic (nominally 20%) or a low (5%) oxygen environment. Each construct was cultured in an individual test dish (Figure 3-1) on a 3 mm agarose bed to allow greater, more homogenous oxygen distribution throughout the construct. Constructs were held firmly in position by the surrounding agarose bed to ensure that their position and orientation remained constant throughout the culture period. Complete medium exchanges were performed twice weekly. A replicate study with a new set of donors was performed upon completion of the initial study to verify findings.

3.3.3 *Experimental design*

Three studies were performed:

1. Hydrogels were seeded with **BMSCs**, **FPSCs** or **CCs** at a cell density of 20×10^6 cells/mL and cultured in 20% oxygen to investigate the influence cell source on oxygen gradients and matrix deposition.
2. Hydrogels were seeded with BMSCs at a cell density of 20×10^6 cells/mL and cultured in either **20%** or **5%** oxygen to investigate the influence of external oxygen environment.
3. Hydrogels were seeded with BMSCs at a cell density of either **20×10^6** or **40×10^6** cells/mL and cultured in 20% oxygen to investigate the influence of the cell seeding density.

3.3.4 Measurement of localised oxygen concentration

A novel test rig (Figure 3-1a) was developed to allow accurate spatial positioning of sensor tips within the engineered constructs. Sample test dishes were prepared by pouring acellular 2% agarose into cell culture dishes (Iwaki, Ø60 mm), thus creating a 4.5 mm deep agarose bed. A recess (1.5 mm deep, Ø5 mm) was created in the centre of the dish, into which samples to be tested were placed (thus leaving a 3 mm deep agarose bed underneath the sample). Sufficient medium was added to the dish to result in a consistent depth of approximately 1 mm of medium above the construct.

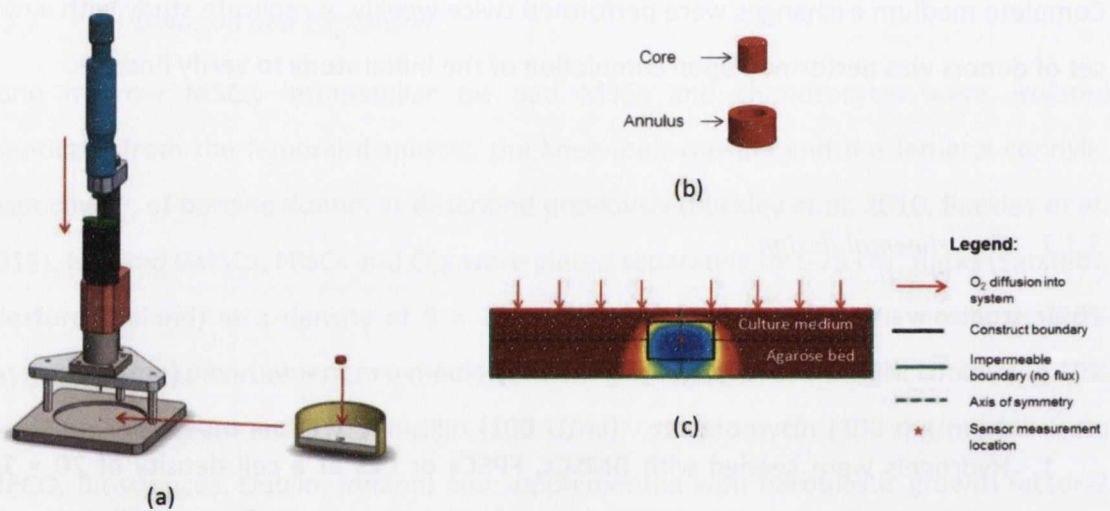


Figure 3-1: The local oxygen tension in the centre of *in vitro* engineered cartilage tissue grafts cultured in chondrogenic media was measured using a custom designed oxygen testing rig, test dish and fibre optic sensor (a). Samples were separated into cores and annuli for spatial biochemical analysis (b). Schematic of the finite element model developed to predict oxygen concentration gradients throughout the rest of the graft (c).

Oxygen tension at the centre of each construct (pO_{2c}) was measured using a fibre optic oxygen transmitter (Microx TX; PreSens – Precision Sensing GmbH, Regensburg, Germany) and implantable, 140 μm tip diameter, fibre optic, oxygen micro-sensors. The sensors were calibrated using a two point calibration in oxygen free (nitrogen saturated and containing sodium sulphite) and air saturated medium at 37°C. At each time-point, a micrometer was used to accurately position the sensor tip at the centre of the construct and the system was allowed to equilibrate for at least 3 hours prior to each oxygen measurement.

3.3.5 Prediction of oxygen concentration within agarose constructs

Oxygen concentration in the constructs and throughout the culture dish was modelled using commercially available finite element modelling software (Comsol Multiphysics 4.3) and is described by a diffusion-reaction type equation. The reaction term followed Michaelis-Menten kinetics:

$$\frac{\partial c}{\partial t} = D \nabla^2 c - \rho \frac{Q_m c}{K_m + c}$$

This equation describes a quasi-steady state reaction, which assumes that after an initial time period, the concentration of the reaction intermediates remain constant with time. Here, c is the oxygen concentration, D is the diffusion coefficient, ρ is the cell density, Q_m is the maximum cellular oxygen consumption rate and K_m is the oxygen concentration at which the reaction rate is at 50% of Q_m . The diffusion coefficient in 2% agarose was determined using the Mackie and Meares relation

$$\frac{D_{ag}}{D_{H_2O}} = \frac{\phi_f^2}{(2 - \phi_f)}$$

so that $D_{ag} = 2.77 \times 10^{-3} \text{ mm}^2/\text{s}$, where ϕ_f ($= 0.98$) is the fluid phase volume fraction (Gu et al. 2003, Sengers et al. 2005). The Michaelis-Menten constant, K_m was set to $63 \text{ } \mu\text{mol}/\text{mm}^3$ (Heywood et al. 2010). The construct was modelled as axisymmetric (Figure 3-1c). Oxygen diffusion through the culture media was accounted for by setting D_{media} ($= 3.0 \times 10^{-3} \text{ mm}^2/\text{s}$) to that of water (Haselgrove et al. 1993, Lightfoot and Duca 1999). The oxygen concentration at the media surface was prescribed as $185 \text{ } \mu\text{M}$, (measured using independently calibrated oxygen sensors) in the case of constructs cultured in the nominally 20% oxygen incubator and $50 \text{ } \mu\text{M}$ for the constructs cultured in the 5% oxygen incubator. At the dish walls, base and at the axis of symmetry, the flux was set to zero. Day 0 time-point simulations were performed for uniform cell concentrations of $p = 20 \times 10^6$ or $40 \times 10^6 \text{ cells}/\text{mL}$ throughout the construct. For day 24 simulations however, the core and outer annular regions were modelled using separate p values, to allow for discrepancies in cell proliferation/death between each region. The percentage change in cell concentration was calculated separately for each sample core and annulus by normalizing day 24 DNA values to mean day 0 DNA values (see Supplementary Figure 3-9a

at end of this chapter). p values for day 24 models were then calculated by multiplying this ratio by the initial (day 0) seeding density, giving cell concentrations in each sample core (p_{core}) and annulus (p_{ann}), thereby accounting for spatial changes in cell number over the study duration. Thus, 3 models per culture condition per time-point were developed, each with unique input parameters for Q_m , p_{core} and p_{ann} . Q_m values ($n=3$) were determined for each cell type and culture condition by systematically adjusting Q_m until the predicted pO_{2c} matched the experimentally observed pO_{2c} . It should be noted that Q_m represents the mean oxygen consumption value for all cells in each construct and, in the case of an experimentally measured pO_{2c} of 0%, the predicted value of Q_m is a minimum value of the oxygen consumption rate required to deplete oxygen to 0% at the centre of the construct. Contour plots were then produced showing predicted oxygen gradients throughout the tissues.

3.3.6 Biochemical analysis

Each construct ($n \geq 3$ per group) was separated into a core and annulus (to allow for spatial biochemical analyses; Figure 3-1b) using a 3 mm biopsy punch and their respective wet weights were measured. Each sample was then used for quantitative analysis of DNA, sulphated glycosaminoglycan (sGAG) and collagen content. For the former analyses, samples were digested with papain (125 $\mu\text{g}/\text{ml}$) in 0.1M sodium acetate, 5mM L-cysteine-HCl, and 0.05M EDTA (pH 6.0, all Sigma-Aldrich) at 60°C under constant rotation for 18 hours. DNA content was quantified using the Hoechst Bisbenzimidazole 33258 dye assay as described previously (Kim et al. 1988), with a calf thymus DNA standard. sGAG content was quantified using the dimethylmethylene blue dye-binding assay (DMMB; Blyscan, Biocolor Ltd., Northern Ireland) with a chondroitin sulphate standard. Collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110 °C for 18 hours in 38% HCl and assayed using a chloramine-T assay with a hydroxyproline : collagen ratio of 1:7.69 (Kafienah and Sims 2004, Ignat'eva et al. 2007). Total sGAG and hydroxyproline values were normalized to DNA values.

3.3.7 *Histology and immunohistochemistry*

At selected time-points, constructs (n = 2) were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C, rinsed with PBS, dehydrated and embedded in paraffin wax. Constructs were then cut in half and sectioned perpendicular to the disc face yielding 8 µm thick sections. Sections were stained with either 1% Alcian Blue 8GX (Sigma-Aldrich, Ireland) in 0.1M HCl for sGAG or Picro-Sirius Red for collagen. The deposition of collagen type II was identified through immunohistochemistry. Briefly, sections were rinsed with PBS and quenched of peroxidase activity for 20 min and treated with chondroitinase ABC (Sigma, 0.25 units/ml) in a humidified environment at 37 °C for 1 h to enhance the permeability of the extracellular matrix by removal of chondroitin sulphate. Slides were again rinsed with PBS and blocked with 10% goat serum for 30 min. Sections were incubated with mouse monoclonal anti-collagen type II diluted 1:100 (Abcam, UK) (concentration 1 mg/ml) for 1 h at room temperature. After washing in PBS, the secondary antibody for type II collagen (Anti-Mouse IgG Biotin antibody produced in goat) (concentration 1 g/L) binding was applied for 1 h. Colour was developed using the Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, UK) for 45 and 5 min exposure to peroxidase DAB substrate kit (Vector laboratories, UK). Slides were dehydrated through ethanol and xylene and mounted with Vectamount medium (Vector Laboratories, UK). Positive and negative controls (porcine cartilage and ligament) were included in the immunohistochemistry staining protocol for each batch.

3.3.8 *Statistical Analysis*

Results are reported as mean ± standard deviation. Groups were analysed by a general linear model for analysis of variance. Tukey's test was used to compare conditions. Unpaired t-tests were used where appropriate to highlight individual significant differences between conditions. Significance was accepted at a level of $P < 0.05$.

3.4 Results

3.4.1 MSCs adopt a chondrocyte-like oxygen consumption rate following agarose hydrogel encapsulation and stimulation with TGF- β 3

Agarose hydrogels seeded with either BMSCs, FPSCs or CCs were cultured for 24 days in chondrogenic medium. The level of oxygen at the centre of the construct (pO_{2c}) was measured at day 0 and 24 (Figure 3-2a). On day 0, the lowest pO_{2c} levels were measured in BMSC seeded constructs, whereas the highest levels were measured in the CC seeded constructs. By day 24, pO_{2c} levels had increased in BMSC constructs, approaching that observed in FPSC seeded constructs. The oxygen levels in CC constructs also increased over the duration of the study, whereas no significant change in oxygen levels were measured in FPSC constructs. Similar results were produced in a replicate study performed with cells isolated from a different donor (Supplementary Figure 3-8a).

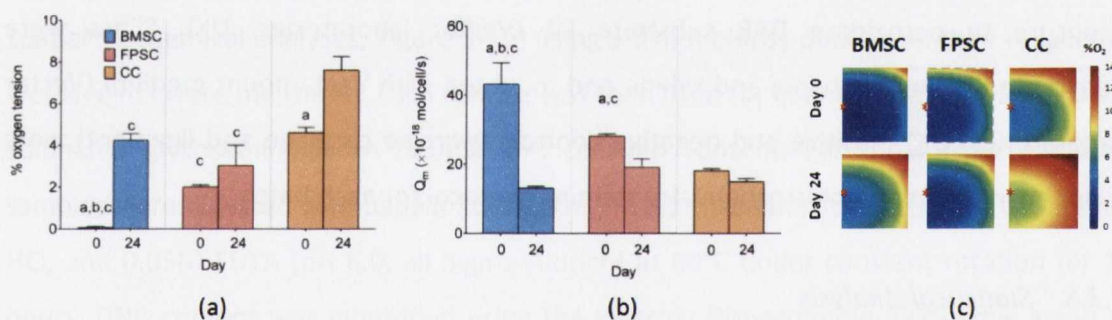


Figure 3-2: Cartilage tissue grafts engineered using various cell sources were cultured for 24 days. Oxygen concentration at centre of cartilage tissue grafts engineered using cells derived from various tissue sources (a). Cellular oxygen consumption rate extrapolated from FE model. (b) FE contour plots showing predicted oxygen concentration throughout the constructs (c). (a, b and c denote statistical difference vs. same cell type at day 24, FPSC constructs at same time-point and CC constructs at same time-point respectively; $P < 0.05$; $n = 3$ * indicates the location of oxygen sensor measurement within the constructs).

While a likely explanation for the observed increases in oxygen levels within the constructs is a change in cellular metabolism as MSCs adopt a more chondrogenic phenotype with time in culture, an alternative explanation could be a change in total number of cells within the engineered tissue due to proliferation or cell death. To investigate this, average DNA content at day 24 was normalized to that of day 0 and the percentage change was calculated (Supplementary Figure 3-9a). Both BMSCs and FPSCs proliferated in both the annuli and the central core section of the samples over the study duration, with a ~40% increase in DNA content. However, a reduction in CC cell numbers was observed, as evidenced by a ~20% decrease in overall DNA content, with cell death being higher in the higher oxygen annular section than in the core. FE modelling of oxygen transport revealed that BM cells at day 0 were found to have highest cellular oxygen consumption rate (Figure 3-2b), but this value dropped by approximately 70% to a level similar to that of CCs by day 24. The CC oxygen consumption rate remained relatively unchanged throughout the study, suggesting that the observed change in oxygen tension in CC constructs can be attributed to cell death, as opposed to altered cell metabolism. Q_m values extrapolated for FPSCs show a significant decrease of approximately 30% in cellular oxygen consumption rate during the 24 days of culture. Despite this, oxygen gradients remained consistent throughout FPSC constructs over the duration of the study, as decreases in FPSC oxygen consumption rate were balanced with increases in cell numbers (Figure 3-2c).

Biochemical analysis of day 24 constructs for cartilage extracellular matrix (ECM) deposition showed that the BMSCs synthesised higher quantities of sGAG on a per-cell basis when compared to FPSCs or CCs (Figure 3-3a). On a wet weight basis, greater levels of sGAG accumulation were observed in FPSC seeded constructs compared to both BMSC seeded constructs and CC seeded constructs (FPSC: 1.75 ± 0.08 %ww; BMSC: 1.29 ± 0.04 %ww; CC: 0.59 ± 0.01 %ww; for whole constructs). Spatial analysis of ECM deposition revealed higher levels of sGAG accumulation in the more hypoxic core than in the outer annulus of the engineered tissues (Figure 3-3 a, b). This difference was also observed in FPSC constructs, but not with CCs (Figure 3-3b). Similar trends were observed for collagen deposition, with BMSCs synthesizing the most collagen when normalized to DNA content (Figure 3-3c), and with the greatest levels of accumulation observed in the core. On a percentage weight basis, comparable levels of total collagen accumulation were observed in FPSC and BMSC seeded constructs, which were higher than those observed in

CC seeded constructs (FPSC: 0.36 ± 0.02 %ww; BMSC: 0.4 ± 0.02 %ww; CC: 0.04 ± 0.01 %ww, for whole constructs). Histological analysis revealed more intense staining for sGAG and collagen in BMSC and FPSC constructs when compared to CCs (Figure 3-3e). Only weak staining for collagen type II was observed in FPSC and CC constructs, with BMSC constructs staining more strongly for type II collagen, particularly in the more hypoxic core region of the engineered tissue.

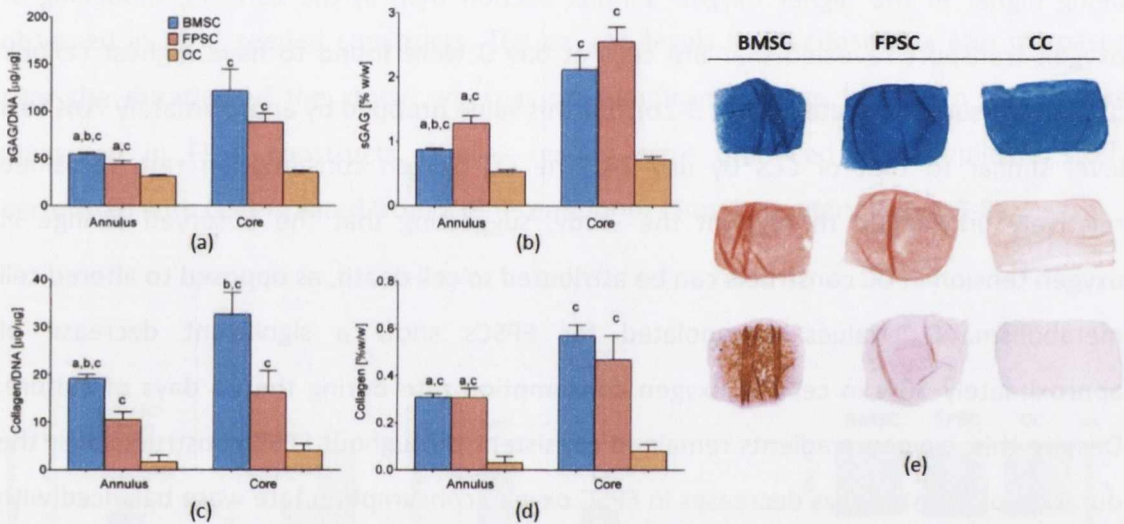


Figure 3-3: Cartilage tissue grafts engineered using various cell sources were cultured for 24 days, separated into cores and annuli, then analysed for DNA, sGAG and collagen content (a-d). Histological and immune-histochemical staining (e) was performed on representative sections for sGAG, collagen and collagen type II (top, middle, bottom respectively). (a, b and c denote statistical difference vs. core of construct using the same cell type, FPSC constructs in the same region and CC constructs in the same region respectively; $P < 0.05$; $n = 3$).

3.4.2 Decreasing the external oxygen tension reduces the rate at which MSCs consume oxygen and leads to increased matrix accumulation.

Although regions of low oxygen tension correlated with increased ECM deposition in stem cell seeded constructs, it was unclear whether these low oxygen conditions were directly enhancing chondrogenesis, or if these regions of low oxygen within the engineered tissues were simply a function of the high levels of oxygen consumption by encapsulated

cells. In an attempt to address this question, BMSC-seeded constructs were cultured in either a normoxic (20%) or a low oxygen (5%) environment and then measured (and subsequently predicted) local levels of oxygen availability in the engineered grafts. pO_{2c} was observed to be close to 0% for both external oxygen conditions at day 0 (Figure 3-4a). However, by day 24, local pO_{2c} levels had increased significantly for both external oxygen environments.

Unsurprisingly, oxygen levels in the core of BMSC constructs cultured in a low oxygen environment were still lower than those in the core of constructs cultured in normoxia at day 24. FE simulations predicted higher concentrations of oxygen (ranging from 0-14% O_2) throughout the constructs maintained in normoxic conditions compared to those maintained in low oxygen conditions, where the oxygen levels throughout most of the construct were less than 2% (Figure 3-4c). Q_m was shown to decrease in both cases over the culture period duration, but was significantly lower in the 5% oxygen ($Q_m = 8.8 \pm 0.8 \times 10^{-18}$ mol/cell/s) condition than in 20% oxygen ($Q_m = 13.1 \pm 0.5 \times 10^{-18}$ mol/cell/s) by day 24 (Figure 3-4b). Although decreasing the external oxygen tension to 5% had no effect on sGAG accumulation in the already low oxygen core, sGAG accumulation in the annulus of the construct maintained at an external oxygen tension of 5% was approximately twice that measured in the annulus of the construct maintained in normoxic conditions (Figure 3-5, a & b).

Collagen deposition also increased in the annulus of the constructs maintained at 5% oxygen, but decreased in the core (Figure 3-5, c & d). The average predicted oxygen level in the annulus was 9.0% for constructs maintained in normoxia and 2.3% for those maintained in hypoxia, while the average predicted oxygen level in the core was 6.4% for constructs in normoxia and 1.7% for those in hypoxia. This suggests that while environments of less than 2% oxygen tension can continue to support sGAG synthesis, collagen synthesis can be negatively impacted. Positive Alcian Blue, Picrosirius Red and type II collagen staining was observed in all constructs (Figure 3-5e), with the latter staining maximally in the normoxia construct core.

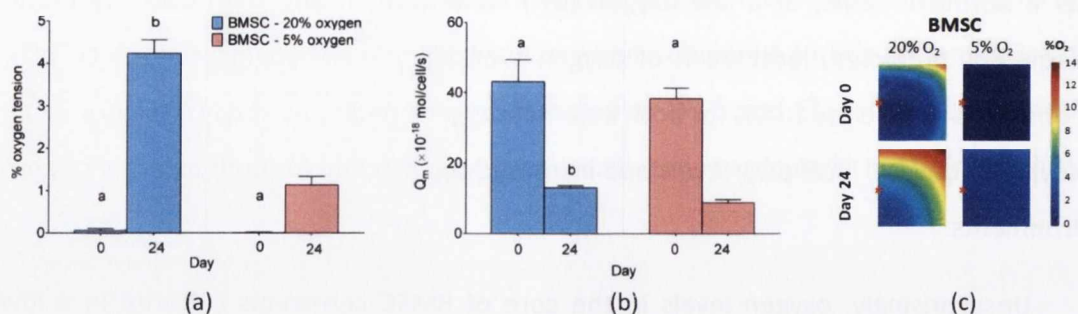


Figure 3-4: Cartilage tissue grafts engineered using bone marrow MSCs were cultured in either a normoxic (20%) or low (5%) external oxygen environment for 24 days. Oxygen concentration measured at centre of the constructs (a). Cellular oxygen consumption rate extrapolated from FE model (b). FE contour plots showing predicted oxygen concentration throughout the constructs (c). (a, b denote statistical difference vs. same culture condition group at day 24 and 5% oxygen environment group at the same time point respectively; $P < 0.05$; $n = 3$. * indicates the location of oxygen measurement within the constructs).

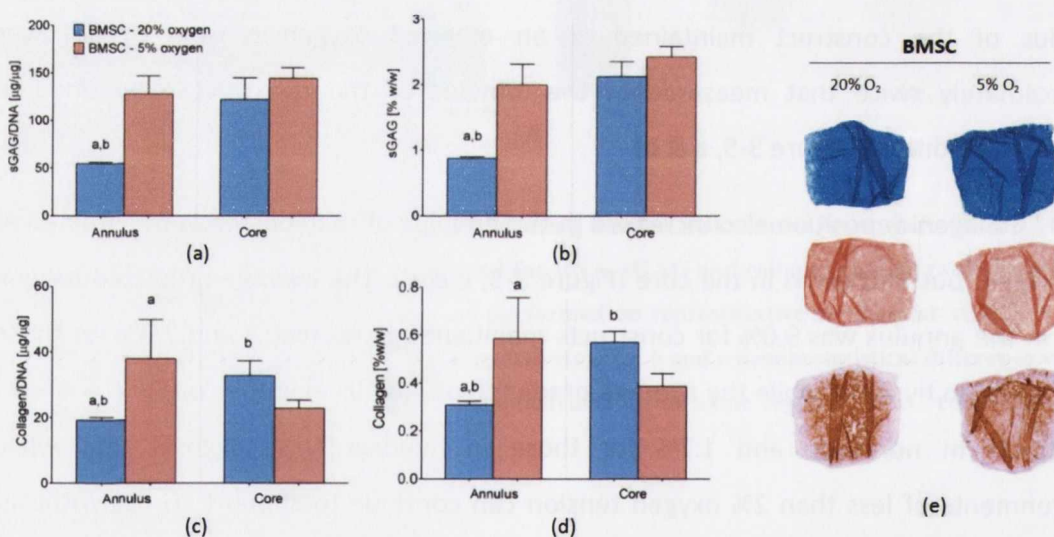


Figure 3-5: Cartilage tissue grafts engineered using various cell sources were cultured for 24 days, separated into cores and annuli, then analysed for DNA, sGAG and collagen content (a-d). Histological and immuno-histochemical staining (e) was performed on representative sections for sGAG, collagen and collagen type II (top, middle, bottom respectively). (a, b and c denote statistical difference vs. core of construct using the same cell type, FPSC constructs in the same region and CC constructs in the same region respectively; $P < 0.05$; $n = 3$).

3.4.3 *Increasing the cell seeding density lowers oxygen levels in tissue engineered cartilage and increases extracellular matrix synthesis.*

To investigate the influence of cell seeding density (SD) on engineered cartilage tissue development, constructs were seeded at either 20 or 40 million cells/mL and cultured in chondrogenic medium for 24 days. On day 0, pO_{2c} was measured to be approximately 0% in both groups (Figure 3-6a). This value increased for both conditions over the study period despite significant cell proliferation (Figure 3-9c), but was significantly greater in the lower SD group by day 24. A higher cell SD was predicted to lead to the development of a much larger hypoxic core region on both day 0 and 24 (Figure 3-6c), although the size of this region was not as large as that for the lower cell SD when maintained at 5% external oxygen tension (compare Figure 3-4c and Figure 3-6c). Extrapolated values for the average cellular oxygen consumption rate at day 24 were similar for both conditions (Figure 3-6b).

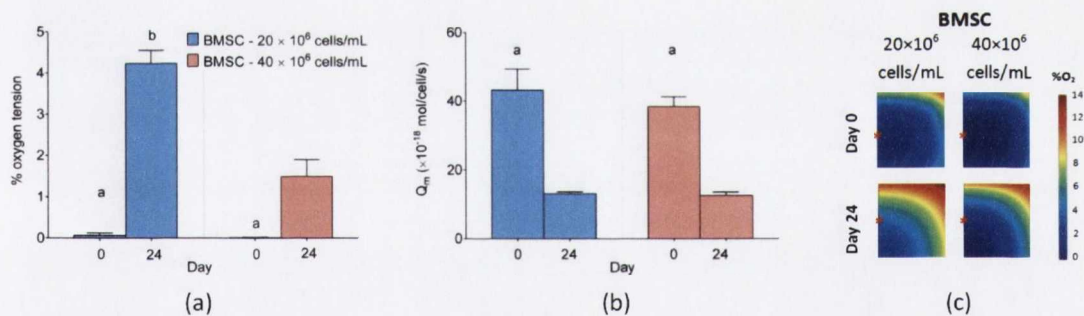


Figure 3-6: Cartilage tissue grafts engineered using bone marrow stem cells were seeded initially at a density of either 20 or 40 million cells/mL and cultured for 24 days. Oxygen concentration measured at the centre of constructs (a). Cellular oxygen consumption rate extrapolated from FE model (b). FE contour plots showing predicted oxygen concentration throughout the constructs (c). (a, b denote statistical difference vs. same initial seeding density group at day 24 and 40 million cell group at the same time point; $P < 0.05$; $n = 3$. * indicates the location of oxygen measurement within the constructs).

Spatial biochemical analysis revealed that doubling the initial SD resulted in a large increase in sGAG synthesis (on a per cell basis) and accumulation in the construct annulus, as measured by increases in sGAG/DNA ($\times 3.5$ times, Figure 3-7a) and sGAG as a % of wet weight ($\times 6.7$ times, Figure 3-7b). Increasing the SD also lead to higher sGAG accumulation in the core region, but not when sGAG accumulation was normalized to DNA content. Collagen synthesis was also enhanced in the annulus with increasing SD, but was reduced in the core region (Figure 3-7, c & d). More intense staining for sGAG was observed throughout the high SD constructs (Figure 3-7e), while collagen staining was located primarily in the annular region. Immuno-histochemical staining for collagen type II clearly showed the effects of varying the SD, with collagen II concentrated in the core of the low SD constructs and around the periphery of the high SD constructs.

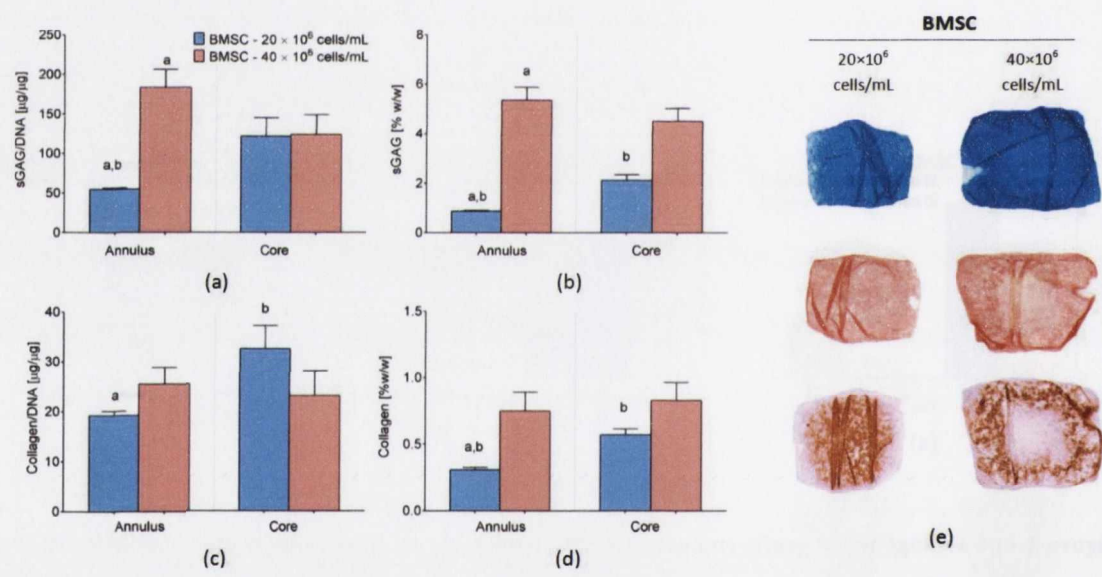


Figure 3-7: Cartilage tissue grafts engineered using bone marrow MSCs were seeded initially at a density of either 20 or 40 million cells/mL for 24 days, then separated into cores and annuli and analysed for DNA, sGAG and collagen content (a-d). Histological and immuno-histochemical staining (e) was performed on representative sections for sGAG, collagen and collagen type II (top, middle, bottom respectively). (a, b denote statistical difference vs. core of construct cultured in a similar environment and same region of construct seeded at 40 million cells/mL respectively; $P < 0.05$; $n = 3$).

3.5 Discussion

It has previously been demonstrated that the external oxygen tension can influence MSC metabolism, differentiation and matrix synthesis (Fehrer et al. 2007, Buckley et al. 2010, Meyer et al. 2010, Pattappa et al. 2013). Successful engineering of 3-dimensional cartilage tissue grafts of a clinically relevant scale will likely involve a thorough understanding and control of oxygen and nutrient gradients throughout the tissue. In the current study, a combined experimental and numerical approach was utilised to characterise the spatial oxygen environment within cartilage tissues engineered using various sources of cells. Oxygen levels measured within tissues seeded with BMSCs and CCs increased significantly over the study duration. However, biochemical analysis and finite element modelling revealed different causes for these increases: MSCs were found to possess a high initial oxygen uptake rate, but to adopt a more chondrocyte-like oxygen consumption profile with the onset of chondrogenic differentiation, whereas increases in oxygen concentration within CC constructs were attributed to cell death. Spatial biochemical analysis of revealed a correlation between low oxygen regions and enhanced levels of cartilage specific extra cellular matrix deposition.

Direct measurements of oxygen levels within the constructs provided an insight into the local cellular environment during chondrogenesis in 3D hydrogels. BMSCs and FPSCs were found to have proliferated significantly over the study duration, while CCs population reduced. Using this data, it was estimated the average oxygen consumption rate of BMSCs to be 43.2×10^{-18} mol/cell/s prior to chondrogenic differentiation, which is comparable to those previously reported for BMSCs expanded in normoxia (33×10^{-18} mol/cell/s, Pattappa et al. 2013). FPSCs were estimated to use oxygen at a rate of 28.1×10^{-18} mol/cell/s at day 0. CC oxygen consumption did not change significantly throughout the study, with an average rate of 16.5×10^{-18} mol/cell/s, which is reasonably consistent with previously reported values (19.4×10^{-18} mol/cell/s, Heywood and Lee 2010). However, by day 24, both BMSCs and FPSCs had adopted a similar oxygen uptake (13.1 and 19.1×10^{-18} mol/cell/s, respectively) to that of CCs. MSCs are known to possess the ability to adapt their oxygen consumption to changes in the oxygen environment (Pattappa et al. 2013). The apparent switch to a more glycolytic metabolism reported in the current study has also been observed previously in a MSC pellet culture study

(Pattappa et al. 2011), whereas increases in oxidative metabolism has been associated with a loss of chondrocyte phenotype (Heywood and Lee 2008).

In the current study, it was demonstrated that BMSCs possess a higher initial oxygen uptake rate than FPSCs. A possible explanation for this is that these cells occupy differentially oxygenated niches *in vivo* and have adapted metabolisms to reflect this. Also, the isolated cell populations will be heterogeneous with significant cell-cell variability (Wagner et al. 2006, Russell et al. 2010). Despite this, both groups were successfully induced to adopt a more chondrogenic oxygen consumption rate and synthesize cartilage specific extracellular matrix. Consistent with previously reported findings (Meyer et al. 2010, Buckley et al. 2011), matrix deposition was generally maximal in regions of low oxygen tension. Even when cultured in normoxia, MSC seeded constructs developed a hypoxic core region (Figure 3-2c), where matrix deposition was greatest. Culturing in a low oxygen environment effectively expanded this hypoxic region to include the entirety of the construct (Figure 3-4c), thereby enhancing matrix synthesis within the annular region also. However, this also had the effect of further reducing the oxygen tension in the core from an average concentration (by volume) of 6.4% to 1.7% oxygen, which, while having no effect on sGAG content, appeared to inhibit collagen synthesis. This suggests that, while both sGAG and collagen synthesis is enhanced by culture in hypoxia, very low oxygen environments can be detrimental to collagen production. However, since MSCs have been observed to increase their glucose uptake when maintained in hypoxia (Pattappa et al. 2013, Naqvi and Buckley 2015), the lower oxygen environment predicted throughout the entirety of the constructs cultured in hypoxia will also likely lead to a depletion of glucose in the core region, thus potentially inhibiting collagen synthesis. Indeed, it has been reported that, while collagen deposition of MSCs remains unaffected in either low oxygen or low glucose environments, hypoxia combined with deprivation of glucose leads to significantly reduce collagen synthesis, whereas sGAG synthesis is enhanced under these conditions, when compared to normoxic controls (Naqvi and Buckley 2015).

Increasing the cell seeding density also had the effect of expanding the hypoxia core region (Figure 3-6c) and again enhancing matrix accumulation in the annulus, at the cost of reduced collagen synthesis (on a per cell basis) in the core. As discussed previously, this may be due to low oxygen conditions or glucose depletion in gels seeded

at high densities. However, as native cartilage consists of a zonal architecture, with gradients in biochemical content and functional properties throughout the tissue (Chen et al. 2001, Julkunen et al. 2009, Gannon et al. 2012), introducing nutrient gradients, and even selective near deficiencies, may be ultimately desirable when attempting to recapitulate native-like tissue architecture.

These findings point to the importance of not only environmental oxygen tension, but also construct scale and geometry in cartilage tissue engineering. Previous studies from our group have employed numerical modelling techniques to investigate the influence of construct scale on tissue development (Buckley et al. 2011). These models assumed constant medium oxygen saturation equal to that of the culture environment atmosphere. However, the current study demonstrates the importance of including the surrounding media in the model, as increases in medium height above the construct will result in a longer oxygen diffusion path, and hence, lower oxygen availability at the construct periphery. The current study also highlights the value of experimental observations of oxygen levels, as this serves to increase the validity of the model. However, while cell density was estimated separately for each core and annulus region, cell density gradients, which are not fully accounted for, will be present within these regions. Also, the K_m value used for all cell types was a value reported for bovine articular chondrocytes and was assumed not to vary throughout the study. Furthermore, oxygen tension was measured only at the centre of each construct, whereas multiple measurement locations would increase the model validity (Thorpe et al. 2013). Finally, when investigating cellular metabolism, no model that excludes glucose transport and cellular uptake can present a full picture. Therefore, it is proposed that future studies should involve accurately measuring cell density gradients, quantifying temporal changes in K_m for each culture condition, monitoring both oxygen and glucose concentrations at multiple locations throughout the tissues and development of dual-species oxygen/glucose diffusion-reaction numerical models.

3.6 Conclusion

Understanding how the local cellular environment influences cell metabolism, phenotype and matrix synthesis will be crucial to engineering functional cartilage tissue grafts of a clinically relevant scale. Oxygen and nutrient gradients and consumption rates within tissue grafts can be influenced by varying such factors as culture environment oxygen tension, medium depth above constructs, cell seeding density and construct scale. However, these gradients will be often in a state of flux, varying both spatially and temporally; a feedback loop where the local environment will influence cell metabolism, which in turn will influence the cells environment. These studies present a combined experimental and numerical approach to characterising these gradients and cellular responses and, potentially provides a framework which in the future may allow tissue engineers to control these gradients to engineer native-like articular cartilage. It was demonstrated that MSCs undergoing chondrogenesis adapt their oxygen consumption profile to that similar to chondrocytes. Therefore, determining if MSCs used to engineer cartilage grafts adopt a metabolic profile similar to chondrocytes might be considered a key determinant of the success of stem cell based cartilage tissue engineering strategies.

Having examined and characterized the local oxygen gradients within cartilaginous tissue grafts, and the influence of this environment on MSC phenotype, it was decided to next examine the influence of other joint-related stimuli. Therefore, several bioreactor systems were developed and utilized to investigate how the various modes of mechanical loading that occur within the articular cartilage environment (as discussed in Chapter 2) can direct MSC fate.

3.7 Supplementary figures

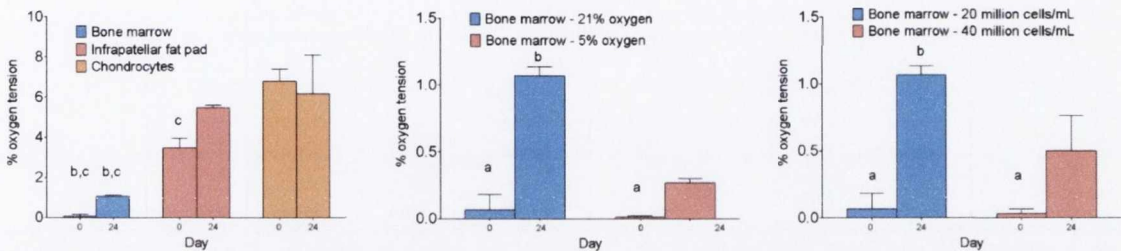


Figure 3-8: A replicate study was performed using cells from a separate set of donors and oxygen tension in the centre of each construct was measured. Data is arranged (a) by cell source, (b) by external oxygen environment and (c) by initial cell seeding density.

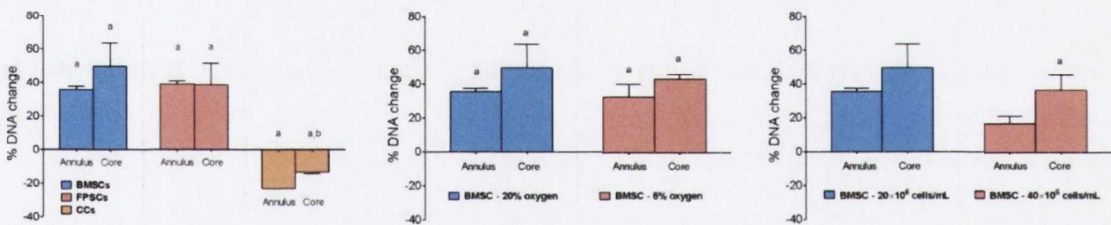


Figure 3-9: The percentage change in cell density in sample cores and annuli after 24 days of culture. Cell density at day 24 was estimated by normalizing day 24 DNA values to the average day 0 values, which were assumed to be representative of the initial cell seeding density, for each group. Data is arranged (a) by cell source, (b) by external oxygen environment and (c) by initial cell seeding density (a, b denote a statistically significant different DNA content vs. day 0 samples, and vs the DNA content in the core region of the same samples respectively, $P < 0.05$; $n = 3$).

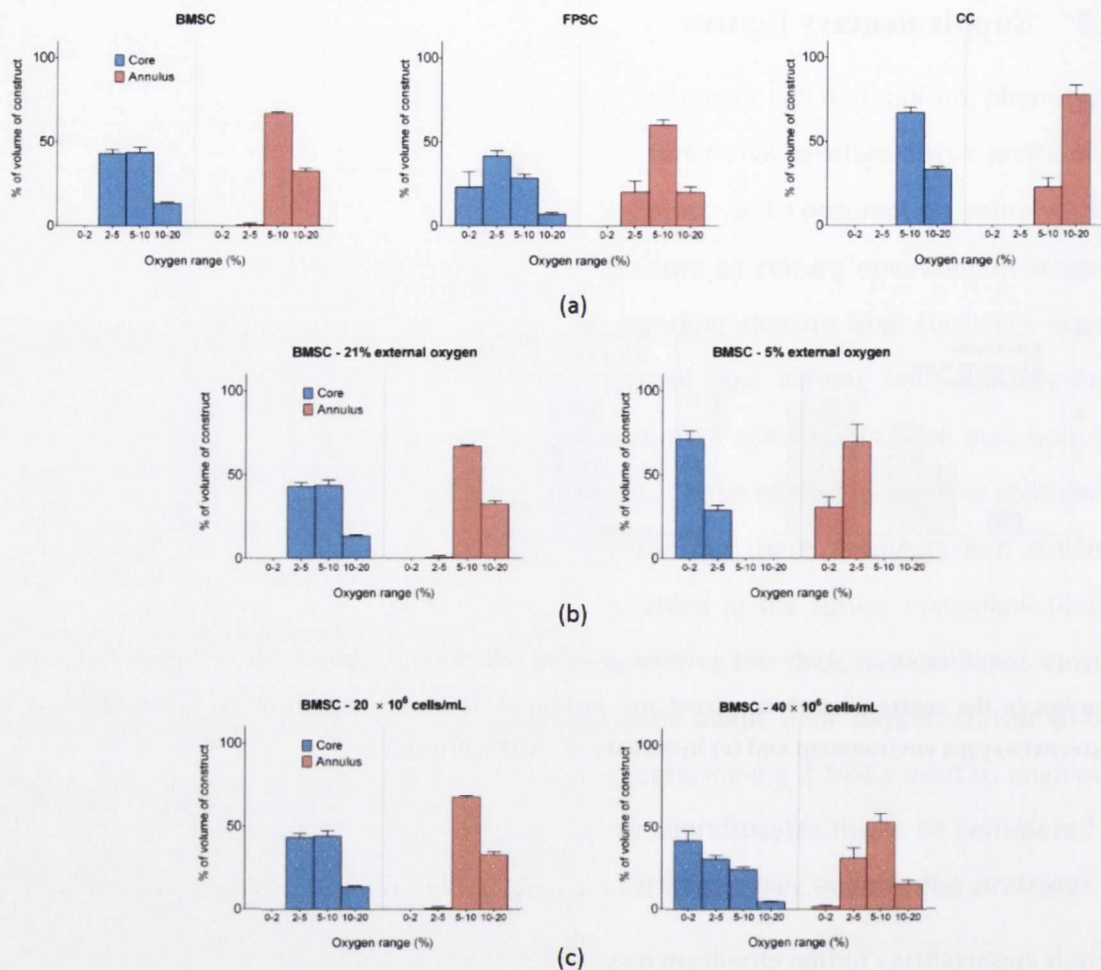


Figure 3-10: FEA models were analysed to determine the percentage volume of each sample that lay within the specified oxygen ranges. Data is arranged (a) by cell source, (b) by external oxygen environment and (c) by initial cell seeding density (n = 3).

Chapter 4 Cyclic hydrostatic pressure promotes a stable cartilage phenotype and enhances the functional development of cartilaginous grafts engineered using MSCs of different tissue origins

For the purposes of this thesis, the study presented is adapted from the original article published in Journal of Biomechanics, June 2014, Vol. 47, No. 9 pp. 2115-2121 (Carroll et al. 2014).

4.1 Abstract

The objective of this study was to investigate how joint specific biomechanical loading influences the functional development and phenotypic stability of cartilage grafts engineered *in vitro* using stem/progenitor cells isolated from different source tissues. Porcine bone marrow derived multipotent stromal cells (BMSCs) and infrapatellar fat pad derived multipotent stromal cells (FPSCs) were seeded in agarose hydrogels and cultured in chondrogenic medium, while simultaneously subjected to 10MPa of cyclic hydrostatic pressure (HP). To mimic the endochondral phenotype observed *in vivo* with cartilaginous tissues engineered using BMSCs, the culture media was additionally supplemented with hypertrophic factors, while the loss of phenotype observed *in vivo* with FPSCs was induced by withdrawing transforming growth factor (TGF)- β 3 from the media. The application of HP was found to enhance the functional development of cartilaginous tissues engineered using both BMSCs and FPSCs. In addition, HP was found to suppress calcification of tissues engineered using BMSCs cultured in chondrogenic conditions and acted to maintain a chondrogenic phenotype in cartilaginous grafts engineered using FPSCs. The results of this study point to the importance of *in vivo* specific mechanical cues for determining the terminal phenotype of chondrogenically primed multipotent stromal cells. Furthermore, demonstrating that stem or progenitor cells will appropriately differentiate in response to such biophysical cues might also be considered as an additional functional assay for evaluating their therapeutic potential.

4.2 Introduction

Articular cartilage possesses a limited capacity for self-repair. Current clinical therapies for cartilage defect repair such as autologous chondrocyte implantation suffer from significant drawbacks, such as donor site morbidity and dedifferentiation of chondrocytes during monolayer expansion. MSCs possess the ability to proliferate extensively *ex vivo* whilst maintaining their multipotent differentiation capabilities (Bruder et al. 1997, Kadiyala et al. 1997) and can be induced to differentiate towards a chondrogenic lineage (Caplan 1991, Johnstone et al. 1998, Mackay et al. 1998, Pittenger et al. 1999, Sheehy et al. 2012, Vinardell et al. 2012), making them an attractive alternative cell type for cartilage repair strategies. Multipotent cells can be isolated from numerous different tissues. There are, however, significant differences in their properties depending on cell source, with joint tissue derived stem cells having a gene expression profile closer to that of chondrocytes when compared to extra-articular tissue derived stem cells (Segawa et al. 2009). There is also evidence to suggest that joint tissue derived multipotent stromal cells, such as those isolated from the infrapatellar fat pad (IFP) or synovium, may possess a superior chondrogenic potential to those derived from alternative sources (Sakaguchi et al. 2005, Vinardell et al. 2012). In spite of this, the *in vivo* phenotypic stability of cartilaginous tissues engineered using such cells, irrespective of their origin, is by no means assured (De Bari et al. 2004, Pelttari et al. 2006, Hennig et al. 2007, Dickhut et al. 2008). Previous studies have shown that following *in vivo* subcutaneous implantation, IFP derived multipotent stromal cells (FPSCs) tend to undergo fibrous dedifferentiation or resorption, as evidenced by increased collagen type I and reduced sulphated glycosaminoglycan (sGAG) content, whereas bone marrow derived multipotent stromal cells (BMSCs) tend to follow an endochondral pathway, with increased mineralization and vascularization of the engineered tissue (Pelttari et al. 2006, Vinardell et al. 2012). This highlights the potential challenges associated with using any such cell type for successfully regenerating articular cartilage.

Within the orthotopic environment of a full thickness cartilage defect, it has been demonstrated that hypertrophy of implanted multipotent stromal cells (MSCs) is restricted to a region of the regenerating tissue close to the bone, demonstrating that *in vivo* signalling molecules and biomechanical stimuli play a key role in regulating the chondrogenic phenotypic (Steck et al. 2009). Identifying how such *in vivo* specific

environmental cues regulate the terminal phenotype of MSCs may play a key role in realising their full therapeutic potential. Mechanical signals, such as hydrostatic pressure (HP), are a key component of the *in vivo* joint environment and have been shown to play a significant role in regulating the chondrogenic differentiation of MSCs. Previous studies have shown varying and occasionally conflicting results (Parkkinen et al. 1993, Carver and Heath 1999, Suh et al. 1999, Carver and Heath 2000, Jortikka et al. 2000, Smith et al. 2000, Smith et al. 2005, Hu and Athanasiou 2006, Finger et al. 2007, Ogawa et al. 2009, Meyer et al. 2011, Liu et al. 2012, Maxson and Burg 2012, Puetzer et al. 2012, Safshekan et al. 2012, Steward et al. 2012, Vinardell et al. 2012, Steward et al. 2013), but it would appear that the application of physiological levels (3 - 10 MPa) of intermittent HP can enhance proteoglycan and collagen synthesis and upregulate aggrecan and collagen II mRNA expression in chondrocytes and stem/progenitor cells isolated from bone marrow, synovial tissues and adipose tissues. There is also evidence to suggest that dynamic HP can act to stabilise the phenotype of chondrogenically primed joint tissue derived MSCs (Vinardell et al. 2012). A better understanding of how mechanical loading influences terminal differentiation of different tissue-specific MSCs should facilitate efforts to tissue engineer phenotypically stable cartilaginous grafts using such cells.

While an extensive body of work exists comparing the biochemically driven differentiation of MSCs isolated from different tissues, little is known about how these unique cell populations will respond to biophysical stimuli. The objectives of this study were to compare how chondrogenically primed BMSCs and FPSCs respond to the application of physiological levels of HP, and to investigate how such joint-specific biomechanical loading influences the stability and functional development of cartilage grafts engineered using cells from both sources. It was hypothesized that the application of cyclic HP would (i) enhance the functional development of cartilage tissues engineered using both BMSCs and FPSCs, (ii) reduce hypertrophic differentiation and endochondral ossification of cartilaginous grafts engineered using BMSCs and (iii) stabilize and maintain the chondrogenic phenotype in cartilaginous grafts engineered using FPSCs. To mimic the endochondral phenotype observed in tissues engineered using BMSCs, the culture media was supplemented with hypertrophic factors (Sheehy et al. 2012) and the constructs were simultaneously subjected to 10 MPa of cyclic HP. To mimic the fibrous dedifferentiation observed in tissues engineered using FPSCs, the chondrogenic growth factor transforming growth factor (TGF)- β 3 was withdrawn from the culture medium (Vinardell et al. 2012)

and again constructs were subjected to 10 MPa of cyclic HP. In this way, it was possible to mimic how physiological loading can impact the phenotypic stability of tissue engineered cartilaginous grafts using a well-controlled *in vitro* model.

4.3 Methods

4.3.1 Agarose gel constructs fabrication and culture

BMSC and FPSCs were isolated and expanded as described in the supplementary material section. Both cell types were mixed with 4% agarose (Type VII; Sigma-Aldrich, Ireland) at a ratio of 1:1 at ~40°C to yield a final gel concentration of 2% at a density of 20 million cells/mL. Each group consisted of cells from a single donor (i.e. donors were not pooled). The agarose-cell constructs (Ø5×3 mm) were encapsulated within an acellular agarose block (as described below) and maintained in a chemically defined chondrogenic medium consisting of high glucose DMEM supplemented with 100 U/mL penicillin/streptomycin (both from Gibco), 100 mg/mL sodium pyruvate, 40 mg/mL L-proline, 50 mg/mL L-ascorbic acid-2-phosphate, 1.5 mg/mL bovine serum albumin, 1 × insulin–transferrin–selenium, 100 nM dexamethasone (all Sigma-Aldrich) and 10 ng/mL of transforming growth factor-β3 (TGF-β3, ProSpec-Tany TechnoGene, Ltd.). On day 21, selected BMSC groups were exposed to hypertrophic medium for 14 days, by changing the following parameters: TGF-β3 was withdrawn from the medium, which was then supplemented with 1 nM L-thyroxine (Sigma-Aldrich) and 20 µg ml⁻¹ β-glycerophosphate (Sigma-Aldrich), and the level of dexamethasone was reduced to 1 nM. Also, on day 21, additional FPSC groups were maintained in chondrogenic medium (as described above) without TGF-β3 for 14 days. Constructs were allowed to equilibrate overnight before the initiation of hydrostatic pressure.

4.3.2 Application of hydrostatic pressure

Cell seeded agarose constructs were encapsulated, evenly spaced, in an agarose block to improve homogeneity of oxygen and nutrient availability and to provide protection from

handling damage when the constructs were being placed into and out of the HP bioreactor. Constructs were then inserted into heat-sealed, gas-permeable, water-tight, sterile bags (EVO120, Quest Biomedical, UK) with 4 mL of medium per construct and the air was removed via a needle free port. Cyclic HP was applied via a water filled, custom-made bioreactor within a 37°C incubator as described previously (Meyer et al. 2011). The sealed bags exposed to HP were placed into the pressure vessel, while the free swelling controls were placed into an open water bath next to the pressure vessel. HP was applied at an amplitude of 10 MPa and a frequency of 1 Hz for a duration of 4 hours per day, 5 days per week for 5 weeks. The bags were returned to a culture incubator (37°C, 5% CO₂) between loading periods and suspended separately in an upright position for homogenous gas transfer. Media changes were performed twice weekly via the needle free port.

4.3.3 Mechanical, histological and biochemical assessment of tissues

The mechanical properties of the engineered constructs were determined as described previously (Buckley and Kelly 2012). Stress relaxation tests were performed to determine the equilibrium modulus of the tissue, which consisted of a ramp and hold cycle, with a ramp displacement of 1 µm/s until 10% strain was obtained and then maintained until equilibrium was reached. Dynamic tests, which involved applying a cyclic strain of 1% at a frequency of 1 Hz, were performed immediately after the stress relaxation cycle to determine the dynamic modulus of the tissue. Constructs were also analysed biochemically to determine their DNA, sulphated glycosaminoglycan (sGAG), collagen and calcium content. Sections were stained with Alcian Blue for sGAG, Picro-Sirius Red for collagen and Alizarin Red for mineral deposition, while the localized deposition of collagen types I & II was identified through immunohistochemistry. See supplementary material section for further details of biochemical and histological assays/methodologies.

4.3.4 Experimental design

To test the hypotheses, three studies were performed. In study 1, BMSC and FPSC seeded constructs were cultured in chondrogenic medium for 5 weeks. In study 2, BMSC

constructs were cultured in chondrogenic medium for 3 weeks, followed by 2 weeks culture in hypertrophic medium. In study 3, FPSC constructs were cultured in chondrogenic medium containing TGF- β 3 for 3 weeks, followed by 2 weeks culture in chondrogenic medium in the presence or absence of TGF- β 3. All study groups consisted of a hydrostatically loaded group (HP) and a free-swelling unloaded control (FS).

4.3.5 Statistical Analysis

All statistical analyses were carried out using Minitab 15.1. Results are reported as mean $\pm$ standard deviation. Groups were analysed using a general linear model for analysis of variance. Tukey's test was used to compare conditions. Significance was accepted at a level of $P < 0.05$.

4.4 Results

4.4.1 *Dynamic hydrostatic pressure enhances the functional development of cartilage tissue engineered using both BMSCs and FPSCs.*

BMSC and FPSC seeded constructs were cultured for 5 weeks in chondrogenic medium in the presence or absence of HP. The application of HP had no effect on the DNA content of either BMSC or FPSC seeded constructs (data not shown). HP significantly enhanced sGAG accumulation in both BMSC ($P<0.0001$) and FPSC ($P<0.0006$) seeded hydrogels (Figure 4-1, a-b), but only increased collagen accumulation in BMSC seeded constructs ($P<0.0001$, Figure 4-1, c-d). Histological staining (Figure 4-1e) revealed a high degree of spatial symmetry in ECM deposition for all groups. Alcian Blue staining revealed greater sGAG accumulation in the pericellular region of BMSC constructs (Figure 4-1e).

sGAG accumulation was more diffuse in FPSC seeded constructs and, at the tissue level, more localised to the peripheral region of the engineered tissue. Interestingly, in FPSC seeded constructs only, a narrow, well-defined boundary of collagen deposition was observed primarily between the outer annulus (where sGAG synthesis was maximised) and the central region of low ECM deposition. This is perhaps indicative of a region of optimized nutrient availability for collagen synthesis and warrants further investigation. For both cell types, application of HP led to more intense Alcian Blue staining, with more intense Picrosirius Red staining observed in BMSC constructs. While HP had little influence on collagen type I deposition, collagen type II accumulation was enhanced throughout BMSC constructs when exposed to HP. Although FPSC constructs followed a similar trend, staining for collagen II was less intense than BMSC seeded constructs. Increases in cartilage specific ECM accumulation were accompanied by improvements in the functional properties of both BMSC and FPSC derived grafts, as evidenced by significantly increased equilibrium and dynamic moduli following application of hydrostatic pressure (Figure 4-2).

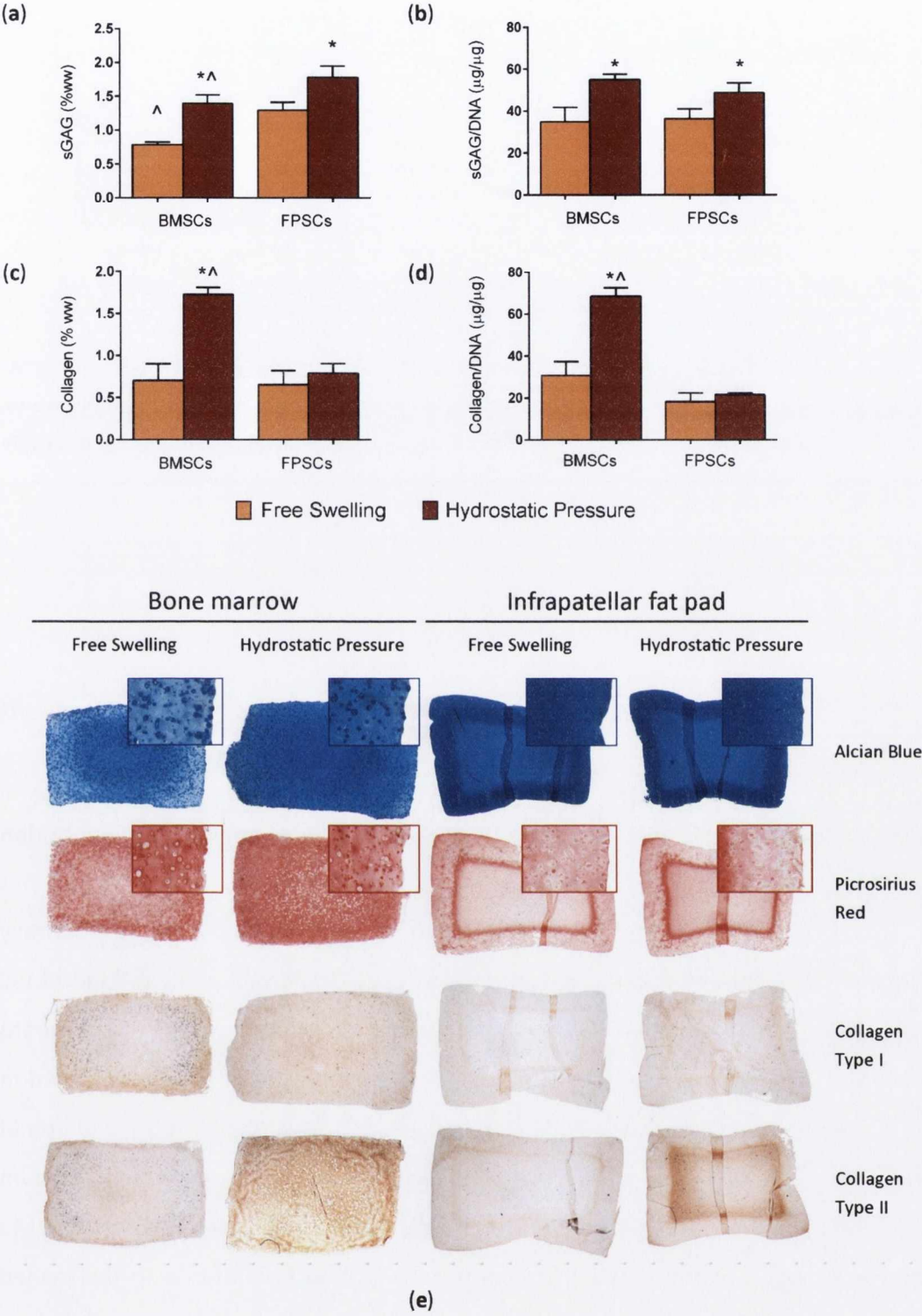


Figure 4-1: Dynamic hydrostatic pressure enhances the accumulation of cartilage specific ECM in both BMSC and FPSC constructs when cultured in chondrogenic medium for 5 weeks, as shown by biochemical analyses (a-d) and histological/immune-histological staining (e.) *, ^ denote statistical significance vs. free swelling group of similar cell type and vs. FPSCs under similar loading conditions respectively (P<0.002; n = 4).

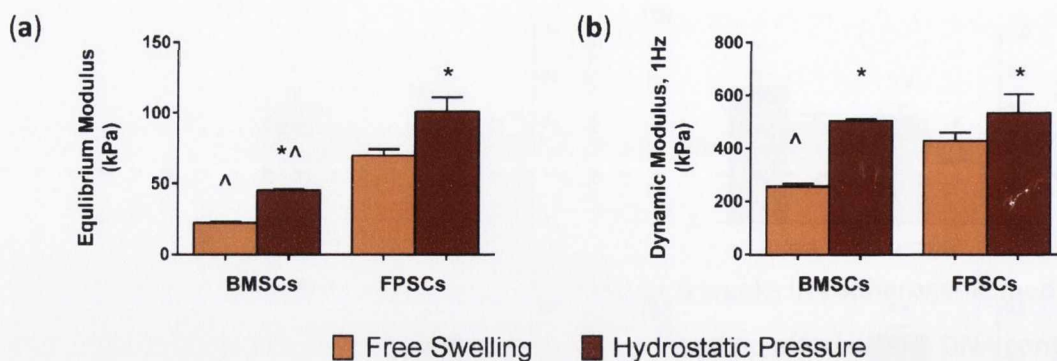


Figure 4-2: Dynamic hydrostatic pressure enhances the functional properties of both BMSC and FPSC constructs when cultured in chondrogenic medium for 5 weeks. *, ^ denote statistical significance vs. free swelling group of similar cell type and vs. FPSCs under similar loading conditions respectively ($P < 0.002$; $n = 4$).

4.4.2 *Dynamic hydrostatic pressure suppresses calcification of cartilage grafts engineered using BMSCs*

The application of HP resulted in significantly ($P < 0.002$) reduced calcium deposition within BMSC seeded constructs maintained in chondrogenic medium (Figure 4-3a). This result was confirmed by Alizarin Red staining (Figure 4-3b), which showed dramatically reduced staining in the constructs exposed to HP. Next, chondrogenically primed BMSC constructs were cultured in the presence of hypertrophic factors in order to accelerate the onset and progression of endochondral ossification. No difference was observed in calcium content between the loaded and FS constructs (Figure 4-4a), with the addition of hypertrophic factors leading to a decrease in cell viability and a large increase in calcium deposition on a per cell basis. Alizarin Red staining (Figure 4-4b), which was restricted to the peripheral region of these constructs, appeared slightly less intense in the loaded constructs.

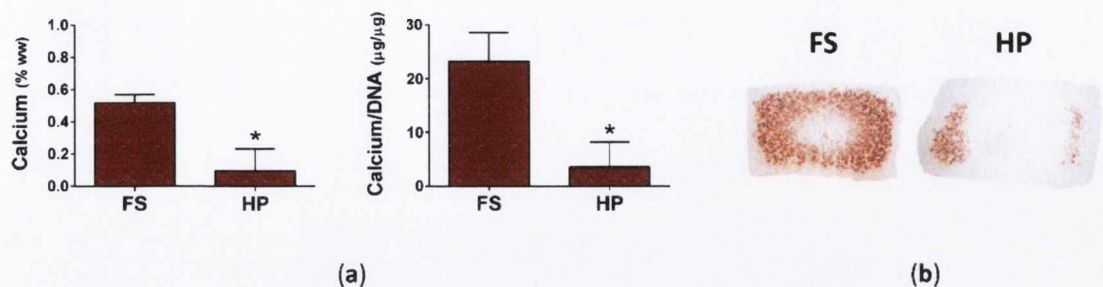


Figure 4-3: Dynamic hydrostatic pressure suppresses calcification of BMSC constructs when cultured in chondrogenic medium for 5 weeks, as shown by biochemical analysis of calcium content of BMSC constructs (a) and Alizarin Red staining of BMSC constructs for calcium deposition (b). FS and HP indicate free swelling and loaded respectively. * denotes statistical significance vs. free swelling group of similar cell type ($P < 0.002$; $n = 4$).

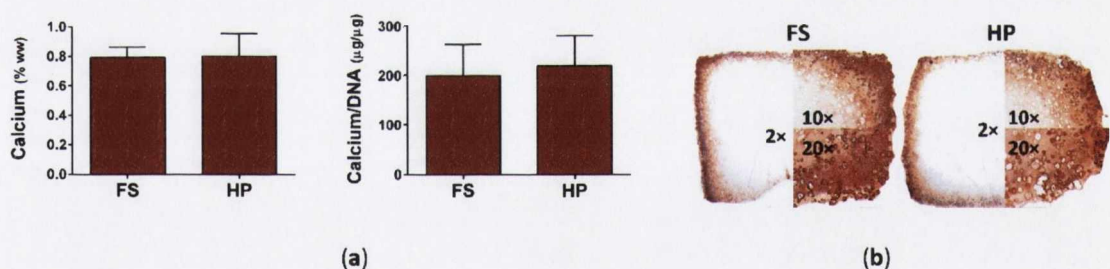


Figure 4-4: Dynamic hydrostatic pressure has no significant effect on calcification of BMSC constructs when cultured in chondrogenic medium for 3 weeks, followed by 2 weeks culture in hypertrophic medium, as shown by biochemical analysis ($n = 4$) of calcium content of BMSC constructs (a) and Alizarin Red staining of BMSC constructs for calcium deposition (b). FS and HP indicate free swelling and loaded respectively. Original image magnification is shown inset.

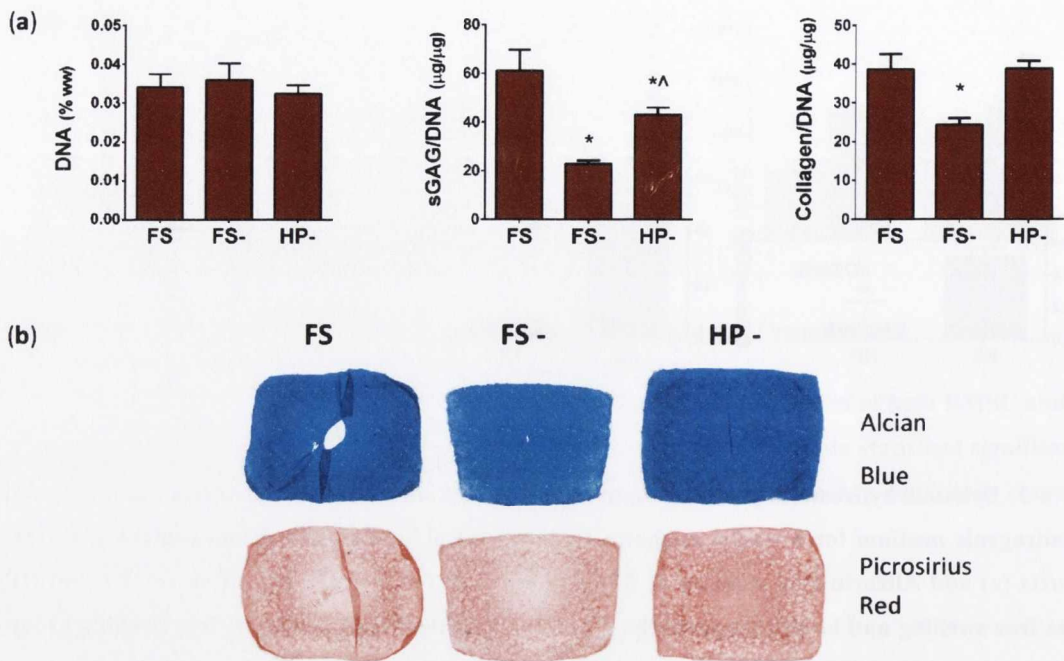


Figure 4-5: Dynamic hydrostatic pressure acts to maintain a chondrogenic phenotype in FPSC constructs when cultured for 3 weeks in chondrogenic medium containing TGF- β 3, followed by 2 weeks in the presence or absence of TGF- β 3, as shown by biochemical analyses (a) and histological staining (b) of constructs. FS, FS- and HP- indicate free swelling with continuous TGF- β 3 supplementation, free swelling without TGF- β 3 and loaded without TGF- β 3 respectively. * and ^ denote statistical significance vs. FS and FS- groups respectively ($P < 0.001$; $n = 4$).

4.4.3 *Dynamic hydrostatic pressure acts to maintain a chondrogenic phenotype in cartilaginous grafts engineered using FPSCs*

No evidence of calcium deposition was observed in cartilaginous tissues engineered using FPSCs (data not shown). To mimic the fibrous dedifferentiation observed in cartilaginous grafts engineered using FPSCs following subcutaneous implantation (Vinardell et al. 2012), constructs were cultured for 3 weeks in chondrogenic medium containing TGF- β 3, followed by 2 weeks where TGF- β 3 was withdrawn from the culture media of half of the groups, in the presence or absence of HP. The withdrawal of TGF- β 3 from FPSC seeded constructs after 3 weeks (FS-) had no effect on the DNA content of the engineered tissues (Figure 4-5a), when compared to continuous TGF- β 3 supplementation (FS). However, TGF- β 3 withdrawal resulted in a significant decrease in sGAG and collagen deposition by week 5. Interestingly, in the absence of TGF- β 3, the application of hydrostatic pressure

(HP-) partially mitigated this reduction, with regards to sGAG synthesis, and fully restored collagen levels to that observed in the non-loaded, TGF- β 3 supplemented constructs. Histological staining (Figure 4-5b) demonstrated greater levels of sGAG and collagen accumulation in the loaded group when compared to the free swelling group, with a more homogenous distribution of extra cellular matrix (ECM) throughout the construct.

4.5 Discussion

Understanding how joint specific environmental factors regulate the phenotype of MSCs is central to developing novel cell based therapies for cartilage regeneration. In this study, a custom HP bioreactor was employed to firstly elucidate how this stimulus can be used to enhance the functional development of cartilage constructs engineered using multipotent cells derived from different tissue sources, and secondly, as a model system to understand how joint specific environmental factors such as HP will regulate phenotypic stability of such cartilage grafts. In agreement with previous studies (Meyer et al. 2011), the application of HP was found to enhance the mechanical functionality of cartilaginous grafts engineered using BMSCs and, furthermore, to suppress the inherent tendency of these tissues to mineralise and progress along the endochondral route. In addition, HP would also appear to play a role in maintaining the chondrogenic phenotype of FPSCs, as indicated by the findings that, upon removal of TGF- β 3, HP also acted to enhance cartilage-specific extracellular matrix development. These results suggest that cartilaginous tissues engineered using either cell source have the ability to terminally differentiate towards a stable chondrogenic phenotype within an environment where the constructs are subjected to high magnitude cyclic HP.

This study adds to the growing body of literature pointing to the beneficial effects of mechanical stimuli, which appear to improve the functionality of cartilage grafts engineered using MSCs. Articular cartilage is a multiphasic tissue, the solid phase of which is composed predominately of a collagen (mainly type II) fibrillar network (around 10–22% by wet weight) enmeshing proteoglycan macromolecules (2–7% by wet weight) (Maroudas et al. 1969, Muir et al. 1970, Bayliss and Ali 1978, Inerot et al. 1978, Maroudas et al. 1980, Zirn et al. 1984, Mow et al. 1992, Rieppo et al. 2009, Gannon et al. 2012). Previous studies have demonstrated that the application of dynamic compression (Huang

et al. 2010, Bian et al. 2012, Thorpe et al. 2013) or hydrostatic pressure (Miyanishi et al. 2006, Wagner et al. 2008, Ogawa et al. 2009, Huang et al. 2010, Meyer et al. 2011, Correia et al. 2012, Liu et al. 2012, Steward et al. 2012, Vinardell et al. 2012, Liu et al. 2013) can enhance the sGAG and collagen content as well as the mechanical functionality of cartilage constructs engineered using MSCs. However, these studies often fail to produce cartilage grafts with mechanical functionality or ECM content approaching that of native cartilage. It appears that the efficacy of pre-implantation mechanical stimulation is dependent on many variables, such as substrate stiffness (Steward et al. 2013, Steward et al. 2014), magnitude and duration of applied loading (Ikenoue et al. 2003, Correia et al. 2012), time of initiation of loading during culture period (Thorpe et al. 2010), cell donor source (Meyer et al. 2011) and, as demonstrated in the current study, the tissue source of MSCs. It remains to be determined how mechanically functional a tissue needs to be to ensure regeneration of hyaline tissue, although computational studies suggest the likelihood of success will improve with increases in pre-implantation functionality (Nagel and Kelly 2013).

The mean equilibrium modulus of FPSC constructs was found to be greater than that of BMSC constructs. This can be attributed, at least in part, to the higher levels of sGAG accumulation (as a percentage of wet weight) within FPSC constructs. The differences in spatial matrix accumulation may also play a role in determining the apparent mechanical properties of the engineered tissues (Kelly and Prendergast 2004, Khoshgoftar et al. 2013). Following the application of HP, there was no difference in the dynamic modulus of BMSC and FPSC constructs. This can be attributed to the large increase in collagen deposition observed in BMSC constructs following the application of HP. While the equilibrium modulus of engineered cartilage is known to be strongly correlated to the sGAG content (Kelly et al. 2007, Vinardell et al. 2011), the dynamic modulus also strongly depends on the functionality of the collagen network, as it plays a key role in enabling the tissue to generate fluid load support (Ateshian 2009).

Within full thickness cartilage defects, it has been demonstrated that hypertrophy of implanted MSCs is restricted to a region of the regenerating tissue close to the bone, suggesting that biomechanical stimuli play a key role in determining the stability of the chondrogenic phenotype (Steck et al. 2009). The results of this study support this hypothesis, with the application of HP found to dramatically reduce the mineralization of

BMSC constructs cultured in a chondro-inductive medium. This effect was not observed, however, when constructs were maintained in medium supplemented with hypertrophic factors. A possible reason for this is that these factors may provide an unphysiological, potent chemical cue that mechanical stimulation cannot override. The *in vivo* mechanical environment is complex, and MSCs will not only experience high levels of HP, but also shape changing deformations as the tissue is subjected to tension and compression during normal joint loading. Indeed, previous studies from our group and others have demonstrated that other such mechanical cues, such as dynamic compression, can also suppress hypertrophy of MSCs (Bian et al. 2012, Thorpe et al. 2013). In addition to mechanical loading, MSCs in the joint defect environment will also experience other phenotype defining stimuli, such as hypoxic conditions, which have also been shown to suppress hypertrophy and endochondral ossification of chondrogenically primed MSCs (Sheehy et al. 2012). Understanding how MSCs integrate the multiple mechanical and biochemical cues they will experience *in vivo* and how these cues direct their phenotype is a central challenge in the field of regenerative medicine.

The mechanism by which HP enhances cartilage-specific matrix synthesis and suppresses hypertrophy of MSCs has not yet been fully elucidated. According to the biphasic theory for cartilage, the solid matrix of the tissue can be considered to be intrinsically incompressible when loaded within the normal physiologic range of pressures and, unlike with dynamic compression, tissue deformation will be minimal under pure hydrostatic pressurization (Bachrach et al. 1998). Because of this, alternative mechanisms of HP mechanotransduction have been explored. There is evidence to suggest that HP affects the cell membrane ion channels (Browning et al. 1999), possibly via changes in the conformations of the transmembrane proteins (Kornblatt and Kornblatt 2002). The resulting changes in intracellular ion concentrations can then, in turn, result in changes in cellular gene expression and protein synthesis (Horowitz and Lau 1988). Several studies have also indicated that HP can trigger the release of intracellular calcium stores (Browning et al. 2004, Mizuno 2005), but further investigation will be necessary to determine whether such factors regulate the onset of hypertrophy in BMSCs. What has been demonstrated is that a stiffer substrate promotes a more endochondral phenotype in BMSCs (Bian et al. 2013). Since the pericellular environment will become stiffer as ECM content increases with time in culture, this may contribute to the development of an endochondral phenotype in BMSC constructs. However, it has also been shown that HP

can override the influence of a stiffening microenvironment on chondrogenesis of MSCs (Steward et al. 2013), possibly by modulating changes to the cytoskeleton. Taken together, these results raise the possibility that HP may act to suppress a substrate stiffness mediated progression of hypertrophy in BMSCs, although further work is clearly required to directly test this hypothesis.

In conclusion, the results of this study demonstrate that the application of physiological levels of HP to cartilage grafts engineered using different types of MSCs not only improves their mechanical functionality, but also leads to the development of a more stable cartilaginous phenotype. A better understanding of stem cell mechanobiology should open up many exciting new possibilities for the field of articular cartilage tissue engineering and regenerative medicine. It may allow for the development of *in vitro* bioreactor stimulation protocols to engineer not only more mechanically functional grafts, but also tissues that are resistant to hypertrophy and endochondral ossification. Perhaps more importantly, a more complete understanding of stem cell mechanobiology may ultimately allow us to manipulate the *in vivo* mechanical environment (for example, by modulating the mechanical properties of scaffolds/engineered tissues or through appropriate post-operative rehabilitation regimes) to ensure such cells terminally differentiate towards a desired phenotype upon implantation into the body.

The findings presented in this chapter add to the growing body of evidence indicating that, as with dynamic compressive loading, the application of HP can act as a pro-chondrogenic stimulus in MSCs. As discussed in Chapter 2, chondrocytes are subjected to a complex local mechanical environment *in vivo*, consisting of multiple modes of loading, such as fluid pressure, compression and tension. Less is known, however, about the influence of dynamic tensile loading in guiding MSC fate in 3D implantable matrices. Therefore, next, a novel bioreactor was developed and employed to investigate the influence of cyclic tensile strains on both the chondrogenic/myogenic switch that has been previously observed in MSCs (Gao et al. 2010, Thorpe et al. 2012) (Chapter 5) and the tendency of bone marrow-derived MSCs, as demonstrated above, to progress along an osteogenic pathway (Chapter 6).

Chapter 5 The influence of cyclic tensile strain on MSC differentiation in 3D hydrogels is dependent on cell morphology and substrate stiffness

5.1 Abstract

The objectives of this study were (i) to develop a custom bioreactor for the application of uniaxial cyclic tensile strain (CTS) to stem cell-seeded 3D hydrogels, (ii) to investigate the influence of CTS on chondrogenically-primed MSC seeded 3D hydrogel grafts, and (iii) the dependency of this response on matrix stiffness and MSC morphology. Porcine bone marrow derived MSCs were encapsulated in collagen-alginate blended hydrogels, which for allowed tuning of both matrix rigidity (soft vs. stiff) and MSC shape (round vs. spread). Both stiffer substrates and the adoption of a round cell morphology were found to promote MSC chondrogenesis and progression along an endochondral pathway. The novel bioreactor was demonstrated to be a system capable of applying accurate tensile strains to soft 3D hydrogels, whilst maintaining an aseptic, controlled environment and cell viability. The application of CTS was found to upregulate the expression of the myogenic markers smooth muscle actin (ACTA2), heavy chain myosin (MYH1) and calponin (CNN1), but downregulate chondrogenic markers collagen type II (COL2A1) and aggrecan (ACAN) in a substrate stiffness dependent manner. Furthermore, mechanical stimulation was found to synergize with a spread cellular morphology to enhance myogenic gene expression. These studies represent the first investigations into the complex interplay between tensile stimuli, matrix rigidity and cellular morphology when guiding MSC fate in 3D engineered grafts. Taken together, these findings highlight the importance of both extrinsic physiological mechanical loads and intrinsic biomaterial cues in determining MSC lineage commitment.

5.2 Introduction

Due to their relative ease of isolation, ability to proliferate extensively *in vitro* and to differentiate along multiple lineage pathways, MSCs are a promising cell type for tissue regeneration strategies. However, unlocking the potential of these cells requires an understanding of how environmental factors regulate their fate. The influence of growth factors and other soluble cues on MSC fate strongly depends on other environmental factors, such as the stiffness of the local substrate (Engler et al. 2006, Huebsch et al. 2010, Bian et al. 2013), topographical cues that direct cell shape (McBeath et al. 2004, Gao et al. 2010, Thorpe et al. 2012) and the application of external mechanical stimuli such as hydrostatic pressure, fluid flow or cyclic compressive and tensile strain (Huang et al. 2004, Campbell et al. 2006, Li et al. 2009, Connelly et al. 2010, Bian et al. 2012, Steward et al. 2012, Thorpe et al. 2012, Carroll et al. 2014, Steward et al. 2014). For example, it has been shown that lineage commitment can be controlled by tuning substrate stiffness, with softer matrices promoting a neurogenic or adipogenic phenotype, matrices of intermediate stiffness being more myogenic and comparatively rigid matrices promoting osteogenesis (Engler et al. 2006, Rowlands et al. 2008, Chatterjee et al. 2010, Huebsch et al. 2010, Park et al. 2011, Bian et al. 2013). The role of substrate stiffness in regulating chondrogenesis is less clear, with some studies suggesting a soft substrate best supports a chondrogenic phenotype (Park et al. 2011), but others pointing to the benefit of intermediate stiffness (Allen et al. 2012). Cell shape is also known to regulate MSC fate, with a more stellate or spread morphology supporting osteogenesis or myogenesis, while a round shape has been shown to promote chondrogenesis or adipogenesis (McBeath et al. 2004, Gao et al. 2010). Cell shape would also appear to play a role in regulating MSC fate in more physiologically relevant 3D environments. After encapsulation in fibrin hydrogels, MSCs have been shown to spread and adopt a more myogenic phenotype once stimulated with transforming growth factor- β 3 (TGF- β 3) (Thorpe et al. 2012). Interestingly, the differentiation pathway of MSCs in such hydrogels ultimately depends on the external mechanical environment, as they adopted a more chondrogenic phenotype if additionally subjected to dynamic compression (Thorpe et al. 2012). Therefore directing MSCs down either the chondrogenic or myogenic pathway requires careful modulation of the biochemical and biophysical environment.

It is well established that cyclic tensile strain (CTS) can direct MSC differentiation. Multiple studies have demonstrated that uniaxial CTS can enhance the expression of markers associated with osteogenesis, fibrogenesis and myogenesis, and suppress chondrogenesis and adipogenesis in MSCs in monolayer culture (Simmons et al. 2003, Hamilton et al. 2004, Park et al. 2004, Friedl et al. 2007, Chen et al. 2008, Qi et al. 2008, Ghazanfari et al. 2009, Jang et al. 2011, Maul et al. 2011, Huang et al. 2012, Li et al. 2015). These studies also show that in 2D culture, uniaxial CTS leads to cell reorganisation and alignment (Wang and Grood 2000) and that tension induced differentiation signals are dependent on MSC orientation (Kurpinski et al. 2006, Heo et al. 2011, Subramony et al. 2013). However, although there is an abundance of data regarding CTS in 2D cultures, fewer studies have examined the influence of CTS on MSCs seeded in more physiologically relevant 3D matrices, and most naturally focus on the influence of CTS on tenogenic and ligamentogenic differentiation. These studies generally show an upregulation in the expression of genes associated with ligamentous or fibro-cartilaginous differentiation, with increased collagen deposition and enhanced construct mechanical properties following the application of CTS (Juncosa-Melvin et al. 2007, Farng et al. 2008, Kuo and Tuan 2008, Connelly et al. 2010, Doroski et al. 2010, Baker et al. 2011, Kreja et al. 2012, Yang et al. 2012). It has also been shown that CTS can induce osteogenic differentiation in MSCs maintained in 3D hydrogels (Sumanasinghe et al. 2006, Haudenschild et al. 2009).

The role that external mechanical stimuli play in MSC lineage commitment, and its dependency on factors such as matrix stiffness and cell shape, has not yet been fully elucidated. The hypothesis of this study is that CTS will suppress chondrogenesis and promote myogenesis of MSCs encapsulated in physiologically relevant 3D hydrogels. It was further hypothesised that the response of MSCs to external mechanical cues will depend on both the shape they adopt in such hydrogels and the stiffness of the surrounding substrate. To test this hypothesis, MSCs will be encapsulated within blended collagen-alginate hydrogels which have previously been used to modulate both the encapsulated cell morphology and its surrounding substrate stiffness (Gillette et al. 2010, da Cunha et al. 2014). Also described is the development of a novel bioreactor system capable of applying uniaxial cyclic tensile strain to such 3D hydrogels in an aseptic, controlled environment.

5.3 Methods

5.3.1 Cell Isolation and expansion

Bone marrow derived MSCs were isolated aseptically from the femoral diaphysis of a porcine donor and expanded, as described previously (Thorpe et al. 2013, Carroll et al. 2014). Briefly, isolated MSCs were plated and cultured in a standard expansion media (EM) formulation consisting of high glucose Dulbecco's Modified Eagle Medium (DMEM, GlutaMAX™) containing 10% foetal bovine serum and 1% penicillin (100 U/ml)-streptomycin (100 mg/ml) (all from GIBCO, Biosciences, Dublin, Ireland) and supplemented with fibroblast growth factor-2 (FGF-2, 5 ng/mL; ProSpec-Tany TechnoGene Ltd, Israel) and amphotericin B (0.25 mg/mL; Sigma-Aldrich, Arklow, Ireland). Following colony formation, the cells were trypsinised, counted and plated at a seeding density of 5×10^3 cells/cm² at each passage. Complete media exchanges were performed twice weekly.

5.3.2 Collagen - alginate hydrogel fabrication and culture

MSCs were trypsinised and re-suspended in DMEM at the required concentration for encapsulation at a seeding density of $10E^6$ cells/mL in collagen–alginate blended hydrogels. Stock solutions were prepared by diluting collagen type I (#354249, Corning Inc.) to an initial concentration of 6 mg/mL and dissolving sodium alginate powder (ProNova™, NovaMatrix) in deionized water for a concentration of 50 mg/mL. All reagents and implements used were chilled on ice before use to prevent premature gelling of the collagen phase. The MSC cell suspension was mixed with collagen stock solution and 10X DMEM was added to maintain cell viability. Next, pH buffer was added slowly until solution turned pink in colour. For studies involving variation of substrate stiffness, calcium sulphate (CaSO₄) was cryo-milled, then sonicated in solution, before being added at a final concentration of 100 µM to minimise warping of the stiffer constructs due to gradients in cross-linking rates. Finally the required volume of alginate was added and the solution was topped up with deionized water where necessary. The final concentration of collagen was 1.33 mg/mL for all studies presented. A final alginate concentration of 0.8% was used for studies requiring variation of cell shape. However, an

alginate final concentration of 0.6% vs. 2.25% was used for studies requiring variation of substrate stiffness.

Cell/hydrogel solutions were thoroughly, but gently mixed, then transferred by syringe to recesses in moulded flexible silicone strips (Figure 5-2a). These strips were then incubated at 37°C for 80 minutes to allow the collagen to cross-link. Where a spread cell morphology was required, this incubation period was increased to 12 hours. Ionic cross-linking of the alginate phase was then initiated by immersion in EM supplemented with CaCl_2 (50 mM for 30 mins for cell morphology studies and 20 mM for 30 mins, followed by 50 mM for 30 mins for substrate stiffness studies to minimise cross-linking rate gradients and ensure complete cross-linking). Constructs were washed and incubated in EM overnight, then loaded into bioreactors containing differentiation medium (DM). DM consisted of high glucose DMEM supplemented with 100 U/mL penicillin/streptomycin (both from GIBCO), 100 mg/mL sodium pyruvate, 40 mg/mL L-proline, 50 mg/mL L-ascorbic acid-2-phosphate, 1.5 mg/mL bovine serum albumin, 1×insulin–transferrin–selenium, 100 nM dexamethasone (all Sigma-Aldrich) and 10 ng/mL of transforming growth factor- β 3 (TGF- β 3, ProSpec-Tany TechnoGene, Ltd.). For studies requiring variation of substrate stiffness, DM was supplemented with 1 mM CaCl_2 to maintain hydrogel stiffness differential for the duration of the study. All differentiation studies were performed at 5% oxygen and 37°C.

5.3.3 *Mechanical stimulation of hydrogel encapsulated MSCs*

A novel bioreactor system (see Appendix A for technical drawings) was developed for the aseptic application of uniaxial cyclic tensile strain to soft 3D hydrogels. The entire system was housed within a cell culture incubator for the duration of each study. Custom rubber strips (Figure 5-2a) were moulded using a commercially available silicone elastomer kit (Sylgard 184, Dow Corning) by mixing the base with the curing agent at a ratio of 20:1, pouring into a PTFE mould, de-gassing by vacuum and cross-linking for 1 hour at 100°C. Each strip had dog-bone shaped recess to allow moulding in situ of hydrogel samples. The strips were soaked and washed in ethanol and water to minimise leaching of residual curing agent, then autoclaved before use.

Polymer hexagonal strain mounts were fabricated and assembled. Each strain assembly was designed to allow fixation of 6 rubber strips, positioned vertically (Figure 5-2 a[ii]). Strain assembly parts were free to move along a square shaft which allowed uniaxial translation whilst constraining rotational movement about the shaft axis. Magnets were embedded at each end of the strain assembly. A cylindrical bioreactor chamber (Figure 5-2 a[iii]) was developed to house the strain assembly and maintain samples under sterile conditions. Luer adapters were fitted to air inlet/outlet ports and syringe pumps were used to supply filtered air recycled from within the incubator at a rate of 3 mL/minute. When used in conjunction with an oxygen tension (pO_2) controllable incubator, the pumps allowed for gentle agitation and re-oxygenation of the culture medium, thus maintaining uniform dissolved oxygen and nutrient levels at the surface of the samples. A flexible silicone membrane allowed the magnetic adapters outside the chamber to connect to and displace the magnets inside the chamber without compromising the sterility of the chamber. These adapters were fixed to motorized linear actuators (NA08A30, Zaber Technologies Inc., microstep size $<0.1\ \mu\text{m}$). The actuators were mounted on a rig (Figure 5-2 a[iv]), into which the bioreactor chambers could be placed. Strong magnets ($>20\ \text{kg}$ pull force) embedded in the base of this rig below each bioreactor held the lower magnets inside the chamber fixed in position, while the magnets attached to the actuators above the bioreactor allowed vertical displacement of the upper magnets within the chamber. Bioreactor components were sterilized using ethylene oxide gas treatment prior to use.

A custom Matlab (version R215b, Mathworks Inc.) script was developed that allowed precise control of applied strain, frequency and loading duration (Figure 5-1). The system was calibrated by mounting hydrogels marked with ink at the extremities and displacing the actuators in 1 mm increments. Image analyses of hydrogels being strained were performed to correlate hydrogel deformation with actuator displacement (Figure 5-2c). Oxygen levels in the media were monitored near the chamber base using non-invasive oxygen sensors (Fibox 3, PreSens GmbH) and cell viability was monitored over the duration of a one week pilot study.

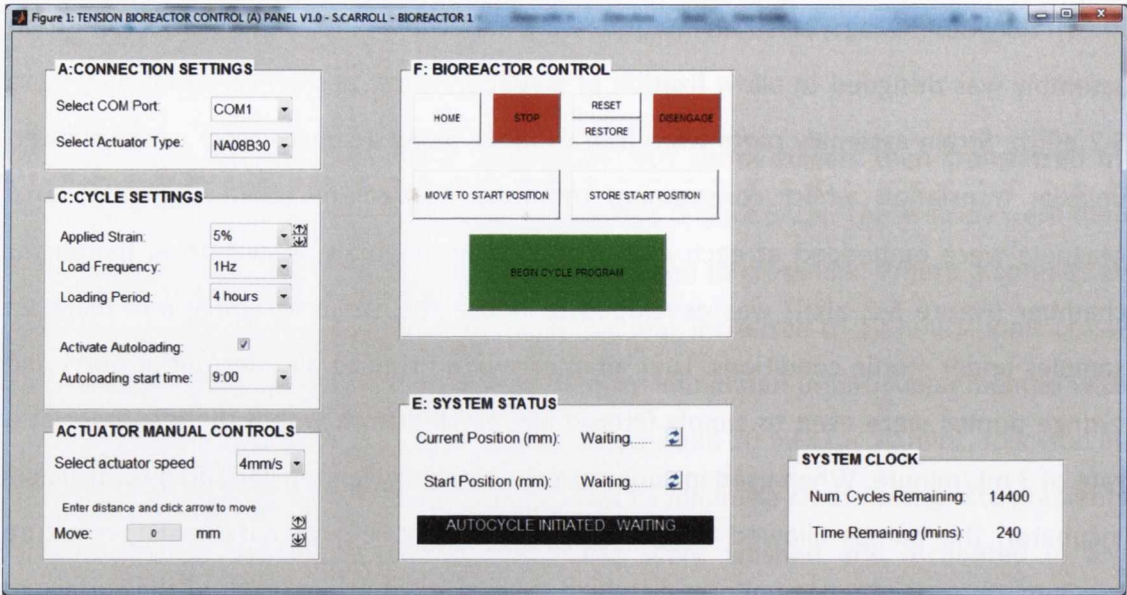


Figure 5-1: Front panel of bioreactor control software developed in Matlab. Multiple instances of the script could be run simultaneously by utilising partitioned virtual environments, thereby allowing multiple bioreactors to run simultaneously from the same PC. The graphical user interface allows control of applied strain magnitude, frequency and duration of loading. Automated loading is facilitated via a script loop that continuously monitored the current time on the system clock, and system information such as current status and cycles remaining is displayed.

5.3.4 Characterization of mechanical properties of collagen-alginate hydrogels

Cylindrical hydrogel constructs were fabricated and mechanical properties were determined via unconfined compression testing, as described elsewhere (Buckley et al. 2009). Briefly, stress relaxation tests were performed to determine the equilibrium modulus of the tissue. These consisted of a ramp and hold cycle, with a ramp displacement of 1 $\mu\text{m/s}$ until 10% compressive strain was obtained. This strain was then maintained until equilibrium was reached, at which point, the stress was noted and the equilibrium modulus calculated. The Young's modulus was calculated from slope of the ramp phase, i.e. the ratio of maximum stress to maximum applied strain. Dynamic tests, which involved applying a cyclic strain of 1% at a frequency of 1 Hz, were performed immediately after the stress relaxation cycle to determine the dynamic modulus of the tissue.

5.3.5 RNA isolation and real-time reverse transcriptase polymerase chain reaction

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used to determine the relative gene expression changes in RNA isolated from MSCs cultured in tension bioreactors. The central sections of dog-bone shape collagen-alginate samples were mechanically disrupted and incubated in alginate dissolving buffer (55 mM sodium citrate, 30 mM EDTA, 150 mM, pH 6.8) at 37°C for 10 min. Cell pellets, obtained via centrifugation, were then re-suspended in Buffer RLT (Qiagen) supplemented with β -mercaptoethanol (10 $\mu\text{l/mL}$) to lyse the cells. Proteinase-K (Qiagen) was added at 10 $\mu\text{l/mL}$, and samples were incubated at 55°C for 10 min to disrupt any collagen present. Samples were then transferred to spin columns (RNeasy Micro Kit, Qiagen). Ethanol was added for a final concentration of 33% EtOH, then RNA was isolated according to manufacturer's instructions and subsequently re-suspended in RNase-free water.

RNA concentrations were quantified using a NanoDropTM (ND-1000) spectrophotometer and RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK). Real-time PCR reactions were performed in 20 μL volumes containing 10 μL Sybr Green Master Mix (Applied Biosystems), 0.4 μM forward primer, 0.4 μM reverse primer and sufficient sample for 50 ng of cDNA. Primers used are listed in Table 1. Reactions were carried out in duplicate on

an ABI 7500 real-time PCR system (Applied Biosystems) with an amplification profile of 50°C for 2 min, 95°C for 10 min, followed by 40-45 cycles of denaturation at 95°C for 15 s. and annealing/amplification at 60°C for 1 min. Quantitative expression of target genes relative to the endogenous control reference gene (GAPDH) and the selected calibrator was carried out using the $2^{-\Delta\Delta CT}$ method as previously described (Livak and Schmittgen 2001).

Table 5-1: Table 1: Primers used for Real-Time Reverse Transcription Polymerase Chain Reaction

<i>Symbol</i>	<i>Gene</i>	<i>Forward primer</i>	<i>Reverse primer</i>
ACAN	Aggrecan	GACCACTTTACTCTTGGTG	TCAGGCTCAGAACTTCTAC
ACTA2	Smooth muscle aortic alpha-actin	CAAAAGAGGAATCCTGACC	CATTGTAGAAAGAGTGGTGC
ALPL	Alkaline Phosphatase	TTTCACTCTTCTTAGTGCTG	CGTTACGGAATGAGGAAAC
BMP2	Bone morphogenic protein-2	ATGTGGAGGCTCTTCAATG	CATGGTCGACCTTAGGAG
CNN1	Calponin 1, Basic, Smooth Muscle	AGATGGCATCATTCTTGC	ATGAAGTTGCCAATGTTCTC
COL10A1	Collagen type 10, alpha-1	GTAGGTGTTGGTATTGCTC	GAGCAATACCAACACCTAC
COL1A1	Collagen type 1, alpha-1	TAGACATGTTCACTTTGTG	GTGGGATGTCTTCTTCTTG
COL2A1	Collagen type 2, alpha-1	CGACGACATAATCTGTGAAG	TCCTTTGGGTCCTACAATATC
COL3A1	Collagen type 3, alpha-1	TCATCCCACTGTTATTTTGG	CTCTATCCGCATAGGACTG
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	TTTAACTCTGGCAAAGTGG	GAACATGTAGACCATGTAGTG
MYH1	Myosin, Heavy Chain 1	GAGTCACTTCCAGCTAAAG	CATTTCATGAGCTCTGGC
OPN	Secreted phosphoprotein 1 (osteopontin)	CTGCAGACCAAGGAAAATC	AGCATCTGTGTAATTTGTTGG
RUNX2	Runt-related transcription factor-2	CCAACAGAGGCATTTAAGG	CCAAAAGAAGTTTGCTGAC
SOX9	SRY (Sex Determining Region Y)-Box-9	CAGACCTTGAGGAGACTTAG	GTTTCGAGTTGCCTTAGTG
TNC	Tenascin-C	ATCTAGTCTTCTCAACTCCG	GAGTAGAATCCAAACCACTTG

5.3.6 Cell viability and F-actin cytoskeleton fluorescent imaging MSC

Fluorescent imaging was performed as described previously (Thorpe et al. 2012). Dog-boned shaped MSC seeded constructs were sectioned along the long axis for imaging through the depth of the sample. To assess cell viability, sections were washed in phenol red free DMEM (DMEM-, Sigma-Aldrich) followed by incubation in 4 mM calcein AM (live cell membrane) and 4 mM ethidium homodimer-1 (dead cell DNA; both Cambridge Bioscience, Cambridge, UK). Sections were again washed and imaged with an Olympus FluoView FV1000 Confocal Microscope.

F-actin cytoskeletal filaments were visualised using rhodamine 110 conjugated phalloidin (VWR). Constructs were sectioned as described above and fixed in 4% paraformaldehyde solution containing sodium cacodylate and barium chloride (all Sigma-Aldrich) to irreversibly cross-link the alginate matrix. Cells were permeabilised in 0.5% Triton-X100 (Sigma-Aldrich) and incubated overnight in DMEM- with 1.5% BSA and rhodamine 110 conjugated phalloidin (1:40; 200 U/mL; VWR). Construct slices were washed in DMEM- and imaged with an Olympus FluoView FV1000 Confocal Microscope.

5.3.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism (Version 4.3) software. Student's t-tests or one-way analyses of variance (ANOVA) were used where appropriate. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $P < 0.05$. Numerical and graphical results are reported in the form of mean $\pm$ standard error (SE) from the mean.

5.4 Results

5.4.1 Bioreactor Validation

Hydrogel strain was correlated with actuator displacement by video analysis of marked hydrogels being strained during tensile testing, and hydrogels were found to deform in a relatively linear manner (R^2 value = 0.996, Figure 5-2c). A 7 day pilot study was performed to monitor oxygen levels and cell viability. Live/dead imaging showed high cell viability throughout the hydrogel for the duration of the study (Figure 5-2b) and that oxygen did not vary significantly from nominal levels (5% O_2) due to the pumped air supply (data not shown).

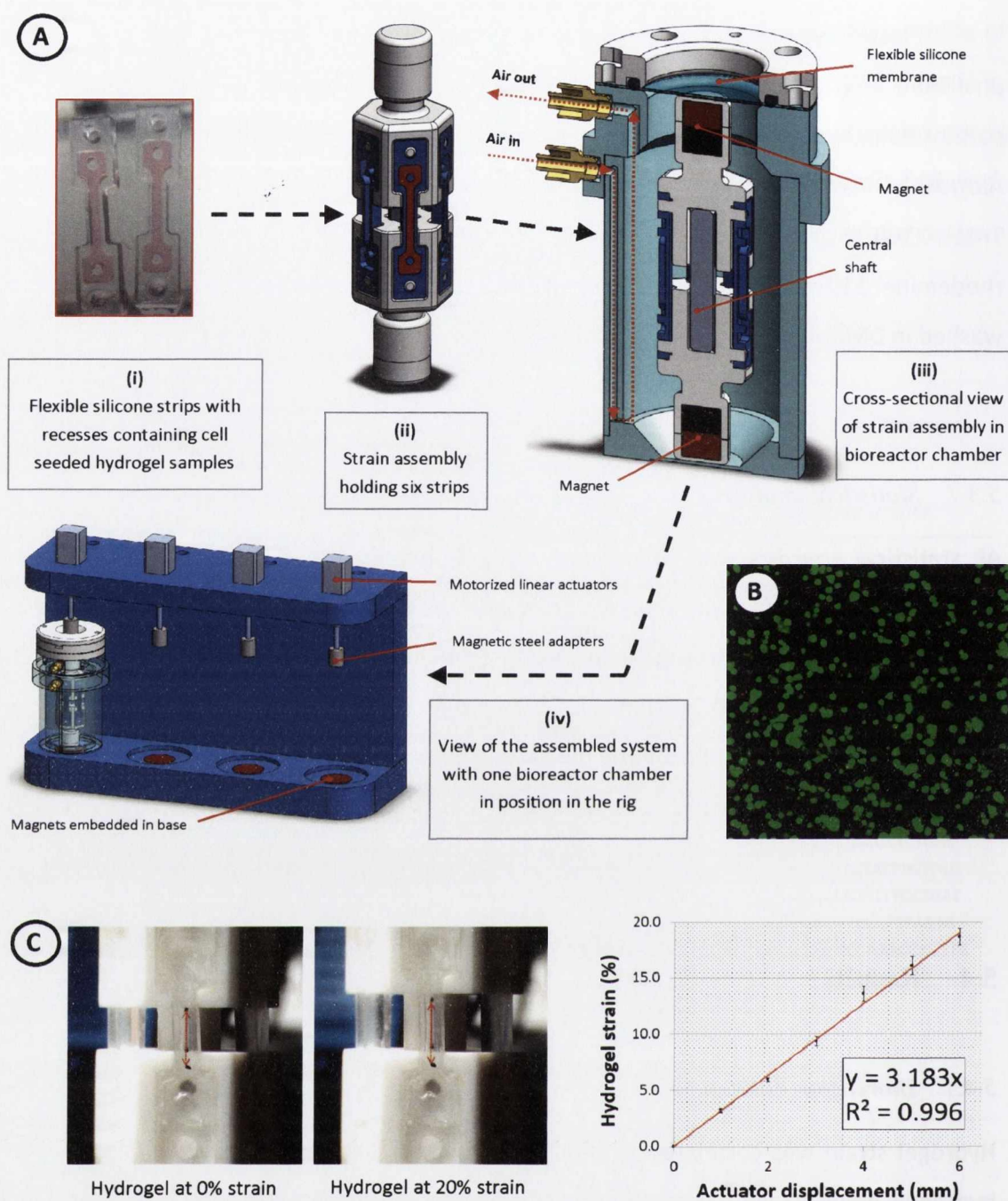


Figure 5-2: A novel bioreactor system for application of uniaxial cyclic tensile strain to soft 3D hydrogels was developed (a). Dog-bone shaped cell-seeded hydrogels constructs were moulded in recesses within flexible silicone strips (i). These strips were mounted on strain assemblies (ii), which were then sealed into the bioreactor chamber (iii) under sterile conditions. The displacement of external actuators (iv) was aseptically transferred to accurately stretch the strips in the bioreactor using magnets, both outside of and within the chamber, connected around a flexible membrane separator. MSCs encapsulated in collagen-alginate blended hydrogels and cultured in the bioreactor remained viable over 7 days (b). Tensile testing image analyses were performed to correlate actuator displacement with hydrogel strain (c). Representative images and data from hydrogel strain validation image analyses are shown.

5.4.2 *Stiffer substrates promote chondrogenesis and progression along the endochondral pathway*

The stiffness of blended collagen-alginate hydrogels were modulated by varying the concentration of alginate within the construct. The Young's Modulus increased from 2.2 ± 0.2 kPa for 0.6% alginate hydrogels to 10.3 ± 2.3 kPa for 2.25% alginate hydrogels; a 4.7 fold difference in measured stiffness (Figure 5-3a, $P=0.003$). These groups were termed "soft" and "stiff" respectively.

MSCs were encapsulated in both soft and stiff hydrogels and cultured under free-swelling conditions for 4 days in media supplemented with the growth factor TGF- β 3, a growth factor known to regulate differentiation and to play a role in promoting both chondrogenic and myogenic differentiation of MSCs (Gao et al. 2010). The stiffer substrates promoted the expression of markers associated with chondrogenesis (COL2A1, ACAN) and osteogenesis (ALPL, BMP2, OPN), but had no effect on markers of myogenic differentiation (ACTA2, MYH1, CNN1; Figure 5-3c).

5.4.3 *Cyclic tensile strain enhances myogenesis but inhibits chondrogenesis of MSCs*

MSC encapsulated in both soft and stiff hydrogels were mounted in bioreactors and subjected to 10% uniaxial CTS. After 4 days of culture, MSCs in all groups maintained a mostly spherical morphology and displayed clearly defined actin cytoskeletons (Figure 5-4a). The application of CTS had an inhibitory effect on the expression of the chondrogenic markers COL2A1 and ACAN (Figure 5-4b) in MSCs seeded in stiffer matrices. This inhibitory effect was not observed, however, in MSCs seeded in soft substrates. Regardless of substrate stiffness, SOX9 expression was not influenced by the application of CTS. CTS was found to significantly upregulate the myogenic marker ACTA2 in stiff hydrogels, and MYH1 and CNN1 in soft hydrogels (Figure 5-4b).

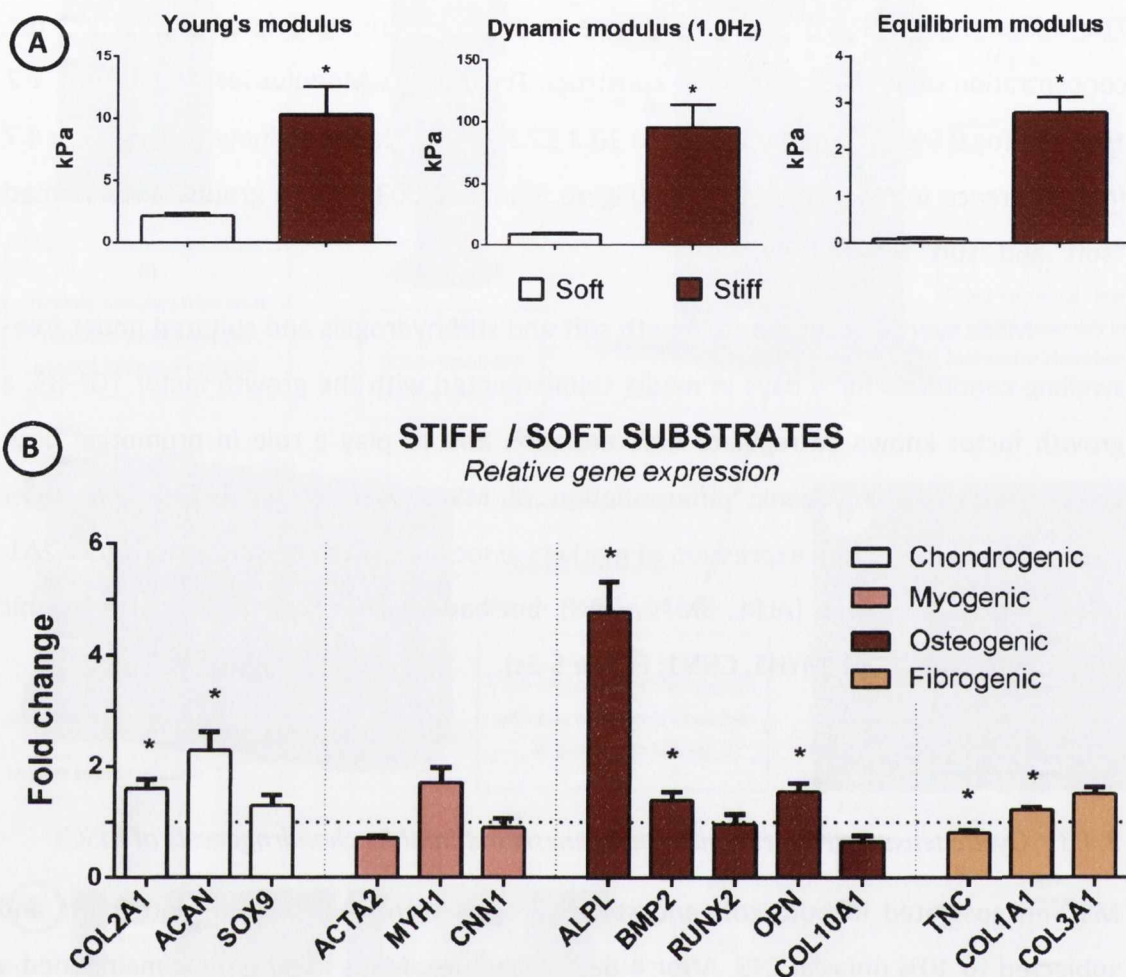


Figure 5-3: Mechanical characterisation of collagen-alginate blended hydrogels created using 0.6% (soft) and 2.25% (stiff) final alginate concentration (a). Relative gene expression in MSCs encapsulated in stiff blended hydrogels (normalized to MSCs in soft hydrogels) after 4 days of culture (b). * indicates a significant difference vs. soft group ($P < 0.05$; $n = 5$).

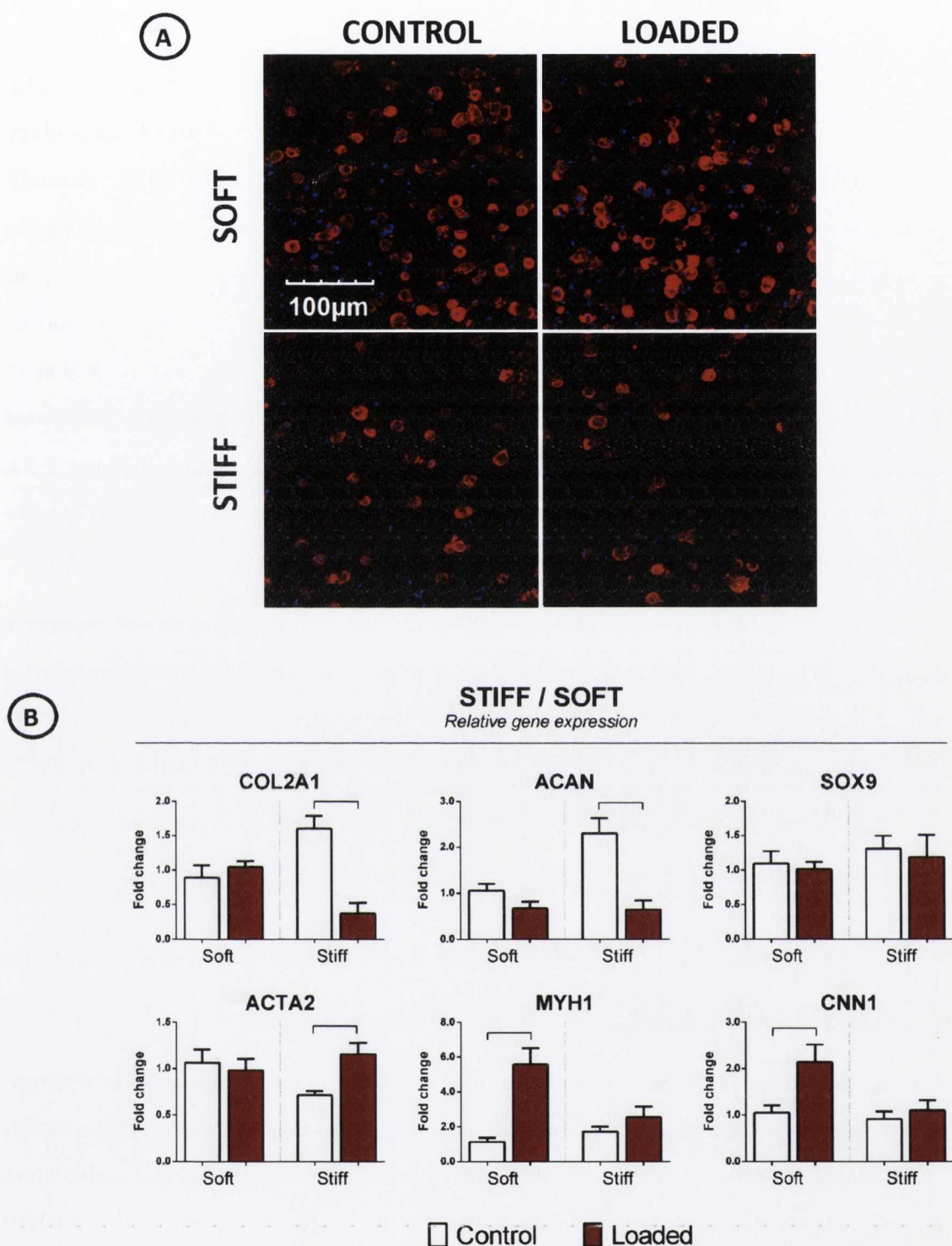


Figure 5-4: F-actin staining of MSCs encapsulated in a soft or stiff hydrogel and cultured in free swelling or loaded conditions for 4 days (a). Cell nuclei are counter-stained with Dapi (blue). Chondrogenic and myogenic relative gene expression at day 4 normalized to the soft/free-swelling control group (b). Significant differences vs. non-loaded control group are indicated ($P < 0.05$; $n = 5$).

5.4.4 *A spread MSC morphology is correlated with suppression of chondrogenesis*

Despite the increases in the expression of myogenic markers with the application of CTS, MSCs generally maintained a round cell morphology. However a more spread morphology is generally associated with a myogenic phenotype. In an effort to further promote myogenesis, cell shape was controlled by encapsulating MSCs in collagen-alginate hydrogels and performing cross-linking with calcium chloride either immediately to promote a spherical cell shape, or after a 12 hours delay period to allow MSCs to spread within the collagen matrix. Live-dead staining 24 hours after cell encapsulation and actin cytoskeleton staining after 4 days of culture under free-swelling conditions confirmed that MSCs in immediately cross-linked hydrogels retained a round shape (Figure 5-5a, top), while cells that were allowed a delay period before cross-linking adopted a more elongated, stellated morphology (Figure 5-5a, bottom).

Cell shape appeared to have no significant effect on the expression of myogenic markers (ACTA1, MYH1, and CNN1) after 4 days of culture in media supplemented with TGF- β 3 (Figure 5-5b). However, cell spreading was correlated with a reduced expression of markers associated with chondrogenesis (SOX9) and endochondral ossification (BMP2 and COL10A1).

5.4.5 *Cyclic tensile strain synergizes with a spread cellular morphology to enhance markers of myogenesis.*

The application of CTS appeared to have little apparent influence on cell morphology, with the MSCs encapsulated in the immediately cross-linked hydrogels displaying a mostly spherical cytoskeleton and cells in the delayed cross-linked group retaining a visibly more elongated, spread morphology (Figure 5-6a). Furthermore, gene expression analysis revealed no significant effect of CTS on chondrogenic gene expression, regardless of cell shape within the hydrogels (Figure 5-6b). In contrast, CTS significantly upregulated both MYH1 and CNN1 expression when applied to MSCs with a spread morphology.

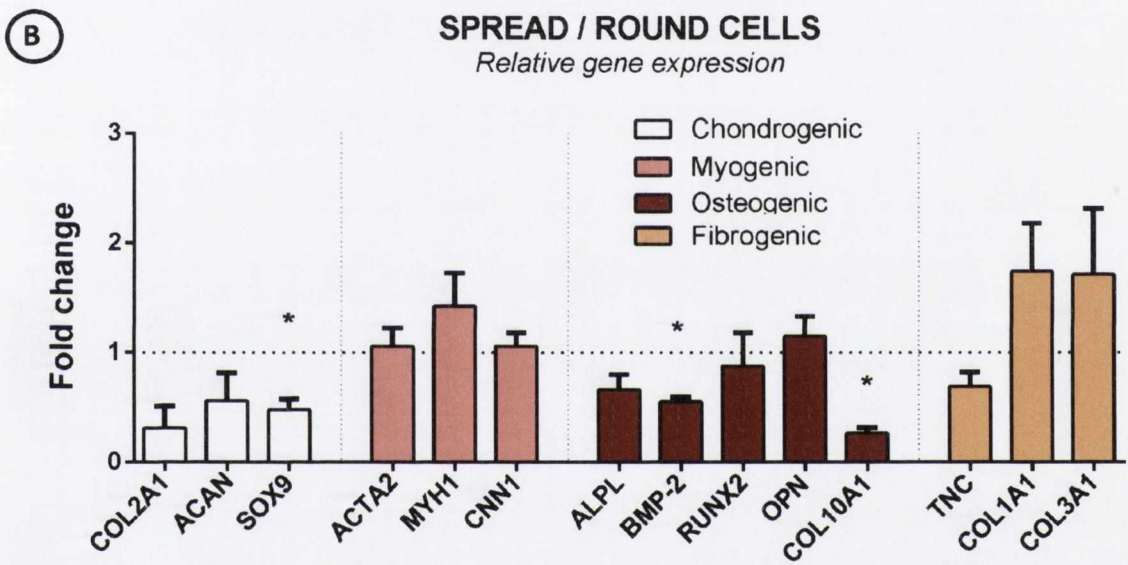
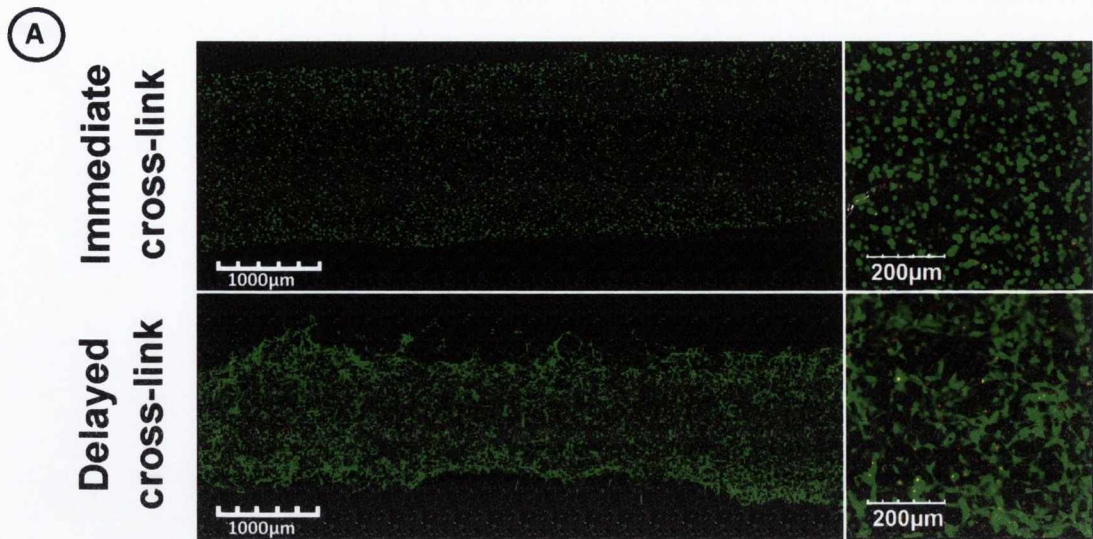


Figure 5-5: Live/dead staining 24 hours after MSCs were encapsulated in an immediately (round) or delayed (spread) cross-linked hydrogel (a). Relative gene expression in spread MSCs (normalized to round MSCs) after 4 days of culture. * indicates a significant difference vs. round group ($P < 0.05$; $n = 5$).

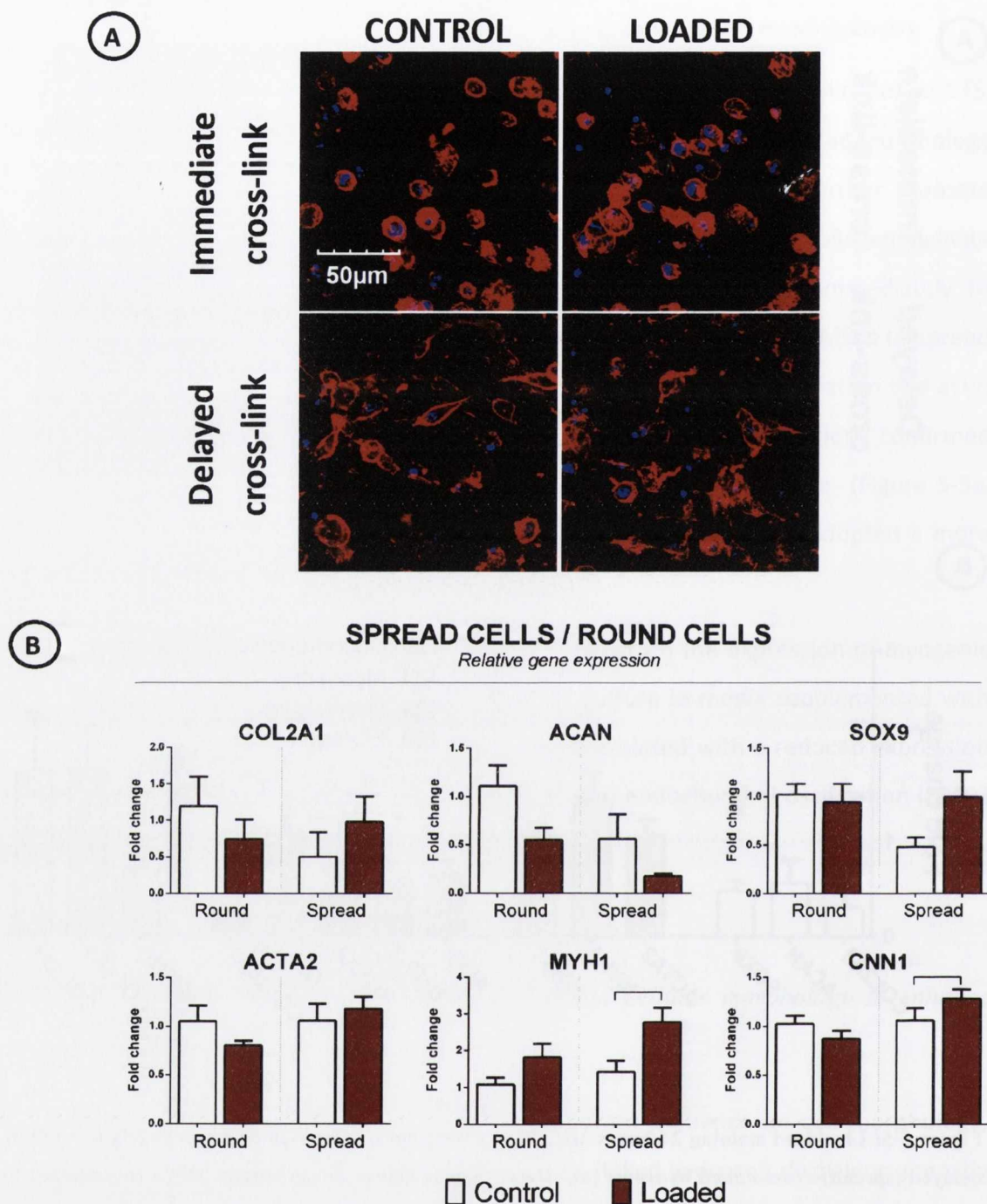


Figure 5-6: F-actin staining of MSCs encapsulated in an immediately (round) or delayed (spread) cross-linked hydrogel and cultured in free swelling (control) or loaded conditions for 4 days (a). Cell nuclei are counter-stained with Dapi (blue). Chondrogenic and myogenic relative gene expression at day 4 normalized to the round/free-swelling group (b). Significant differences vs. non-loaded control group are indicated ($P < 0.05$; $n = 5$).

5.5 Discussion

In a regenerative context, an understanding of how external mechanical stimuli influence MSC lineage commitment will be key to engineering functional tissue grafts. In this study, a novel bioreactor was developed that is capable of applying accurate levels of uniaxial CTS to soft 3D hydrogels in a controlled, aseptic environment. Needle-free ports enabled connection of air pumps to ensure stable, uniform levels of oxygen tension, pH and nutrients throughout the culture media and facilitated simple media changes under sterile conditions. The system was demonstrated to be capable of accurately straining collagen-alginate blended hydrogels whilst maintain cell viability, and is suitable for use with other soft 3D hydrogel systems. This bioreactor was then utilized to investigate the interdependency of mechanical stimuli, matrix stiffness and cell shape on the phenotype of MSCs encapsulated in 3D collagen-alginate grafts. Both stiffer matrices and a spherical cell morphology were found to promote a chondrogenic phenotype, when compared with softer matrices and spread morphology respectively. The application of CTS was found to enhance markers of myogenesis, and a spread cell morphology appeared to synergize with tensile stimulation to enhanced myogenic differentiation of MSCs.

Increasing the stiffness of the collagen-alginate hydrogel from 2.2 kPa to 10.3 kPa was found to enhance the expression of genes associated with both chondrogenesis (COL2A1, ACAN) and osteogenesis (ALPL, BMP2, OPN), whilst having no effect on myogenic markers, suggesting that that stiffer substrates promote a more endochondral phenotype (Figure 5-3c). It has previously been shown that stiffer substrates are correlated with enhanced expression of osteogenic genes and promotion of MSC hypertrophy and endochondral markers (Engler et al. 2006, Bian et al. 2013). However, previous studies have found that increasing substrate stiffness also enhances markers of myogenic differentiation (Engler et al. 2006). On such stiffer 2D substrates, MSCs adopt a more spread, stellate morphology (Rowlands et al. 2008), which is known to be supportive of myogenesis (Gao et al. 2010). In contrast, in immediately cross-linked alginate 3D hydrogels MSCs remain round, regardless of stiffness (Huebsch et al. 2010, Parekh et al. 2011). Collectively, these findings motivated our subsequent studies to explore the role of cell shape in regulating myogenesis in MSCs in 3D environments.

The application of uniaxial CTS was found to suppress chondrogenic gene expression (Figure 5-4b), thus corroborating previously reported findings using MSCs in

3D hydrogels (Haudenschild et al. 2009). However, this inhibitory effect was only observed in stiffer hydrogels. Our group has also previously reported that the application of hydrostatic pressure and dynamic compression enhances chondrogenic differentiation in stiff gels only (Steward et al. 2013, Steward et al. 2014), suggesting that chondrogenically primed MSCs become more mechano-sensitive as the stiffness of their substrate increases. In contrast, CTS was found to promote the expression of different markers of myogenesis regardless of substrate stiffness (Figure 5-4b), with ACTA2 expression found to be upregulated in stiff hydrogels, and MYH1 and CNN1 expression upregulated in soft hydrogels, indicating that not all mechanically mediated changes in gene expression is enhanced in stiffer matrices. The finding that CTS enhances myogenic markers is in agreement with earlier studies that explored the influence of this stimulus on MSCs seeded on 2D substrates (Hamilton et al. 2004, Park et al. 2004, Kurpinski et al. 2006, Ghazanfari et al. 2009, Jang et al. 2011, Maul et al. 2011).

As expected, cells that were constrained in 3D matrices that imposed a spherical cell shape exhibited markers of a more chondrogenic phenotype when compared to MSCs that were permitted to adopt a more spread morphology (Figure 5-5). In 2D culture a spread MSC shape has been associated with osteogenesis (McBeath et al. 2004), however there was no evidence in this study that a spread cell promoted a more endochondral phenotype. Taken together, these findings suggest that a round cell shape may enhance endochondral ossification in 3D hydrogels by facilitating MSCs to undergo chondrogenesis, a necessary precursor to hypertrophy. Somewhat unexpectedly, a spread shape was not correlated with an increase in myogenic gene expression as might be expected based on the findings of previous studies of MSCs in monolayer culture (Gao et al. 2010). However, monolayer culture is not truly representative of a natural 3D *in vivo* environment. A key difference between 2D and 3D culture is that, although both environments may be permissive for cell spreading, differentials exist in the degree and spatial distribution of integrin binding sites and the resultant cytoskeletal traction forces generated (Beningo et al. 2004), and it may be that longer culture periods are required in 3D hydrogels to detect differences in myogenic gene expression in response to alterations in MSC shape.

The application of CTS to spread MSCs was found to lead to enhanced myogenic gene expression, suggesting that cyclic tensile strain may synergize with a spread cellular

morphology to promote myogenesis of MSCs in 3D hydrogels. This finding can be related to previous studies using MSCs in 2D systems permissive to cellular re-organisation and cell-cell interaction (Hamilton et al. 2004, Park et al. 2004, Kurpinski et al. 2006, Ghazanfarj et al. 2009, Jang et al. 2011, Maul et al. 2011). It may be that increased cytoskeletal tension develops within spread cells (Rowlands et al. 2008), which in turn may result in increased mechano-sensitivity to tensile deformation of the local substrate. Furthermore, since direct cell-cell interaction has been reported to be of key importance for myogenesis of MSCs (Fukuhara et al. 2003, Gao et al. 2010), and cell-cell contact has been shown to be sufficient to induce cellular spreading and enhanced actin stress fibre development (Yeung et al. 2005), it may well be the case that cell spreading leads to increased cell-cell contact, similar to monolayer culture systems, thus myogenically priming the MSCs. Indeed, it is tempting to hypothesise that soft gels facilitate greater cell-cell interaction, as do gels that permit cell spreading, which may explain why the application of CTS in both groups enhances myogenic marker expression in MSCs.

Collagen-alginate hydrogel stiffness can typically be tuned by two methods: varying the cross-link density or varying the alginate concentration, both methods have drawbacks. The method utilised in this study (i.e. varying alginate concentration) may subtly influence other features of the local cellular environment, such as cross-link density, pore interconnectivity and availability of collagen integrin binding sites. Changes in cross-link density, for example, have been reported to influence cell motility, spreading and migration (Kyburz and Anseth 2013) and may subsequently effect cell-cell interactions, which are known to be key for myogenesis of MSCs (Fukuhara et al. 2003, Gao et al. 2010).

It is clear that extrinsic mechanical stimuli can drive MSC differentiation and guide stem cell lineage commitment. However, the successful engineering of functional tissue grafts for clinical applications will require a more complete understanding of the complex interplay between extrinsic mechanical signals, biomaterial characteristics, biochemical environment and cell phenotype. The current study represents the first investigation into the dependency of MSC response to cyclic tensile deformation on matrix rigidity and cell morphology in clinically relevant 3D grafts.

Chapter 6 Cyclic tensile strain can play a role in directing both intramembranous and endochondral ossification of MSCs in 3D hydrogels

6.1 Abstract

Successfully regenerating damaged or diseased bone and other joint tissues will require a detailed understanding of how joint specific environmental cues regulate the fate of cells that are recruited or delivered to the site of injury. Recognising that bone can form and heal through either an intramembranous or an endochondral pathway, the objectives of this study were (i) to explore the influence of cyclic tensile strain (CTS) on the differentiation of MSCs in the absence of any specific growth factor, and (ii) examine the influence of the long-term application of this mechanical stimulus on markers of endochondral ossification in MSCs maintained in chondrogenic culture conditions. A custom bioreactor was used to apply uniaxial tensile deformation to bone marrow-derived MSCs encapsulated within physiological relevant 3D fibrin hydrogels. Mechanical loading, applied in the absence of soluble differentiation factors, was found to enhance the expression of both tenogenic (TNC, COL1A1) and osteogenic markers (BMP2, RUNX2 and ALPL), while suppressing markers of adipogenesis. During longer term culture in the presence of a chondrogenic growth factor, CTS was shown to induce MSC re-organization and alignment, and to enhance the expression of markers associated with endochondral ossification (BMP2, RUNX2, ALPL, OPN, COL10A1) in a strain magnitude dependent manner. Taken together, these findings indicate that tensile loading may play a key role in promoting both intramembranous and endochondral ossification of MSCs in a context dependent manner. This would appear to be mediated, at least in part, by loading induced increases in the expression of osteogenic growth factors such as BMP2. The results of this study demonstrate the potent role that extrinsic mechanical loading plays in guiding stem cell fate, which must be carefully considered in the design of cell and tissue engineering therapies if they are to realize their clinical potential.

6.2 Introduction

Understanding the etiology of diseases such as osteoarthritis requires an in-depth understanding of the role that joint specific environmental factors play in the maintenance of cartilage and its conversion into bone. Furthermore, successfully regenerating damaged or diseased bone and other joint tissues also requires an understanding of how such environmental factors regulate the fate of cells that are recruited or delivered to the site of injury. MSCs isolated from bone marrow can be induced to differentiate towards multiple lineages, making them an attractive cell type for cartilage and bone tissue engineering (Caplan 1991, Vinardell et al. 2012). Bone development occurs via two distinct mechanisms - intramembranous or endochondral ossification. Intramembranous ossification involves the direct differentiation of MSCs into osteoblasts, whereas endochondral ossification first involves differentiation of precursor cells into chondrocytes, the formation of a cartilage template and its subsequent replacement by bone (as reviewed by Frohlich et al. 2008). The latter is the process by which all long bones are formed. It has long been suggested that endochondral ossification during limb development is mechano-regulated, with early studies linking femoral ossification to the regions of maximal tensile strains (Carey 1922). It has also been reported that, during bone healing, callus distraction can produce a systemic increase in concentrations of several bone growth factors (Sato et al. 1999, Weiss et al. 2002). Clearly mechanical cues are integral to directing bone growth, development and healing, however the underlying mechanisms remain poorly understood. A more complete understanding of these processes will benefit not only the fields of cartilage and bone tissue engineering, but will also be integral to the development of novel therapeutic strategies to treat complex diseases such as osteoarthritis.

There has been increased interest in the use of *in vitro* bioreactor systems to examine how different mechanical stimuli regulate both osteogenesis and chondrogenesis of stem cells (Kelly and Jacobs 2010). It has been demonstrated that the application of uniaxial cyclic tensile strain (CTS) to MSCs cultured on 2D substrates can induce osteogenic and fibrous gene expression (Simmons et al. 2003, Friedl et al. 2007, Qi et al. 2008, Diederichs et al. 2009). The magnitude of tensile loading has also been shown to be important, with low strains inducing osteogenesis and higher strains promoting the expression of fibrous or myogenic markers (Chen et al. 2008, Jang et al. 2011). These 2D

culture systems are generally not considered truly representative of the physiological environment of cells *in vivo*, and MSCs have been shown to respond differentially to tensile loading when seeded on 2D substrates compared to when encapsulated within 3D matrices (Rathbone et al. 2012). Of the studies that have explored the influence of tensile strain on directing the differentiation of MSCs in 3D matrices, many have concluded that the application of uniaxial CTS promotes a more ligamentous or fibro-chondrogenic phenotype in MSCs (Altman et al. 2002, Juncosa-Melvin et al. 2007, Farnig et al. 2008, Kuo and Tuan 2008, Connelly et al. 2010, Doroski et al. 2010, Baker et al. 2011, Kreja et al. 2012, Yang et al. 2012). However it has also been demonstrated that the application of CTS to MSCs in 3D collagen gels in the absence of osteogenic supplements can lead to the upregulation of BMP2 (Sumanasinghe et al. 2006), suggesting that this mechanical stimulus may also be osteoinductive. Furthermore, it has also been reported that tensile strain and dynamic compression differentially regulate MSC differentiation (Haudenschild et al. 2009), with CTS upregulating osteogenic markers BMP1, ALP and NELL1, whereas compression was found to promote chondrogenesis of MSCs encapsulated in 3D alginate matrices. This latter finding agrees with a number of separate studies demonstrating that compressive loading can enhance chondrogenesis of MSCs (Huang et al. 2004, Campbell et al. 2006, Thorpe et al. 2012, Steward et al. 2014, Luo et al. 2015). Furthermore, it has been demonstrated that high magnitudes of cyclic hydrostatic pressure and dynamic compression can suppress hypertrophy and endochondral ossification of MSCs (Bian et al. 2012, Thorpe et al. 2013, Carroll et al. 2014, Luo et al. 2015, Zhang et al. 2015). Collectively, these studies point to the integral role that mechanical cues play in directing both the initial phenotype of MSCs and in determining their ultimate fate.

Despite the fact that long bones form and regenerate through the process of endochondral ossification, relatively little is known about the role mechanical cues can play in directing this process. Motivated by previous work pointing to a role for tensile strain in directing bone development and healing, the objective of this study was to explore the role of CTS in directing osteogenesis of MSCs within physiologically relevant 3D hydrogels using a custom developed bioreactor system. Recognising that bone can form and heal through either an intramembranous or an endochondral pathway, we first sought to explore the influence of CTS on the differentiation of MSCs in the absence of specific growth factors to determine if loading alone could initiate osteogenesis. We then

examined the influence of the long-term application of this mechanical stimulus on markers of endochondral ossification in MSCs maintained in chondrogenic culture conditions. The hypothesis was that CTS would enhance endochondral ossification in chondrogenically primed MSCs. The dependency of this process on magnitude and frequency of the applied strain was also examined.

6.3 Methods

6.3.1 Cell Isolation and Expansion

Bone marrow derived MSCs were isolated aseptically from the femoral diaphysis of a porcine donor and expanded, as described previously (Thorpe et al. 2013, Carroll et al. 2014). Briefly, isolated MSCs were plated and cultured in a standard expansion media (EM) formulation consisting of high glucose Dulbecco's Modified Eagle Medium (DMEM, GlutaMAX™) containing 10% foetal bovine serum and 1% penicillin (100 U/ml)-streptomycin (100 mg/ml) (all from GIBCO, Biosciences, Dublin, Ireland) and supplemented with fibroblast growth factor-2 (FGF-2, 5 ng/mL; ProSpec-Tany TechnoGene Ltd, Israel) and amphotericin B (0.25 mg/mL; Sigma-Aldrich, Arklow, Ireland). Following colony formation, the cells were trypsinised, counted and plated at a seeding density of 5×10^3 cells/cm² at each passage. Complete media exchanges were performed twice weekly.

6.3.2 Fibrin hydrogel fabrication and culture

MSCs were suspended in 10,000 KIU/mL aprotinin solution (Nordic Pharma) containing 19 mg/mL sodium chloride and 100 mg/mL bovine fibrinogen type I-S (60–85% protein, 10% sodium citrate and 15% sodium chloride; Sigma-Aldrich), which was combined 1:1 with 5 U/mL thrombin in 40 mM CaCl₂ (pH 7) and transferred by syringe to recesses in custom designed flexible silicone strips. These strips were then incubated at 37°C for 45 minutes to allow cross-linking to complete, producing dog-boned shaped constructs with final concentrations of 50 mg/mL fibrinogen, 2.5 U/mL thrombin, 5000 KIU/mL aprotinin, 17

mg/mL sodium chloride, 20 mM CaCl₂ and 10×10⁶ cells/mL. The constructs were then loaded into bioreactors containing differentiation medium (DM), consisting of DMEM supplemented with 100 U/mL penicillin/streptomycin (both from Gibco), 100 mg/mL sodium pyruvate, 40 mg/mL L-proline, 50 mg/mL L-ascorbic acid-2-phosphate, 1.5 mg/mL bovine serum albumin, 1×insulin–transferrin–selenium and 100 nM dexamethasone (all Sigma-Aldrich). For studies requiring chondro-inductive stimuli, constructs were culture in chondrogenic medium (DM supplemented with 10 ng/mL of transforming growth factor-β3 (TGF-β3, ProSpec-Tany TechnoGene, Ltd., Israel). Media changes were performed twice weekly.

6.3.3 *Mechanical stimulation of hydrogel encapsulated MSCs*

Uniaxial cyclic tensile strain was applied to MSC seeded hydrogels in a custom design bioreactor system (as described in the previous chapter). All bioreactors were housed in incubators set at 5% oxygen, 37°C and a reciprocating syringe air pump system was employed to pass air bubbles (2 mL/min) through the culture medium, thereby gently agitating the system and maintaining a uniform dissolved oxygen content of 5% pO₂. In the initial study, hydrogels were deformed at a strain magnitude of 10% and frequency of 0.5 Hz for 4 hours each day for 7 days. In the second, longer term study, the influence of strain magnitude and frequency was investigated. Therefore, this study involved four experimental groups (non-loaded control, 5% strain at 0.5 Hz, 10% strain at 0.5 Hz and 10% strain at 1 Hz) cultured in chondrogenic medium (+TGF-β3) for 21 days.

6.3.4 *Biochemical analysis*

The central (strained) sections of dog-bone constructs (n ≥ 3) were used for quantitative analysis of DNA, sulphated glycosaminoglycan (sGAG) and collagen content. The wet weight of each section was measured, then the samples were digested with papain (125 µg/ml) in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, and 0.05 M EDTA (pH 6.0, all Sigma-Aldrich) at 60°C under constant rotation for 18 hours. DNA content was quantified using the Hoechst Bisbenzimidazole 33258 dye assay as described previously (Kim et al. 1988), with a calf thymus DNA standard. Sulphated glycosaminoglycan (GAG) content was

quantified using the dimethylmethylen blue dye-binding assay (DMMB; Blyscan, Biocolor Ltd., Northern Ireland) with a chondroitin sulphate standard. Collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110 °C for 18 hours in 38% HCl and assayed using a chloramine-T assay with a hydroxyproline : collagen ratio of 1:7.69 (Kafienah and Sims 2004, Ignat'eva et al. 2007). Total sGAG and collagen values and values normalized to DNA content are reported.

6.3.5 RNA isolation and real-time reverse transcriptase polymerase chain reaction

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used to determine the relative gene expression changes in RNA isolated from MSCs cultured in tension bioreactors. The central sections of dog-bone shape collagen-alginate samples were mechanically disrupted in Buffer RLT (Qiagen) supplemented with β -mercaptoethanol (10 μ L/mL), frozen in liquid nitrogen and stored at -80°C prior to analysis. Thawed samples were then transferred to spin columns for homogenization (QIAshredder, Qiagen), followed by RNA isolation (RNeasy Micro Kit, Qiagen) according to manufacturer's instructions, and subsequently re-suspended in RNase-free water.

RNA concentrations were quantified using a NanoDropTM (ND-1000) spectrophotometer and RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK). Real-time PCR reactions were performed in 20 μ L volumes containing 10 μ L Sybr Green Master Mix (Applied Biosystems), 0.4 μ M forward primer, 0.4 μ M reverse primer, RNase-free water and sufficient sample for 50 ng of cDNA. Primers used and gene abbreviations are listed in Table 1. Reactions were carried out in duplicate on an ABI 7500 real-time PCR system (Applied Biosystems) with an amplification profile of 50°C for 2 min, 95°C for 10 min, followed by 40-45 cycles of denaturation at 95°C for 15 sec and annealing/amplification at 60°C for 1 min. Quantitative expression of target genes relative to the endogenous control reference gene (GAPDH) and the selected calibrator was carried out using the $2^{-\Delta\Delta CT}$ method as previously described (Livak and Schmittgen 2001).

Table 6-1: Primers used for Real-Time Reverse Transcription Polymerase Chain Reactions

Symbol	Gene	Forward primer	Reverse primer
ACAN	Aggrecan	GACCACTTTACTCTTGGTG	TCAGGCTCAGAACTTCTAC
ACTA2	Smooth muscle aortic alpha-actin	CAAAAGAGGAATCCTGACC	CATTGTAGAAAGAGTGGTGC
ALPL	Alkaline Phosphatase	TTTCACTCTTCTTAGTGCTG	CGTTACGGAATGAGGAAAC
BMP2	Bone morphogenic protein-2	ATGTGGAGGCTCTTTCAATG	CATGGTCGACCTTTAGGAG
CNN1	Calponin 1, Basic, Smooth Muscle	AGATGGCATCATTCTTGC	ATGAAGTTGCCAATGTTCTC
COL10A1	Collagen type 10, alpha-1	GTAGGTGTTTGGTATTGCTC	GAGCAATACCAAAACACCTAC
COL1A1	Collagen type 1, alpha-1	TAGACATGTTCAGCTTTGTG	GTGGGATGTCTTCTCTTG
COL2A1	Collagen type 2, alpha-1	CGACGACATAATCTGTGAAG	TCCTTTGGGTCACAAATATC
COL3A1	Collagen type 3, alpha-1	TCATCCCACTGTTATTTTGG	CTCTATCCGCATAGGACTG
FABP4	Fatty acid binding protein-4	CTGAAGAGAGTCATTGCAC	CATTTTGTGAGCACTCTAGG
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	TTTAACTCTGGCAAAGTGG	GAACATGTAGACCATGTAGTG
LECT1	Leukocyte cell derived chemotaxin-1	ACCTTTAAAATGGGAAACGG	GCTTTGATGTAGCACTTCTC
LPL	Lipoprotein lipase	ACCTAACTCGAGTATGCAG	GGTGAATGTGTGAAGACG
MYH1	Myosin, Heavy Chain 1	GAGTCACTTTCAGCTAAAG	CATTTCAATGAGCTCTGGC
OPN	Secreted phosphoprotein 1 (Osteopontin)	CTGCAGACCAAGGAAAATC	AGCATCTGTGTATTTGTTGG
RUNX2	Runt-related transcription factor-2	CCAACAGAGGCATTTAAGG	CCAAAAGAAGTTTGTCTGAC
SOX9	SRY (Sex Determining Region Y)-Box-9	CAGACCTTGAGGAGACTTAG	GTTTCGAGTTGCCTTTATGTG
TNC	Tenascin-C	ATCTAGTCTTTCTCAACTCCG	GAGTAGAATCCAAACCAAGTTG

6.3.6 *Fluorescent staining and quantification of cellular anisotropy of MSC filamentous actin*

Fluorescent images were taken from the central (strained) section only. F-actin cytoskeletal filaments were visualised using rhodamine 110 conjugated phalloidin (VWR). Dog-boned shaped MSC seeded constructs were sectioned for imaging through the depth of the sample and fixed in 4% paraformaldehyde solution. Cells were permeabilised in 0.5% Triton-X100 (Sigma-Aldrich), then incubated overnight in phosphate buffer saline (PBS) with 1.5% BSA and rhodamine 110 conjugated phalloidin (1:40; 200 U/mL; VWR). Construct slices were washed in PBS and imaged with an Olympus FluoView FV1000 Confocal Microscope. Anisotropy of actin filaments was quantified by performing analysis of confocal images (n=4) using the ImageJ FibrilTool plugin (Boudaoud et al. 2014).

6.3.7 *Statistical analysis*

All statistical analyses were performed using GraphPad Prism (Version 4.3) software. Student's t-tests were used where appropriate and one-way analyses of variance (ANOVA) with Tukey's post-test were used to compare multiple conditions. Numerical and graphical results are reported in the form of mean $\pm$ standard error (SE) from the mean. Significance was accepted at a level of $P < 0.05$. Gene expression data are present as fold-differences normalized to the mean of the free-swelling (non-loaded) control group and fold-increase or decreases, where relevant, are indicated in the text below by $\uparrow$ or $\downarrow$ respectively.

6.4 Results

6.4.1 *Cyclic tensile strain in the absence of soluble differentiation factors promotes the expression of tenogenic and osteogenic markers while inhibiting adipogenesis*

MSCs encapsulated in fibrin hydrogels were cultured in bioreactors in the absence of chondrogenic growth factors for 7 days. The application of 10% CTS at 0.5 Hz was found to upregulate the tenogenic markers TNC and COL1A1 ($\uparrow 54.56 \pm 10.83$, and $\uparrow 3.11 \pm 0.34$ respectively; $P < 0.003$) and to suppress the expression of the adipogenic marker LPL ($\downarrow 6.84 \pm 0.62$, $P = 0.0001$). Mechanical stimulation had no effect on the expression of the myogenic markers ACTA2, CNN1 and MYH1 (Figure 6-1). CTS was also found to enhance the expression of a number of markers of osteogenesis, including ALPL and BMP-2 ($\uparrow 7.09 \pm 1.46$ and $\uparrow 4.61 \pm 0.74$; $P < 0.003$). Furthermore, COL10A1 was found to be expressed in all loaded samples, but was not detected in samples in the non-loaded control group (data not shown). Since no expression of chondrogenic markers COL2A1 or SOX9 was detected, while osteogenic markers ALPL, and BMP2 were upregulated following mechanical loading, it would appear that CTS may have a positive role in promoting (intramembraneous) osteogenesis of MSCs in the absence of exogenously supplied growth factors. However, since long bones are known to develop and heal via the more indirect process of endochondral ossification, we therefore next sought to explore the role of CTS on MSCs maintained in chondro-inductive medium for longer term culture duration.

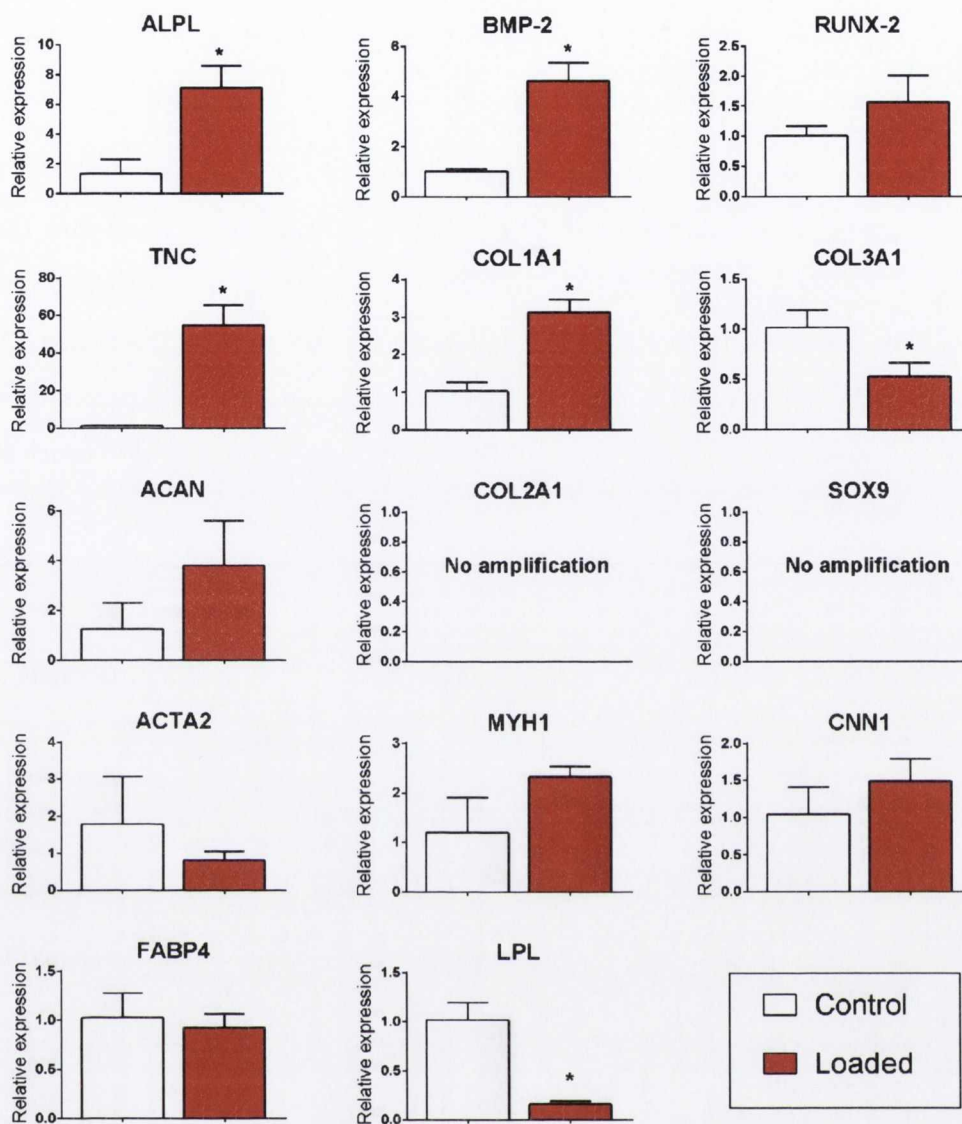


Figure 6-1: Relative gene expression of MSCs encapsulated in fibrin hydrogels and cultured in tensile strain bioreactor in the absence of growth factors for 7 days. The application of 10% cyclic tensile strain at 0.5 Hz was found to promote intramembranous ossification and inhibit adipogenic differentiation of MSCs. Data are presented as fold-changes, relative to the non-loaded (control) group. * indicates a significant difference vs. free swelling control group ($P < 0.05$; $n = 5$). See Table 1 for details of gene symbols.

6.4.2 Cyclic tensile strain promotes endochondral ossification of chondrogenically primed MSCs in a magnitude dependent manner

MSCs encapsulated within fibrin hydrogels and maintained in media supplemented with the chondrogenic growth factor TGF- β 3 for 21 days were found to synthesize cartilage extracellular matrix components (Figure 6-2) and express genes associated with a chondrogenic phenotype (Figure 6-3). The application of 10% strain at 0.5Hz was found to promote cellular proliferation, which led to increased overall GAG and collagen synthesis (Figure 6-2). However, when normalized to DNA content, there was no difference in ECM synthesis rates on a per cell basis.

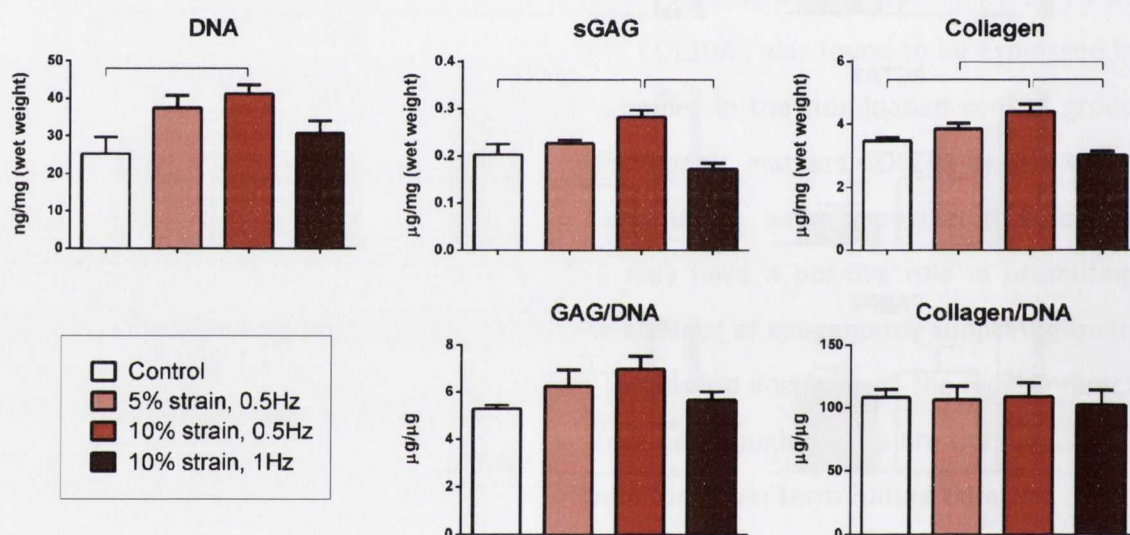


Figure 6-2: Biochemical analysis of MSC encapsulated fibrin hydrogels cultured in tensile strain bioreactors in media supplemented with chondrogenic growth factor TGF- β 3 for 21 days. The application of 10% cyclic tensile strain at 0.5 Hz was found to promote cellular proliferation, which led to an increase in total cartilage matrix deposition. Significant differences between groups are indicated where $P < 0.05$ ($n = 5$).

10% CTS at 1Hz was also found to significantly enhance several markers of chondrogenesis (ACAN and SOX9), but inhibit COL2A1 expression in MSCs cultured in chondrogenic media for 3 weeks (Figure 6-3).

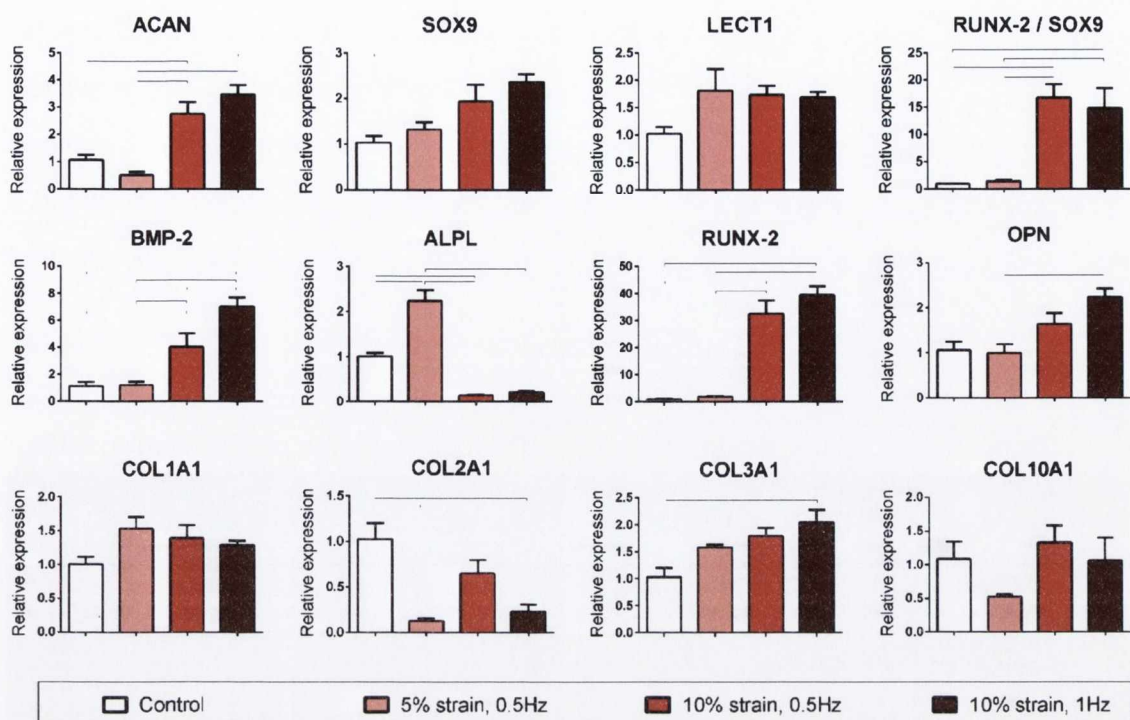


Figure 6-3: Chondrogenic and osteogenic gene expression of MSCs encapsulated in fibrin hydrogels and cultured in media supplemented with chondrogenic growth factor TGF- β 3 for 21 days. Cyclic tensile strain was found to promote endochondral ossification of chondrogenically primed MSCs in a magnitude and frequency dependent manner. Data are presented as fold-changes, relative to the non-loaded (control) group. RUNX-2/SOX9 ratio is presented as fold change/fold change. Significant differences between groups are indicated where $P < 0.05$ ($n = 5$). See Table 1 for explanation of gene symbols.

CTS was also found to enhance the expression of genetic markers associated with an osteogenic phenotype in a magnitude and frequency dependent manner (Figure 6-3). Application of smaller strains appeared to be insufficient to evoke a response in many of the osteogenic markers investigated, with only ALPL upregulated following application of 5% CTS ($\uparrow 2.24 \pm 0.18$, $P < 0.0001$). Of the loading regimens investigated, a tensile stimulus of 10% strain at 1Hz was observed to evoke the largest increase in osteogenic gene expression and resulted in a significant upregulation of BMP-2, RUNX2, COL3A1 and OPN ($\uparrow 7.02 \pm 0.92$, $\uparrow 39.41 \pm 0.434$, $\uparrow 2.05 \pm 0.25$, and $\uparrow 2.24 \pm 0.31$; $P < 0.015$) when compared to the non-loaded control group. Furthermore, the ratio of RUNX2 expression to SOX9 expression was dramatically increased in the 10% CTS groups (Figure 6-3). The magnitude of strain appeared to effect gene expression to a greater degree than

frequency, with the only apparent difference due to strain frequency being a smaller increase in BMP-2 expression in samples strained at 0.5Hz compared to 1Hz. However, BMP-2 levels in this 10% CTS group still remained greater than those in hydrogels strained by both 0 and 5%. In contrast to the 5% CTS group, the application of 10% strain lead to a significant downregulation in ALPL expression after 21 days compared to non-loaded controls.

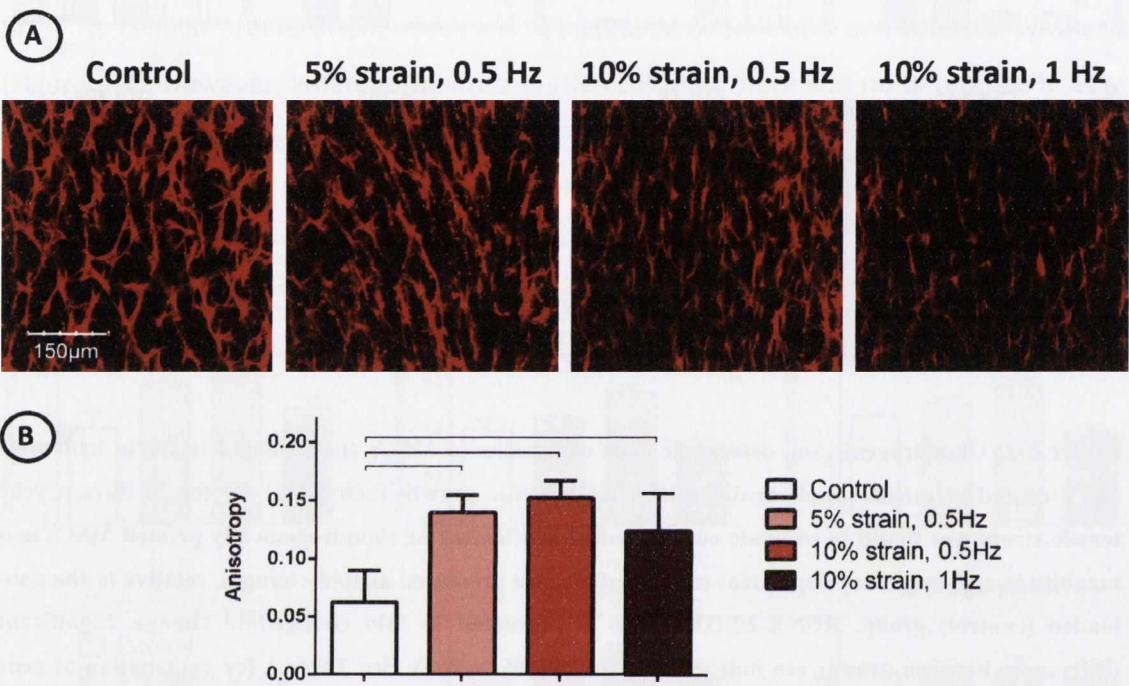


Figure 6-4: F-actin staining (a) and quantification of filament anisotropy (b) of MSCs encapsulated in fibrin hydrogels and cultured in tensile strain bioreactors in media supplemented with chondrogenic growth factor TGF-β3 for 21 days. The application of cyclic tensile strain was found to increase cellular organisation. Significant differences between groups are indicated where $P<0.05$ ($n = 5$).

6.4.3 Cyclic tensile strain promotes cellular alignment of MSCs in 3D fibrin hydrogels

Filamentous-actin staining and confocal imaging of MSC laden 3D hydrogels showed spindle shaped, spread cells with well-developed actin networks in all groups (Figure 6-4a). Image analysis and quantification of cellular organisation revealed that the application of CTS resulted in enhanced anisotropy of the actin network in all groups subjected to mechanical strain (Figure 6-4b). Analysis of images from a replicate study showed a similar trend, but with significantly increased anisotropy in samples subjected to 10% strain only (data not shown).

6.5 Discussion

Our long bones develop and heal through the process of endochondral ossification. The objective of this study was to explore the role of mechanical cues in regulating the initiation and progression of endochondral ossification. The hypothesis was that uniaxial cyclic tensile strain would promote an endochondral phenotype in MSCs encapsulated within physiologically relevant 3D fibrin hydrogels. While it has previously been reported that CTS promotes a fibrochondrocyte phenotype in MSCs seeded in 3D hydrogels (Connelly et al. 2010), the role of this mechanical stimulus in regulating the conversion of cartilage into bone remains poorly understood. The findings of the current study suggest that CTS can promote osteogenesis of MSCs, either directly or indirectly through a cartilage intermediate, and therefore may play a key role in both intramembranous and endochondral ossification of MSCs.

The application of CTS to MSCs cultured in the absence of soluble differentiation factors was found to increase the expression of osteogenic and tenogenic markers. However, the chondrogenic markers COL2A1 and SOX9 were not expressed in either unloaded or loaded conditions (Figure 6-1). Furthermore, in agreement with recently reported findings in 2D culture systems (Li et al. 2015), it was found that mechanical loading suppressed adipogenesis of MSCs. This suggests that CTS is a positive stimulus for intramembranous ossification of MSCs, with tensile loading induced increases in BMP-2 expression a likely mechanism that warrants further investigation. Other loading modalities such as compressive and shear loading have also been shown to lead to increased growth factor expression in MSCs (Huang et al. 2004, Li et al. 2009, Li et al. 2010). Increased osteogenesis on stiff substrates has been linked to cytoskeletal tension and RhoA and RhoA kinase (ROCK) signalling, a pathway which is known to regulate remodelling of the actin cytoskeleton and play a key role as a dynamic mechanosensor (Peyton et al. 2007, Guilak et al. 2009, Khatiwala et al. 2009, Holle and Engler 2011). Mechanical cues such as cyclic stretch and fluid flow have also been shown to promote cell stiffening, enhance cytoskeletal tension and be dependent upon RhoA/ROCK signalling pathways, suggesting common molecular mechanisms translating diverse mechanical cues into increases in osteogenesis (Chiquet et al. 2004, Lee et al. 2006, Matthews et al. 2006, Arnsdorf et al. 2009). These findings add to the growing body of work pointing to the role of different mechanical cues such as substrate stiffness

(Huebsch et al. 2010, Park et al. 2011) in directing either an osteogenic or adipogenic phenotype in MSCs.

Given that long bones form and generally regenerate via endochondral ossification, we examined the influence of longer-term CTS on chondrogenically primed MSC laden 3D hydrogels to explore how this mechanical cue regulates the process of endochondral ossification. The application of CTS was found to influence the expression of both chondrogenic and osteogenic genes in a strain magnitude and frequency dependent manner. 10% tensile strain at 1Hz was found to promote both chondrogenic (ACAN, SOX9) and osteogenic (BMP-2, RUNX-2, OPN, COL3A1) gene expression, whereas 5% strain was determined to be insufficient to induce upregulation of many of these markers (Figure 6-3). Interestingly, mechanical stimulation was observed to lead to an inhibition of COL2A1 expression. COL2A1 expression has previously been shown to follow a temporal profile during endochondral ossification, with peak expression observed at day 7 after BMP-2 induced bone formation (Clancy et al. 2003). MSCs in hydrogels strained at 10% also exhibited a downregulation in ALPL expression after 21 days, whereas those strained at 5% showed enhanced ALPL expression (Figure 6-3). ALPL is considered to be an early marker of osteogenesis in MSCs and is upregulated during the differentiation phase and then downregulated prior to mineralization (Kulterer et al. 2007). Therefore, the downregulation in ALPL expression observed in MSCs subjected to higher magnitude strains may further suggest that CTS is accelerating the process of endochondral ossification. Furthermore, the ratio of RUNX-2 to SOX9 was dramatically increased (>14 fold increase) following application of 10% CTS. SOX9 has been shown to be highly expressed in pre-hypertrophic chondrocytes, but not in hypertrophic chondrocytes (Zhao et al. 1997, Hattori et al. 2010). It exerts an inhibitory effect on RUNX2 function (Zhou et al. 2006) and therefore has been reported to be a negative regulator of bone marrow formation and endochondral ossification (Hattori et al. 2010). There is also evidence to suggest that increased expression of RUNX2 combined with SOX9 downregulation in hypertrophic chondrocytes is a prerequisite to initiation of the cartilage-bone transition in the growth plate (Hattori et al. 2010), and the RUNX2 : SOX9 ratio has been reported to be a positive indicator of osteogenic potential in MSCs (Loebel et al. 2014).

The application of uniaxial CTS was also found to promote re-organization of actin filaments and reduce overall anisotropy in the actin network (Figure 6-4), consistent with previously reported studies of MSCs cultured on both 2D (Hamilton et al. 2004, Chen et al. 2008) and 3D substrates (Altman et al. 2002). Anisotropic cellular and matrix organisation is a characteristic of many developing biological tissues. For example, it has been shown that collagen orientation in the perichondrium of long bones align with the direction of growth, and it has been suggested that this is due to the combination of mechanical tissue strain and the synthesis of new tissue matrix (Foolen et al. 2008). Growth plate tissue is also highly anisotropic, with distinct cellular organization; chondrocytes are arranged into vertical columns which act as functional units of longitudinal bone growth (Hunziker 1994). Since chondrocyte alignment is a hallmark of hypertrophy and endochondral ossification in developing limbs, it is likely that novel cell culture systems that reduce cellular anisotropy and guide structural alignment will be required when engineering grafts to repair structurally complex tissues.

While it is clear that the application of extrinsic mechanical loading can guide stem cell fate, a more complete understanding of how they respond to specific mechanical signals is needed for successful cell based strategies in musculoskeletal medicine. The data presented here suggest that CTS may be a positive stimulus for the promotion of both intramembranous and endochondral ossification of MSC depending on the context in which the stimulus is applied. This should inform the development of novel tissue engineering strategies for cartilage and bone regeneration.

Chapter 7 Discussion

7.1 Overview of research findings

The studies presented in this dissertation aimed to investigate how joint-specific *in vivo* environmental cues can be replicated and manipulated *in vitro* to regulate the differentiation of MSCs, their oxygen consumption rate and ultimately the composition of the tissue they produce. The articulating joint is a complex, low oxygen environment, featuring gradients in nutrient availability and extracellular matrix composition and architecture, and subjected to multiple modes of extrinsic mechanical stimuli. These studies aim to isolate and decouple these various stimuli with a view to engineering functional cartilage tissue grafts.

In Chapter 3, it was demonstrated, via a combined experimental and numerical modelling approach, that oxygen availability throughout cell seeded tissue grafts of a clinically relevant scale is drastically effected by the culture environment oxygen tension and cell seeding density of the engineered construct. These gradients were found to significantly influence both cartilage matrix synthesis and distribution; while both sGAG and collagen synthesis were enhanced in regions of hypoxia ($< 5\% \text{ PO}_2$), very low oxygen environments ($< 2\% \text{ PO}_2$) appeared to be detrimental to collagen production. Furthermore, following growth factor induced chondrogenesis, both bone marrow-derived MSCs (BMSCs) and infrapatellar fat pad-derived MSCs (FPSCs) were found to adopt an oxygen consumption rate similar to that of chondrocytes, and it is proposed that this metabolic switch may be considered a key determinant of the success of stem cell based cartilage tissue engineering strategies.

Next, the influence of extrinsic mechanical stimuli on MSC seeded, clinically relevant 3D grafts was examined through the use of various custom designed bioreactors. In Chapter 4, it was demonstrated that the application of physiological levels of cyclic hydrostatic pressure (HP) was found to enhance the functional development of cartilaginous tissues engineered using both BMSCs and FPSCs. In addition, HP was found to suppress calcification of tissues engineered using BMSCs cultured in chondrogenic conditions, thereby suppressing endochondral ossification. It also acted to maintain a

chondrogenic phenotype in cartilaginous grafts engineered using FPSCs after the withdrawal of chondrogenic growth factors.

While dynamic compressive loading (DC) is known to induce a chondrogenic phenotype and enhance cartilage matrix synthesis, while suppressing myogenesis of MSCs, less is known about the influence of cyclic tensile strain (CTS) on MSC lineage commitment in 3D grafts. In Chapter 5, a novel bioreactor system was developed and demonstrated to be capable of applying accurate tensile strains to soft 3D hydrogels, whilst maintaining cell viability within an aseptic, controlled environment. It was then demonstrated that, in contrast to the pro-chondrogenic/anti-myogenic influence of DC (Thorpe et al. 2012), CTS can suppress chondrogenic markers and promote a more myogenic phenotype in BMSC seeded collagen-alginate blended hydrogels. Furthermore, this response was found to be dependent on both matrix stiffness and MSC morphology, thus highlighting the complex interplay between tensile stimuli, matrix rigidity and cellular morphology when guiding MSC fate in 3D engineered grafts.

Finally, in Chapter 6, recognising that bone can form and heal through either an intramembranous or an endochondral pathway, the influence of CTS on these processes was examined. Mechanical loading, applied in the absence of soluble differentiation factors, was found to enhance the expression of osteogenic genetic markers, while inhibiting adipogenesis of BMSCs encapsulated in fibrin grafts, thus suggesting that CTS may have act as a positive stimulus for direct intramembranous ossification. Next, the influence of longer term CTS on hypertrophy of chondrogenically primed MSCs was investigated. The presence of cartilage extracellular matrix and chondrogenic gene expression confirmed the onset of chondrogenesis in all groups, while mechanical loading was found to significantly upregulate the expression of genes associated with osteogenesis and chondrocyte hypertrophy. This suggests that CTS may also promote endochondral ossification of MSCs in 3D fibrin grafts. Furthermore, CTS was shown to reduce anisotropy of the cellular actin network and promote MSC alignment; an outcome which will likely be of benefit when engineering tissues of complex structures.

Taken together these findings demonstrate a complex interplay of biophysical and biochemical stimuli, which must be considered when developing next generation tissue engineering strategies.

7.2 Significance of research findings

Tissue engineering strategies that attempt to recapitulate native-like tissue architecture will likely require a measure of both temporal and spatial control of the local biochemical and biophysical cellular environment, and the findings presented in this dissertation can be employed to guide these strategies. A major consideration when engineering grafts of a clinically relevant scale is oxygen and nutrient availability. While gradients in these factors may be desirable to achieve inhomogeneous and anisotropic (and thus structurally complex) tissue development, care must be taken to avoid introducing unwanted regions of oxygen deprivation. Furthermore, as demonstrated here, as MSCs adopt an oxygen consumption profile similar to that of the target phenotype, this will in turn influence the local oxygen gradients, therefore perhaps necessitating the option of dynamically adjusting and managing the local oxygen environment. However, through careful consideration of initial cell seeding density and construct geometry and knowledge of temporal changes in MSC metabolism, combined with numerical modelling techniques such as those presented here, the optimal gradients may be induced and maintained. Additionally, the minimally invasive spatial oxygen tension measuring techniques described here may also be employed to provide real-time monitoring, thereby allowing the tissue engineer to make informed adjustments to overall oxygen availability. Potential methods of adjusting oxygen availability range from more sophisticated systems, such as real-time oxygen monitoring feedback-controlled, perfusion bioreactors, to the traditional use of N_2 cell culture incubators or, as demonstrated here, simply adjusting the depth of cell culture medium above the graft. Furthermore, since such monitoring techniques could also be used in conjunction with analyses of metabolites to confirm that the target metabolic profile has been achieved. These results can then be paired with genetic profiling techniques and biochemical analyses to verify successful MSC differentiation.

The studies presented here also add to the growing body of evidence indicating that extrinsic mechanical stimuli can be a powerful tool for guiding MSC fate and, depending on the desired outcome, can be used in combination with, or in place of, biochemical stimuli such as soluble growth factors. Abnormal endochondral ossification has been implicated as contributing factor in osteoarthritis, a pathologic condition characterized by loss of cartilage, abnormal mechanical stresses and alterations in the subchondral bone architecture (Griffin and Guilak 2005, Cox et al. 2013). Furthermore, engineering of grafts for the treatment of full-thickness osteochondral defects will require an understanding of, and an ability to influence, the process of endochondral ossification (Sheehy et al. 2012, Mesallati et al. 2015, Sheehy et al. 2015). These studies demonstrated that stiffer substrates, and matrices that impose a more spherical MSC morphology, were optimal for promoting expression of several of the genetic markers associated with the onset of the endochondral process. Additionally, it was demonstrated that the application of physiological levels of HP can suppress the tendency of chondrogenically primed MSCs to undergo endochondral ossification, whereas cyclic tensile stimulation was revealed to enhance the expression of several markers associated with this process. Interestingly, CTS was also found to enhance the expression of genetic markers associated with direct intramembranous ossification in the absence of soluble growth factors. These findings have implications for the development of future regenerative strategies for treatment of osteoarthritis and osteochondral defects. Furthermore, while the main focus of this dissertation is cartilage and bone tissue engineering, CTS was also found to promote expression of genes associated with myogenic differentiation and promoted the development of a smooth muscle phenotype in MSCs encapsulated in 3D grafts. Therefore, the findings presented here may also have implications for *in vitro* engineering of vascular tissue grafts.

7.3 Limitations and future research directions

Due to limited resources, the studies presented here do not fully account for the intra-donor variation that is commonly observed when examining cellular responses to biochemical and biophysical stimuli. Therefore, it is recommended that for future studies investigating these phenomena, multiple donors be examined to confirm the findings stated in this dissertation. Furthermore, in the oxygen consumption profile study presented in Chapter 3, the local oxygen tension was monitored at the centre of each construct, whereas multiple measurement locations would increase the model validity. Also, model input values for the Michaelis-Menten constant (K_m) were taken from previously reported studies of MSC metabolic kinetics, and while the sensitivity of the simulation outcome to this value is very low, accurate measurements of this parameter, for each cell type used, would enhance model output accuracy. DNA quantification was employed to estimate cell density and spatial distribution at final time-points; however live/dead imaging techniques could be employed for improved accuracy. Finally, when investigating cellular metabolism, no model that excludes glucose transport and cellular uptake can present a full picture. Therefore, it is proposed that similar future studies investigating temporal and spatial changes in MSC metabolism in 3D grafts should involve:

- quantifying temporal changes in K_m for each culture condition,
- using live/dead cell imaging techniques (instead of spatial DNA quantifications) to more accurately measure cell density gradients in each graft for use as model inputs,
- monitoring both oxygen and glucose concentrations at multiple locations,
- developing dual-species oxygen/glucose diffusion-reaction numerical models.

Indeed, although not presented here, the author has recently developed a fully automated and programmable system that employs micro-step actuators to gradually move the sensor tip through the construct, thereby monitoring oxygen gradients throughout the tissue depth in real-time. Furthermore, the dual species oxygen-glucose diffusion-reaction numerical models have also been developed. However, following an

extensive review and testing program of currently available implantable glucose biosensors, it appears that, due to the excessive oxygen dependency of current generation electroenzymatic glucose sensors, it is unlikely that the technology currently exists for sufficiently accurate, minimally invasive, spatial glucose monitoring in such tissue constructs. In the meantime, it is suggested that micro-dialysis techniques may offer a potential, if more invasive, solution.

It is also proposed that future development of bioreactors, should be informed by the findings presented both in Chapters 4-6 of the current dissertation and in previous studies, with a view to developing systems that introduce gradients in both the biochemical and biomechanical environment. For example, bioreactor systems that induce the mechanical stimuli with a chondro-inductive (e.g. dynamic compression) and chondro-protective (e.g. intermittent hydrostatic pressure) influence in one region of a graft, while emphasizing endochondral ossification inductive stimuli (e.g. cyclic tensile strain) in the remaining section. While current systems are unlikely to facilitate such fine control, the author would suggest looking to the articulating joint for inspiration for the development of next generation biomimetic culture systems; an innovative articulating bioreactor could conceivably induce various modes of loading simultaneously. The benefits of such systems could then be complemented by employing biomaterials with graduated features, such as mechanical rigidity, integrin binding site availability, pore size and permeability. Furthermore, such a system could perhaps feature perfusion systems, which coupled with variable matrix permeability, may allow a measure of oxygen and nutrient gradient control, while growth factor eluting degradable matrices could potentially introduce tuneable, region-specific cytokine release. Such a system could potentially encourage spatial variants in stem cell phenotype, morphology and distribution tissue grafts seeded throughout with initially similar progenitor cells, and ultimately allow development of structurally complex tissue grafts.

Chapter 8 Conclusion

The objective of the studies presented here was to investigate the influence of joint-specific environmental factors on MSC differentiation and subsequent tissue formation. It was demonstrated that the fate of MSCs may be directed by varying oxygen gradients in tissue grafts and through the application of physiological levels of various modes of mechanical loading. The main findings of this thesis are as follows:

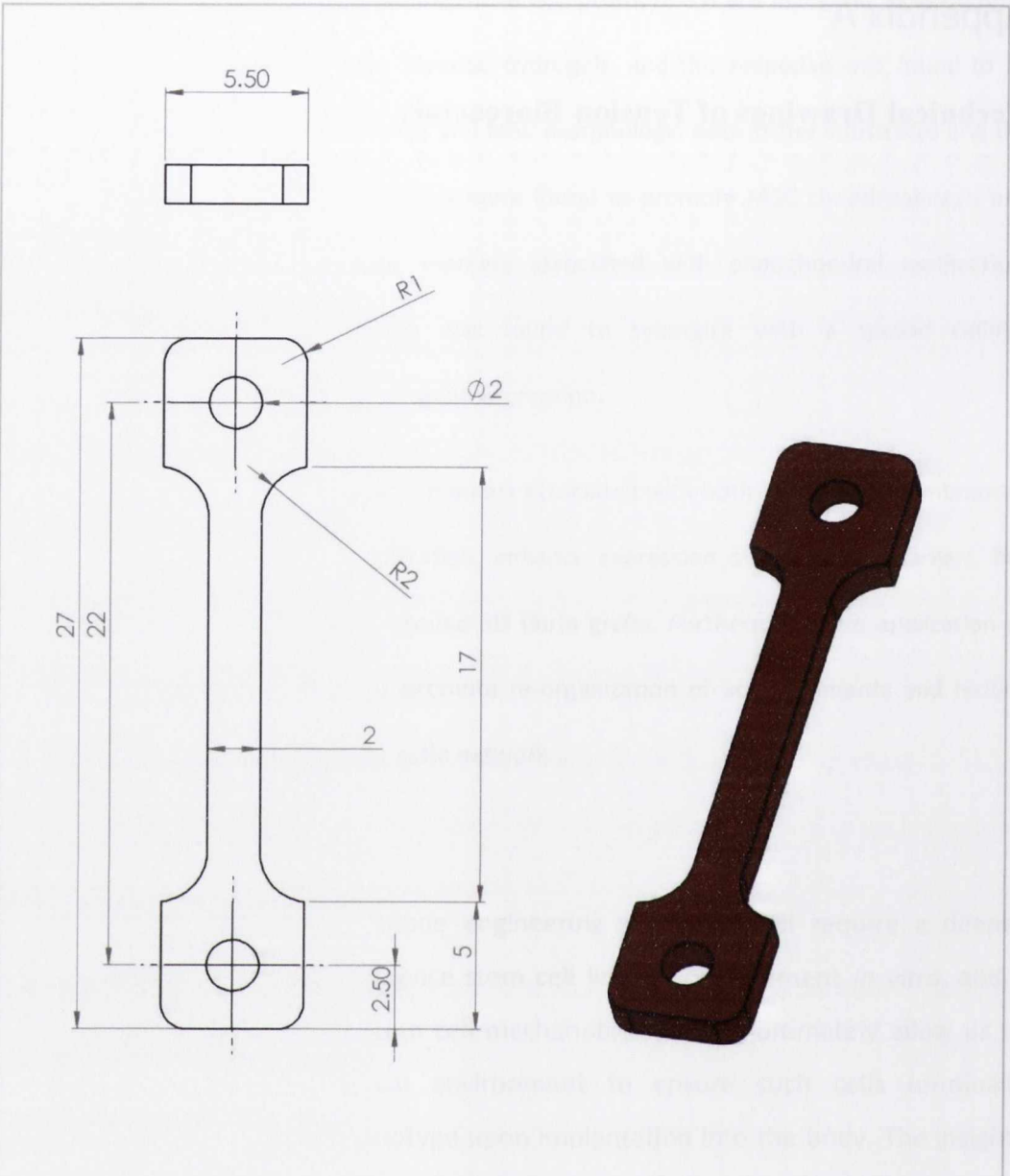
- Following growth factor induced chondrogenesis, both bone marrow-derived MSCs (BMSCs) and infrapatellar fat pad-derived MSCs (FPSCs) adopt an oxygen consumption rate similar to that of chondrocytes.
- Oxygen gradients within cell seeded hydrogels were shown to be dependent on the culture environment oxygen tension and cell seeding density of the engineered construct, and these gradients were found to significantly influence both cartilage matrix synthesis and distribution. While both sGAG and collagen synthesis is enhanced in regions of hypoxia ($< 5\% \text{ PO}_2$), very low oxygen environments ($< 2\% \text{ PO}_2$) appear to be detrimental to collagen production.
- The application of physiological levels of cyclic hydrostatic pressure (HP) was found to enhance the functional development of cartilaginous tissues engineered using both BMSCs and FPSCs, and suppress calcification of tissues engineered using BMSCs cultured in chondrogenic conditions, thereby suppressing endochondral ossification.
- A novel bioreactor system was developed and demonstrated to be capable of applying accurate uniaxial cyclic tensile strains (CTS) to soft 3D hydrogels, whilst maintaining cell viability within an aseptic, controlled environment.

- CTS was found to suppress chondrogenesis and promote a more myogenic phenotype in BMSC seeded collagen-alginate blended hydrogels, and this response was found to be dependent on both matrix stiffness and MSC morphology. Both stiffer substrates and the adoption of a round cell morphology were found to promote MSC chondrogenesis and enhance expression of genetic markers associated with endochondral ossification, whereas mechanical stimulation was found to synergize with a spread cellular morphology to enhance myogenic gene expression.
- CTS was found to promote genetic markers associated with both direct intramembranous and indirect endochondral ossification, enhance expression of tenogenic markers, but suppress adipogenesis of MSC seeded 3D fibrin grafts. Furthermore, the application of uniaxial CTS was also found to promote re-organization of actin filaments and reduce overall anisotropy in the cellular actin network.

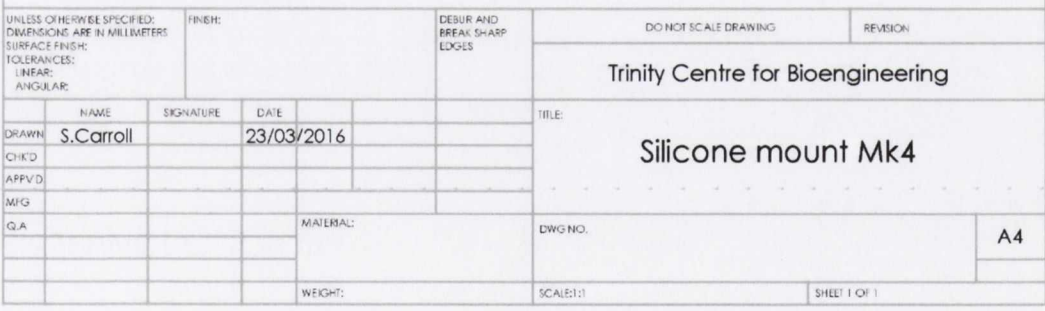
Development of next generation tissue engineering strategies will require a deeper knowledge of how such stimuli influence stem cell lineage commitment *in vitro*, and a more complete understanding of stem cell mechanobiology may ultimately allow us to manipulate the *in vivo* mechanical environment to ensure such cells terminally differentiate towards a desired phenotype upon implantation into the body. The insights provided in this dissertation demonstrate that the biophysical and biochemical environment in 3D grafts can be tailored to optimize MSC lineage commitment and subsequent tissue development.

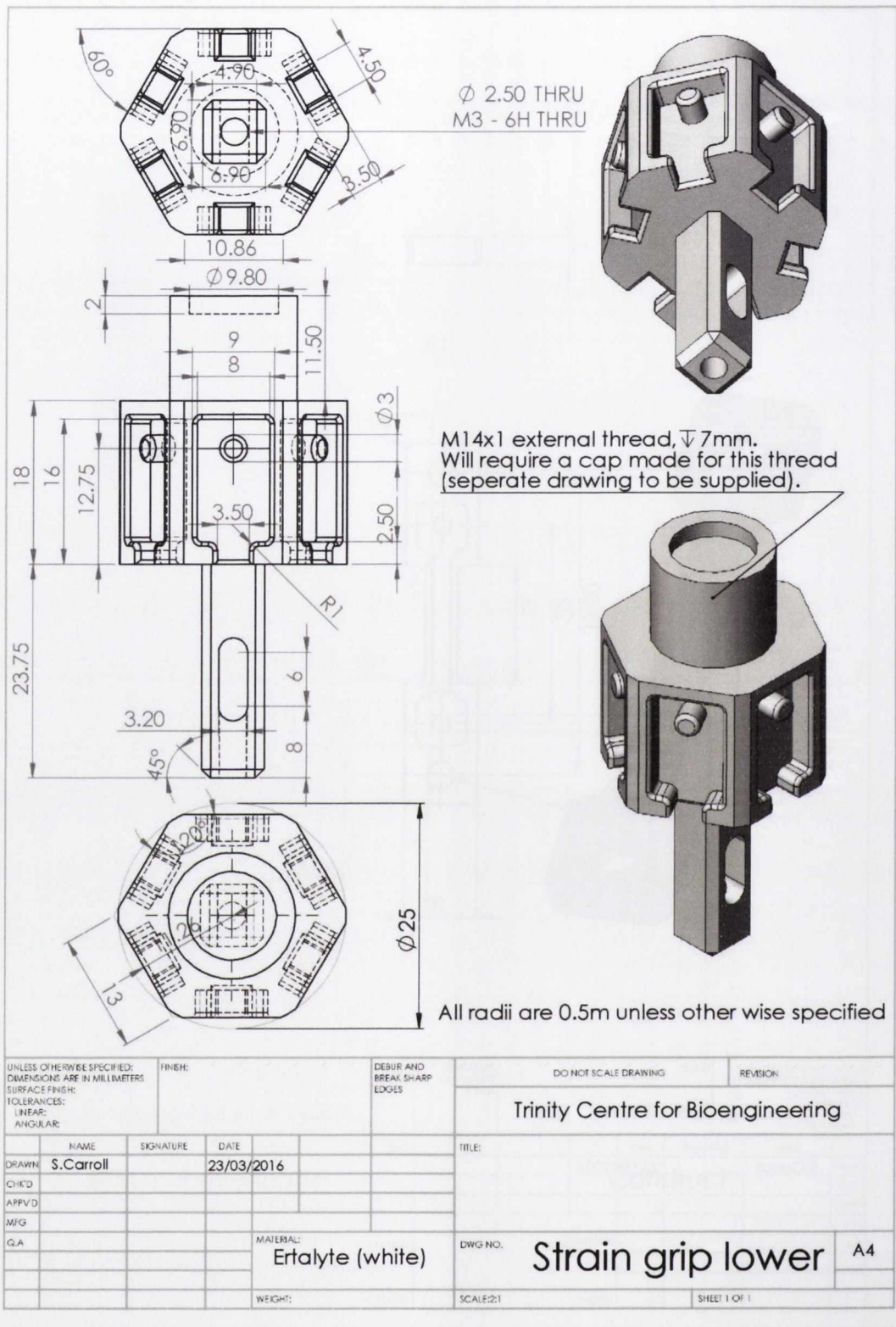
Appendix A

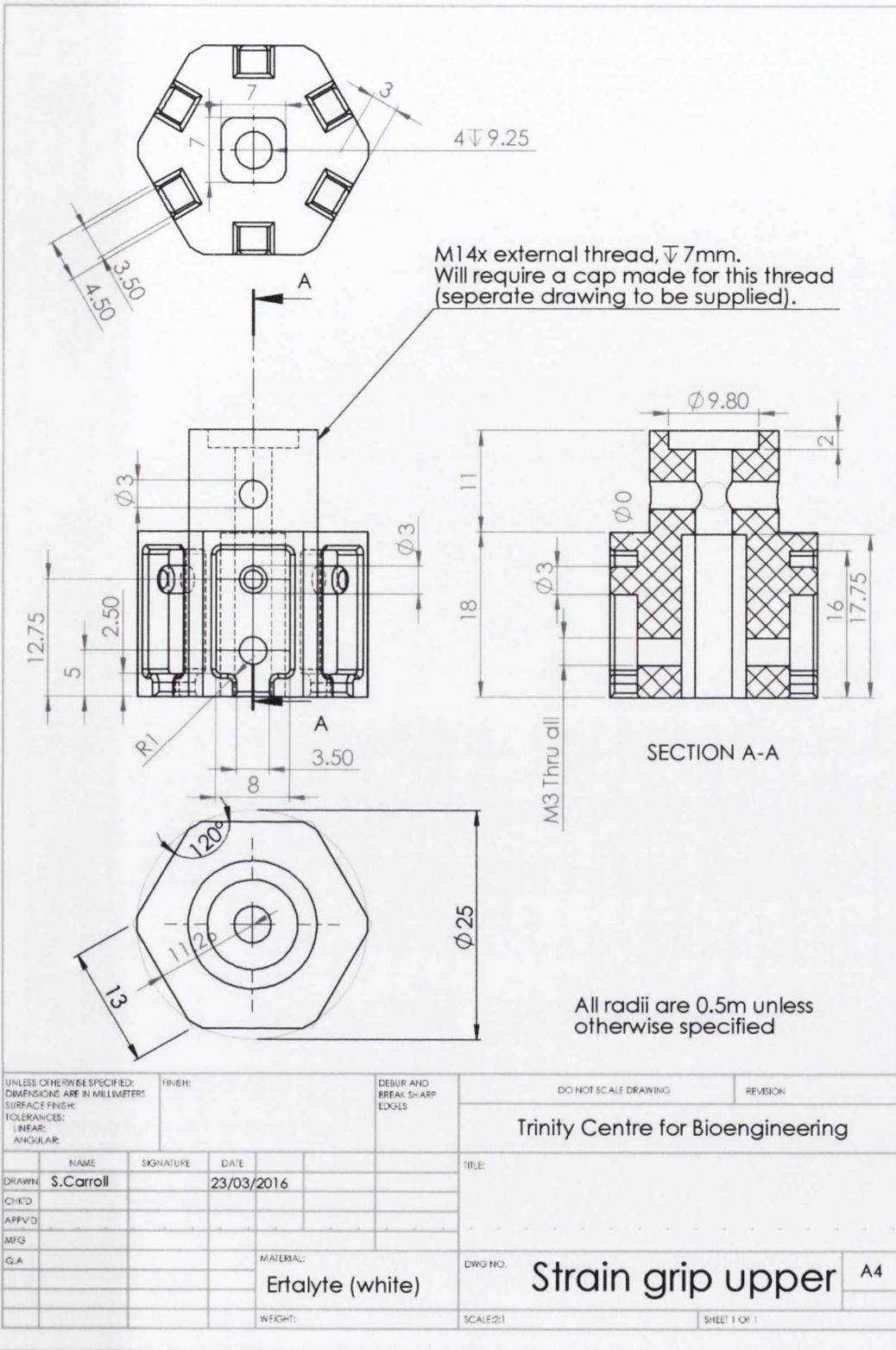
Technical Drawings of Tension Bioreactor.

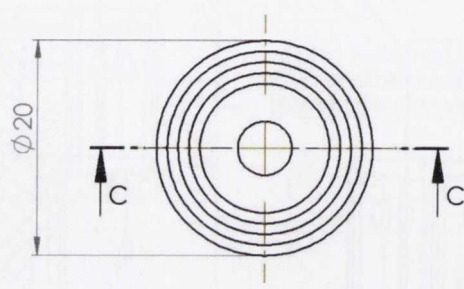
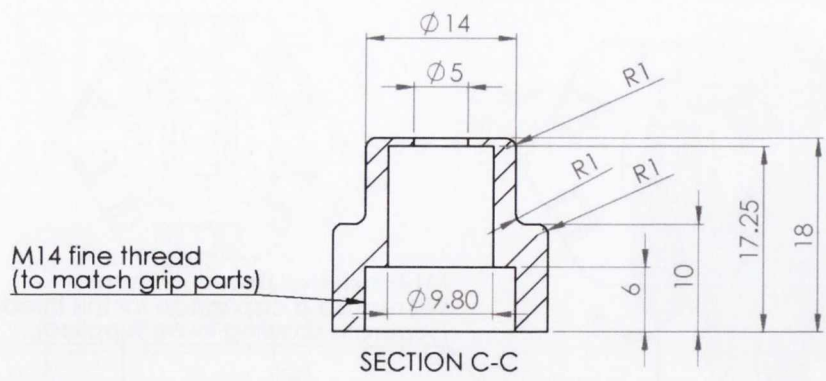


UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN MILLIMETERS SURFACE FINISH: TOLERANCES: LINEAR: ANGULAR:				FINISH:		DEBUR AND BREAK SHARP EDGES		DO NOT SCALE DRAWING		REVISION	
								Trinity Centre for Bioengineering			
								TITLE:			
								Construct			
								DWG NO.			
								A4			
								SHEET 1 OF 1			



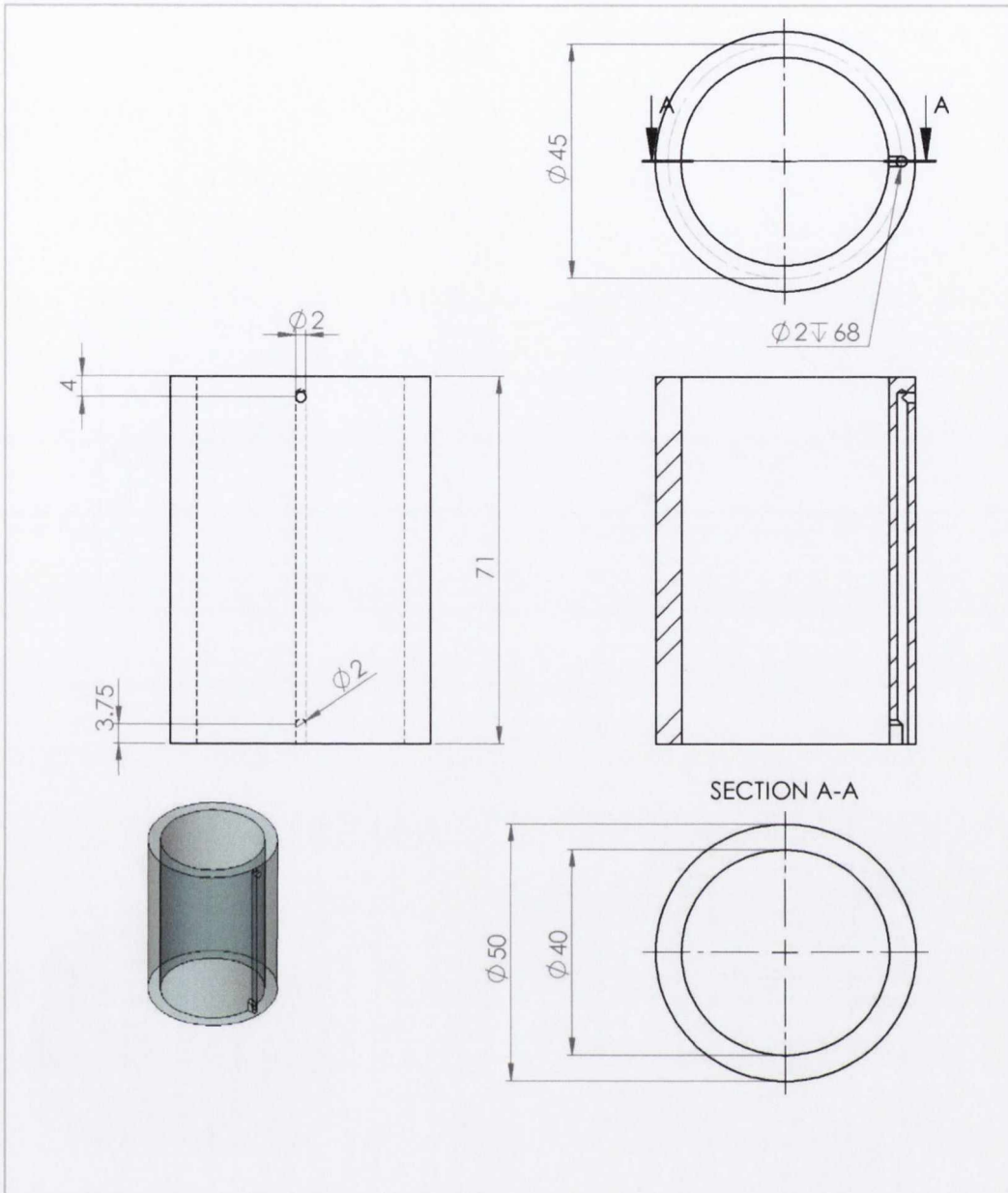




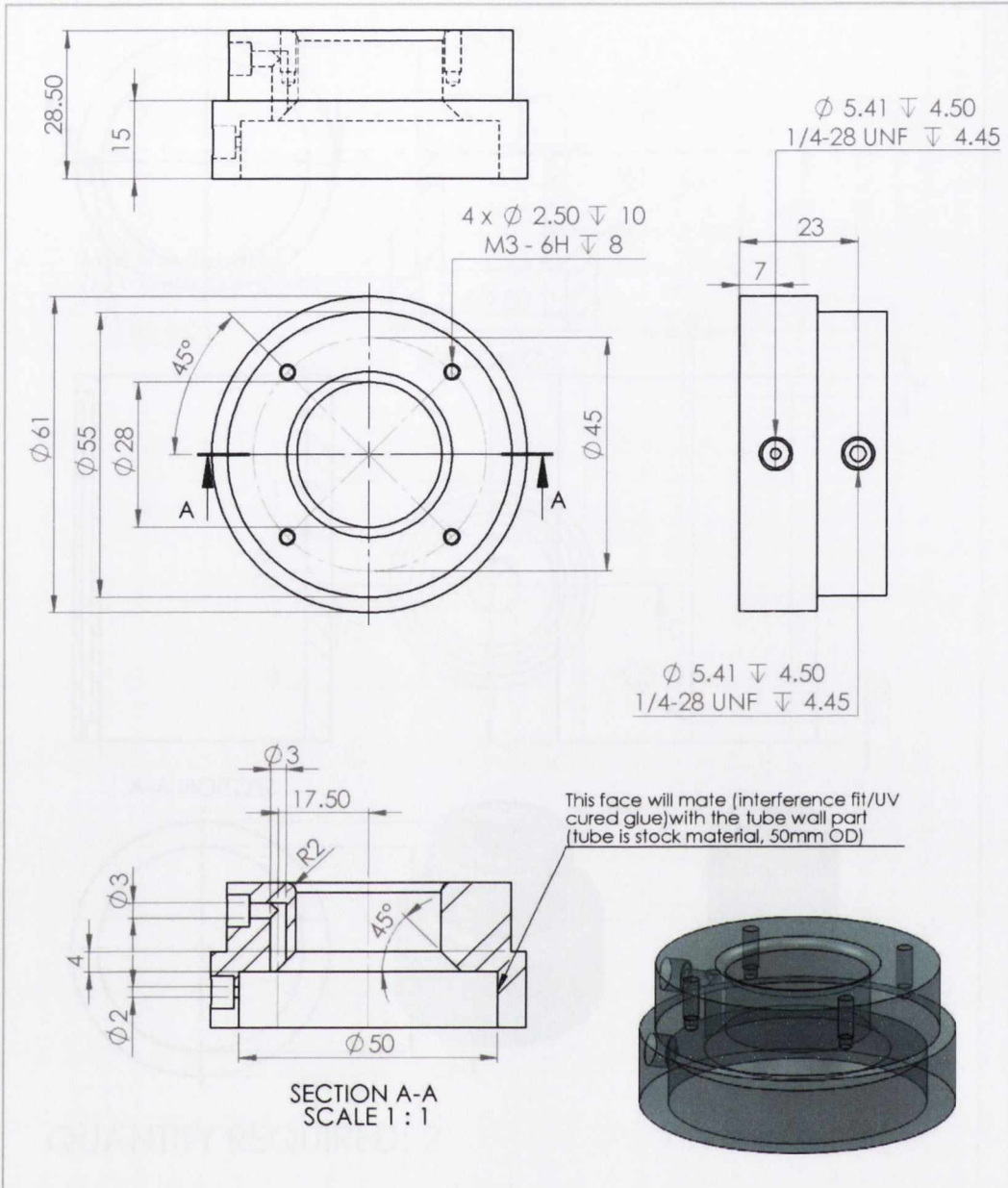


QUANTITY REQUIRED: 2

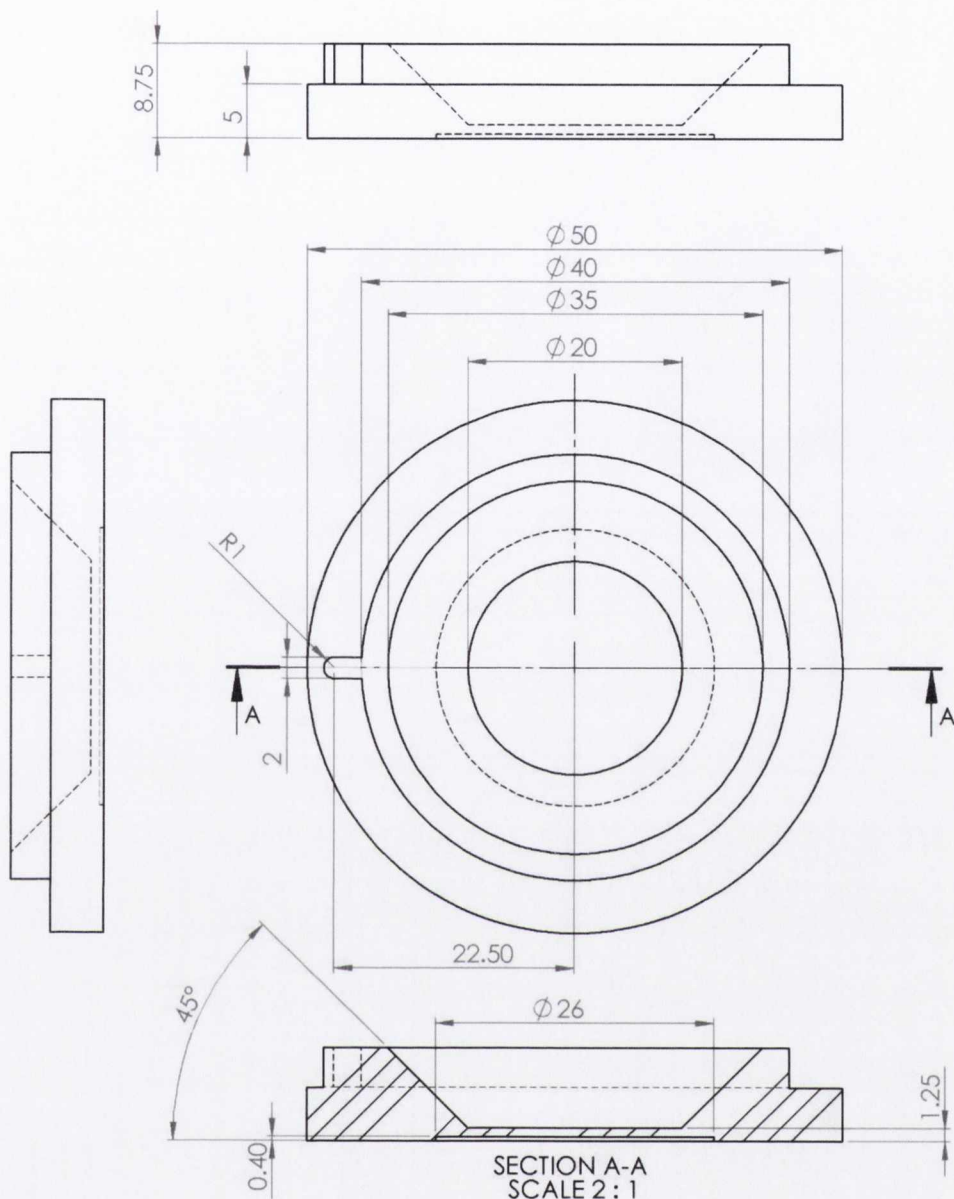
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN MILLIMETERS				FINISH:		DEBUR AND BREAK SHARP EDGES		DO NOT SCALE DRAWING		REVISION	
SURFACE FINISH:								Trinity Centre for Bioengineering			
TOLERANCES:								TITLE:			
LINEAR:								Magnet cap			
ANGULAR:											
NAME		SIGNATURE		DATE							
DRAWN: S.Carroll				31/01/2014							
CHK'D											
APPV'D											
MFG											
QA											
				MATERIAL:		Ertalyte (white)		DWG NO.		A4	
				WEIGHT:				SCALE:1:1		SHEET 1 OF 1	



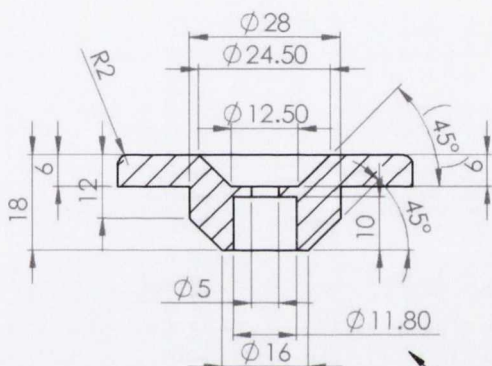
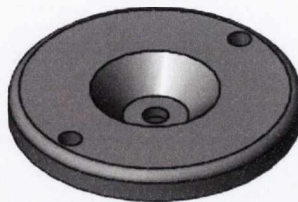
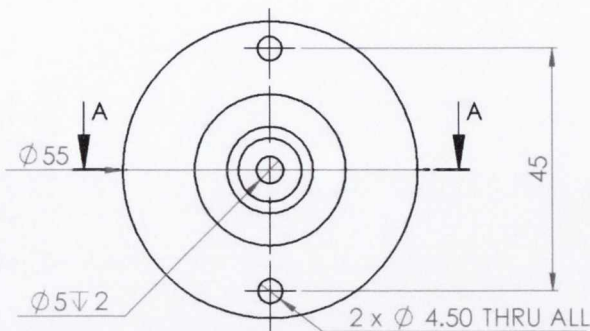
UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN MILLIMETERS				FINISH:		DEBUR AND BREAK SHARP EDGES		DO NOT SCALE DRAWING		REVISION	
SURFACE FINISH:								Trinity Centre for Bioengineering			
TOLERANCES:								TITLE:			
LINEAR:								Tube Wall			
ANGULAR:											
NAME		SIGNATURE		DATE							
DRAWN		S.Carroll		08/10/2014							
CHECKED											
APPROVED											
MFG											
Q.A											
				MATERIAL:		Polycarbonate		DWG NO.			
								A4			
				WEIGHT:				SCALE: 1:1			
								SHEET 1 OF 1			



UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN MILLIMETERS				FINISH:		DEBUR AND BREAK SHARP EDGES		DO NOT SCALE DRAWING		REVISION	
SURFACE FINISH:								Trinity Centre for Bioengineering			
TOLERANCES:											
LINEAR:											
ANGULAR:											
NAME		SIGNATURE		DATE				TITLE:			
DRAWN		S.Carroll		09/10/2014				Tube top			
CHK'D											
APP'VD											
MFG											
Q.A						MATERIAL:		DWG NO.		A4	
						Polycarbonate					
						WEIGHT:		SCALE:1:1		SHEET 1 OF 1	

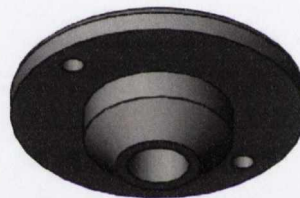


UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN MILLIMETERS				FINISH:		DEBUR AND BREAK SHARP EDGES		DO NOT SCALE DRAWING		REVISION	
SURFACE FINISH:								Trinity Centre for Bioengineering			
TOLERANCES:								TITLE:			
LINEAR:								Tube base			
ANGULAR:											
NAME		SIGNATURE		DATE							
DRAWN: S.Carroll				08/10/2014							
CHK'D											
APPV'D											
MFG											
Q.A											
				MATERIAL:		Polycarbonate		DWG NO.		A4	
				WEIGHT:				SCALE: 2:1		SHEET 1 OF 1	

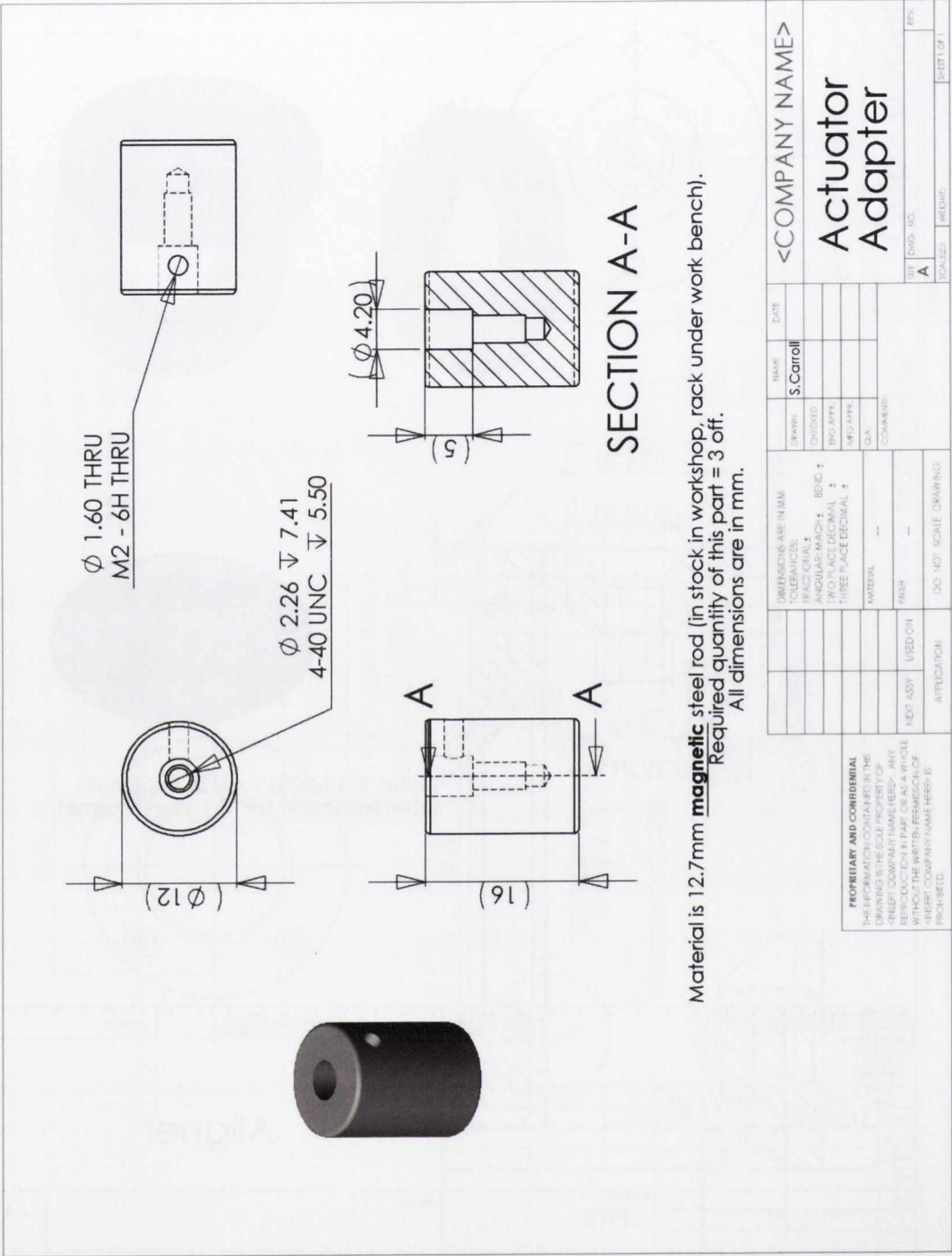


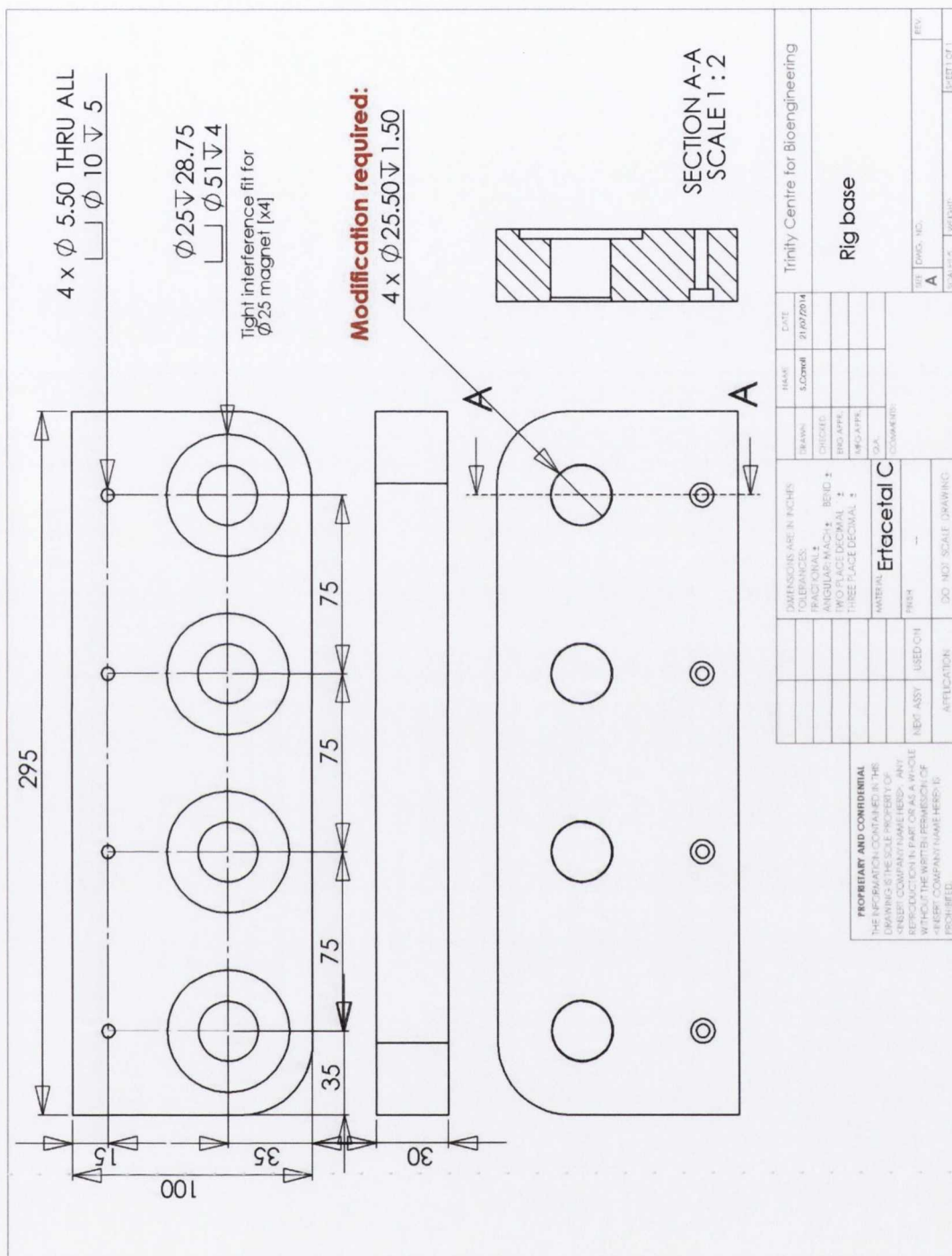
SECTION A-A

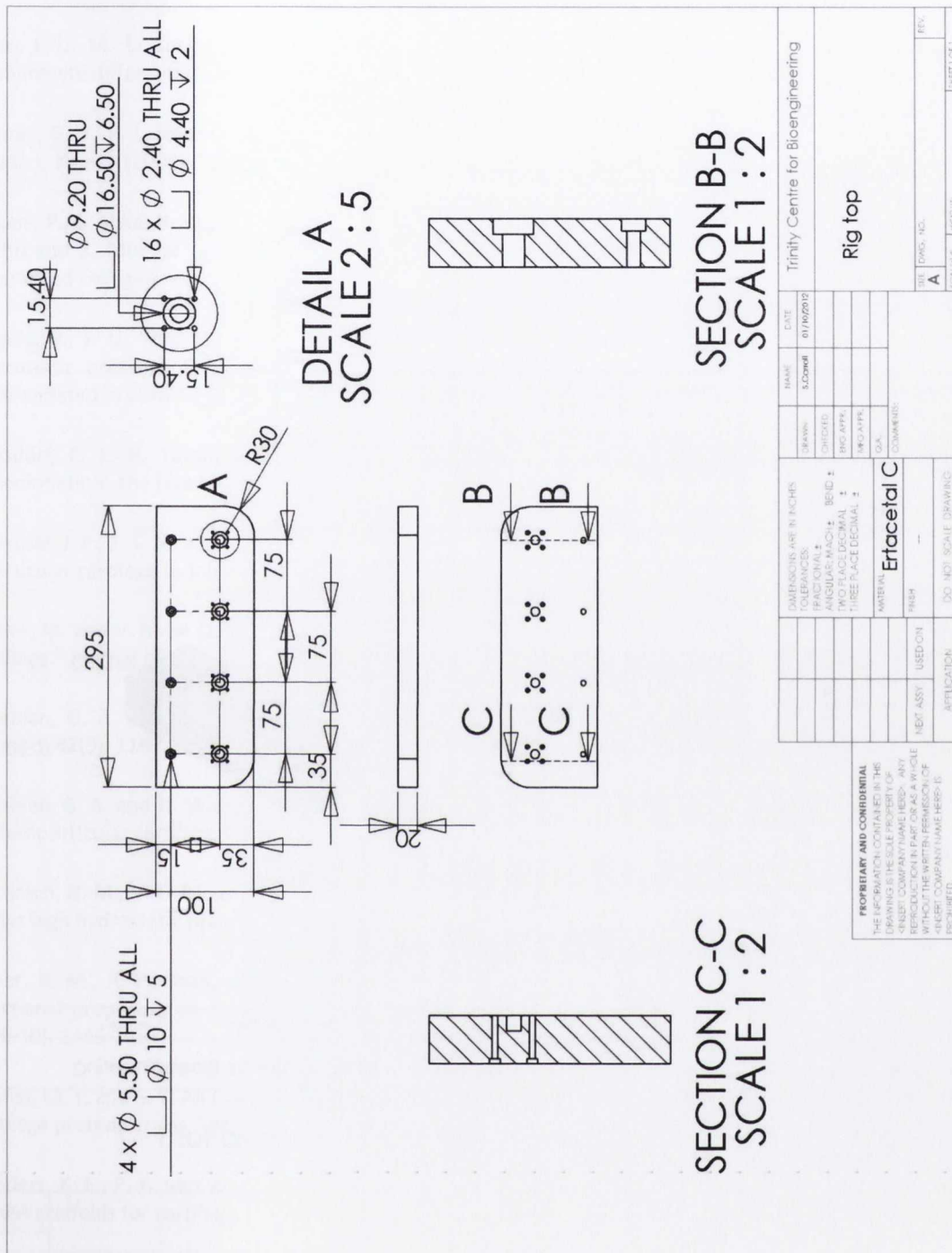
Note: $\phi 11.80$ bore is to be a tight interference fit for $\phi 12\text{mm}$ magnet.

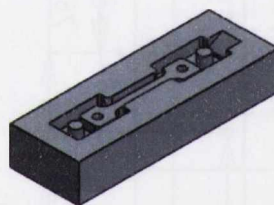
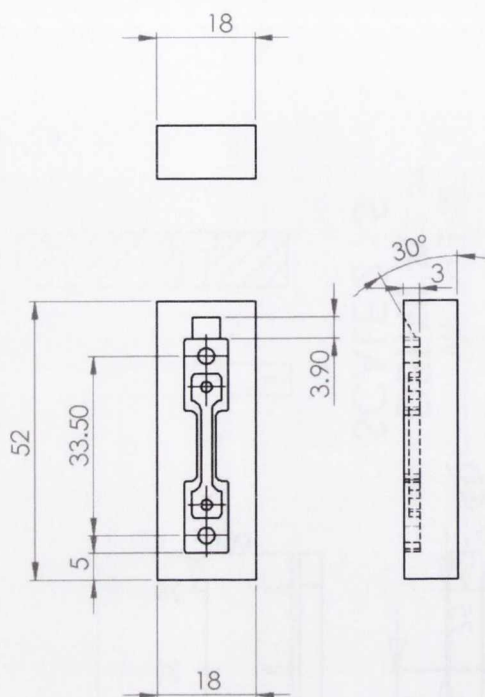


UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN MILLIMETERS		FINISH:		DEBUR AND BREAK SHARP EDGES		DO NOT SCALE DRAWING		REVISION:	
SURFACE FINISH:									
TOLERANCES:									
LINEAR:									
ANGULAR:									
NAME		SIGNATURE		DATE		TITLE:			
DRAWN: S. CARROLL				27/09/2012		Aligner			
CHK'D									
APPV'D									
MFG									
Q.A.									
				MATERIAL:		DWG NO.		A4	
				PTFE					
				WEIGHT:		SCALE: 1:1		SHEET 1 OF 1	









UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN MILLIMETERS SURFACE FINISH: TOLERANCES: LINEAR: ANGULAR:				FINISH:		DEBUR AND BREAK SHARP EDGES		DO NOT SCALE DRAWING		REVISION	
								Trinity Centre for Bioengineering			
								TITLE: Mould for 1			
NAME		SIGNATURE		DATE				DWG NO.		A4	
DRAWN: S.Carroll				23/03/2016							
CHK'D											
APP'D											
MFG											
Q.A						MATERIAL:					
						WEIGHT:		SCALE:1:1		SHEET 1 OF 1	

Bibliography

- Allen, J. L., M. E. Cooke and T. Alliston (2012). "ECM stiffness primes the TGF β pathway to promote chondrocyte differentiation." Molecular biology of the cell **23**(18): 3731-3742.
- Altman, G. H., R. L. Horan, I. Martin, J. Farhadi, P. R. Stark, V. Volloch, J. C. Richmond, G. Vunjak-Novakovic and D. L. Kaplan (2002). "Cell differentiation by mechanical stress." FASEB J **16**(2): 270-272.
- Angele, P., J. Abke, R. Kujat, H. Faltermeier, D. Schumann, M. Nerlich, B. Kinner, C. Englert, Z. Ruszczak, R. Mehrl and R. Mueller (2004). "Influence of different collagen species on physico-chemical properties of crosslinked collagen matrices." Biomaterials **25**(14): 2831-2841.
- Angele, P., J. U. Yoo, C. Smith, J. Mansour, K. J. Jepsen, M. Nerlich and B. Johnstone (2003). "Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated in vitro." J Orthop Res **21**(3): 451-457.
- Arnsdorf, E. J., P. Tummala, R. Y. Kwon and C. R. Jacobs (2009). "Mechanically induced osteogenic differentiation--the role of RhoA, ROCKII and cytoskeletal dynamics." J Cell Sci **122**(Pt 4): 546-553.
- Arokoski, J. P., J. S. Jurvelin, U. Vaatainen and H. J. Helminen (2000). "Normal and pathological adaptations of articular cartilage to joint loading." Scand J Med Sci Sports **10**(4): 186-198.
- Askew, M. and V. Mow (1978). "The biomechanical function of the collagen fibril ultrastructure of articular cartilage." Journal of biomechanical engineering **100**(3): 105-115.
- Ateshian, G. A. (2009). "The role of interstitial fluid pressurization in articular cartilage lubrication." J Biomech **42**(9): 1163-1176.
- Ateshian, G. A. and H. Wang (1995). "A theoretical solution for the frictionless rolling contact of cylindrical biphasic articular cartilage layers." J Biomech **28**(11): 1341-1355.
- Bachrach, N. M., V. C. Mow and F. Guilak (1998). "Incompressibility of the solid matrix of articular cartilage under high hydrostatic pressures." J Biomech **31**(5): 445-451.
- Baker, B. M., R. P. Shah, A. H. Huang and R. L. Mauck (2011). "Dynamic tensile loading improves the functional properties of mesenchymal stem cell-laden nanofiber-based fibrocartilage." Tissue Eng Part A **17**(9-10): 1445-1455.
- Bayliss, M. T. and S. Y. Ali (1978). "Age-related changes in the composition and structure of human articular cartilage proteoglycans." Biochem J **176**(3): 683-693.
- Benders, K. E., P. R. van Weeren, S. F. Badylak, D. B. Saris, W. J. Dhert and J. Malda (2013). "Extracellular matrix scaffolds for cartilage and bone regeneration." Trends Biotechnol **31**(3): 169-176.
- Beningo, K. A., M. Dembo and Y. L. Wang (2004). "Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors." Proc Natl Acad Sci U S A **101**(52): 18024-18029.
- Benya, P. D. and J. D. Shaffer (1982). "Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels." Cell **30**(1): 215-224.
- Bian, L., C. Hou, E. Tous, R. Rai, R. L. Mauck and J. A. Burdick (2013). "The influence of hyaluronic acid hydrogel crosslinking density and macromolecular diffusivity on human MSC chondrogenesis and hypertrophy." Biomaterials **34**(2): 413-421.

Bian, L., D. Y. Zhai, E. C. Zhang, R. L. Mauck and J. A. Burdick (2012). "Dynamic compressive loading enhances cartilage matrix synthesis and distribution and suppresses hypertrophy in hMSC-laden hyaluronic acid hydrogels." Tissue Eng Part A **18**(7-8): 715-724.

Boudaoud, A., A. Burian, D. Borowska-Wykret, M. Uyttewaal, R. Wrzalik, D. Kwiatkowska and O. Hamant (2014). "FibrilTool, an ImageJ plug-in to quantify fibrillar structures in raw microscopy images." Nat Protoc **9**(2): 457-463.

Brama, P. A., J. M. Tekoppele, R. A. Bank, A. Barneveld, E. C. Firth and P. R. van Weeren (2000). "The influence of strenuous exercise on collagen characteristics of articular cartilage in Thoroughbreds age 2 years." Equine Vet J **32**(6): 551-554.

Browning, J., R. Walker, A. Hall and R. Wilkins (1999). "Modulation of Na⁺H⁺ exchange by hydrostatic pressure in isolated bovine articular chondrocytes." Acta Physiologica Scandinavica **166**(1): 39-45.

Browning, J. A., K. Saunders, J. P. Urban and R. J. Wilkins (2004). "The influence and interactions of hydrostatic and osmotic pressures on the intracellular milieu of chondrocytes." Biorheology **41**(3-4): 299-308.

Bruder, S. P., N. Jaiswal and S. E. Haynesworth (1997). "Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation." J Cell Biochem **64**(2): 278-294.

Buckley, C. T. and D. J. Kelly (2012). "Expansion in the presence of FGF-2 enhances the functional development of cartilaginous tissues engineered using infrapatellar fat pad derived MSCs." J Mech Behav Biomed Mater **11**: 102-111.

Buckley, C. T., E. G. Meyer and D. J. Kelly (2011). "The influence of construct scale on the composition and functional properties of cartilaginous tissues engineered using bone marrow-derived mesenchymal stem cells." Tissue Engineering Part A **18**(3-4): 382-396.

Buckley, C. T., S. D. Thorpe, F. J. O'Brien, A. J. Robinson and D. J. Kelly (2009). "The effect of concentration, thermal history and cell seeding density on the initial mechanical properties of agarose hydrogels." J Mech Behav Biomed Mater **2**(5): 512-521.

Buckley, C. T., T. Vinardell and D. J. Kelly (2010). "Oxygen tension differentially regulates the functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived MSCs and articular chondrocytes." Osteoarthritis Cartilage **18**(10): 1345-1354.

Buckwalter, J. and H. Mankin (1997). "Articular cartilage: tissue design and chondrocyte-matrix interactions." Instructional course lectures **47**: 477-486.

Buckwalter, J. A., V. C. Mow and A. Ratcliffe (1994). "Restoration of Injured or Degenerated Articular Cartilage." J Am Acad Orthop Surg **2**(4): 192-201.

Byrne, E. M., E. Farrell, L. A. McMahon, M. G. Haugh, F. J. O'Brien, V. A. Campbell, P. J. Prendergast and B. C. O'Connell (2008). "Gene expression by marrow stromal cells in a porous collagen-glycosaminoglycan scaffold is affected by pore size and mechanical stimulation." Journal of Materials Science: Materials in Medicine **19**(11): 3455-3463.

Bywaters, E. and M. MacKinnon (1937). "The metabolism of joint tissues." The Journal of Pathology and Bacteriology **44**(1): 247-268.

Campbell, J. J., D. A. Lee and D. L. Bader (2006). "Dynamic compressive strain influences chondrogenic gene expression in human mesenchymal stem cells." Biorheology **43**(3-4): 455-470.

Caplan, A. I. (1991). "Mesenchymal stem cells." J Orthop Res **9**(5): 641-650.

Caplan, A. I. (2007). "Adult mesenchymal stem cells for tissue engineering versus regenerative medicine." J Cell Physiol **213**(2): 341-347.

Caplan, A. I. and S. P. Bruder (2001). "Mesenchymal stem cells: building blocks for molecular medicine in the 21st century." Trends Mol Med **7**(6): 259-264.

Carey, E. J. (1922). "Direct observations on the transformation of the mesenchyme in the thigh of the pig embryo (*Sus scrofa*), with especial reference to the genesis of the thigh muscles, of the knee-and hip-joints, and of the primary bone of the femur." Journal of Morphology **37**(1): 1-77.

Carroll, S. F., C. T. Buckley and D. J. Kelly (2014). "Cyclic hydrostatic pressure promotes a stable cartilage phenotype and enhances the functional development of cartilaginous grafts engineered using multipotent stromal cells isolated from bone marrow and infrapatellar fat pad." J Biomech **47**(9): 2115-2121.

Carver, S. E. and C. A. Heath (1999). "Semi-continuous perfusion system for delivering intermittent physiological pressure to regenerating cartilage." Tissue Eng **5**(1): 1-11.

Carver, S. E. and C. A. Heath (2000). "Increasing extracellular matrix production in regenerating cartilage with intermittent physiological pressure." Biotechnol Bioeng **62**(2): 166-174.

Cernanec, J. M., J. B. Weinberg, I. Batinic-Haberle, F. Guilak and B. Fermor (2007). "Influence of oxygen tension on interleukin 1-induced peroxynitrite formation and matrix turnover in articular cartilage." J Rheumatol **34**(2): 401-407.

Chatterjee, K., S. Lin-Gibson, W. E. Wallace, S. H. Parekh, Y. J. Lee, M. T. Cicerone, M. F. Young and C. G. Simon, Jr. (2010). "The effect of 3D hydrogel scaffold modulus on osteoblast differentiation and mineralization revealed by combinatorial screening." Biomaterials **31**(19): 5051-5062.

Chen, S. S., Y. H. Falcovitz, R. Schneiderman, A. Maroudas and R. L. Sah (2001). "Depth-dependent compressive properties of normal aged human femoral head articular cartilage: relationship to fixed charge density." Osteoarthritis Cartilage **9**(6): 561-569.

Chen, Y. J., C. H. Huang, I. C. Lee, Y. T. Lee, M. H. Chen and T. H. Young (2008). "Effects of cyclic mechanical stretching on the mRNA expression of tendon/ligament-related and osteoblast-specific genes in human mesenchymal stem cells." Connect Tissue Res **49**(1): 7-14.

Chiquet, M., A. Sarasa-Renedo and V. Tunc-Civelek (2004). "Induction of tenascin-C by cyclic tensile strain versus growth factors: distinct contributions by Rho/ROCK and MAPK signaling pathways." Biochim Biophys Acta **1693**(3): 193-204.

Clancy, B. M., J. D. Johnson, A.-J. Lambert, S. Rezvankhah, A. Wong, C. Resmini, J. L. Feldman, S. Leppanen and D. D. Pittman (2003). "A gene expression profile for endochondral bone formation: oligonucleotide microarrays establish novel connections between known genes and BMP-2-induced bone formation in mouse quadriceps." Bone **33**(1): 46-63.

Connelly, J. T., E. J. Vanderploeg, J. K. Mouw, C. G. Wilson and M. E. Levenston (2010). "Tensile loading modulates bone marrow stromal cell differentiation and the development of engineered fibrocartilage constructs." Tissue Eng Part A **16**(6): 1913-1923.

Correia, C., A. L. Pereira, A. R. Duarte, A. M. Frias, A. J. Pedro, J. T. Oliveira, R. A. Sousa and R. L. Reis (2012). "Dynamic culturing of cartilage tissue: the significance of hydrostatic pressure." Tissue Eng Part A **18**(19-20): 1979-1991.

Cox, L. G., C. C. van Donkelaar, B. van Rietbergen, P. J. Emans and K. Ito (2013). "Alterations to the subchondral bone architecture during osteoarthritis: bone adaptation vs endochondral bone formation." Osteoarthritis Cartilage **21**(2): 331-338.

Creamer, P. (2000). "Osteoarthritis pain and its treatment." Curr Opin Rheumatol **12**(5): 450-455.

da Cunha, C. B., D. D. Klumpers, W. A. Li, S. T. Koshy, J. C. Weaver, O. Chaudhuri, P. L. Granja and D. J. Mooney (2014). "Influence of the stiffness of three-dimensional alginate/collagen-I interpenetrating networks on fibroblast biology." Biomaterials **35**(32): 8927-8936.

da Silva Meirelles, L., A. I. Caplan and N. B. Nardi (2008). "In search of the in vivo identity of mesenchymal stem cells." Stem Cells **26**(9): 2287-2299.

De Bari, C., F. Dell'Accio and F. P. Luyten (2004). "Failure of in vitro-differentiated mesenchymal stem cells from the synovial membrane to form ectopic stable cartilage in vivo." Arthritis and Rheumatism **50**(1): 142-150.

Demoor, M., D. Ollitrault, T. Gomez-Leduc, M. Bouyoucef, M. Hervieu, H. Fabre, J. Lafont, J. M. Denoix, F. Audigie, F. Mallein-Gerin, F. Legendre and P. Galera (2014). "Cartilage tissue engineering: molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction." Biochim Biophys Acta **1840**(8): 2414-2440.

Dickhut, A., E. Gottwald, E. Steck, C. Heisel and W. Richter (2008). "Chondrogenesis of mesenchymal stem cells in gel-like biomaterials in vitro and in vivo." Front Biosci **13**: 4517-4528.

Diederichs, S., F. Freiburger and M. van Griensven (2009). "Effects of repetitive and short time strain in human bone marrow stromal cells." J Biomed Mater Res A **88**(4): 907-915.

Domm, C., M. Schunke, K. Christesen and B. Kurz (2002). "Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension." Osteoarthritis Cartilage **10**(1): 13-22.

Doroski, D. M., M. E. Levenston and J. S. Temenoff (2010). "Cyclic tensile culture promotes fibroblastic differentiation of marrow stromal cells encapsulated in poly(ethylene glycol)-based hydrogels." Tissue Eng Part A **16**(11): 3457-3466.

Eckstein, F., M. Reiser, K.-H. Englmeier and R. Putz (2001). "In vivo morphometry and functional analysis of human articular cartilage with quantitative magnetic resonance imaging—from image to data, from data to theory." Anatomy and embryology **203**(3): 147-173.

Engler, A. J., S. Sen, H. L. Sweeney and D. E. Discher (2006). "Matrix elasticity directs stem cell lineage specification." Cell **126**(4): 677-689.

Enomoto-Iwamoto, M., M. Iwamoto, Y. Mukudai, Y. Kawakami, T. Nohno, Y. Higuchi, S. Takemoto, H. Ohuchi, S. Noji and K. Kurisu (1998). "Bone morphogenetic protein signaling is required for maintenance of differentiated phenotype, control of proliferation, and hypertrophy in chondrocytes." The Journal of cell biology **140**(2): 409-418.

Erickson, I. E., A. H. Huang, C. Chung, R. T. Li, J. A. Burdick and R. L. Mauck (2009). "Differential maturation and structure-function relationships in mesenchymal stem cell- and chondrocyte-seeded hydrogels." Tissue Eng Part A **15**(5): 1041-1052.

Eyrich, D., F. Brandl, B. Appel, H. Wiese, G. Maier, M. Wenzel, R. Staudenmaier, A. Goepferich and T. Blunk (2007). "Long-term stable fibrin gels for cartilage engineering." Biomaterials **28**(1): 55-65.

Farng, E., A. R. Urdaneta, D. Barba, S. Esmende and D. R. McAllister (2008). "The effects of GDF-5 and uniaxial strain on mesenchymal stem cells in 3-D culture." Clin Orthop Relat Res **466**(8): 1930-1937.

Farrell, M. J., E. S. Comeau and R. L. Mauck (2012). "Mesenchymal stem cells produce functional cartilage matrix in three-dimensional culture in regions of optimal nutrient supply." Eur Cell Mater **23**(425): 425-440.

- Farrell, M. J., M. B. Fisher, A. H. Huang, J. I. Shin, K. M. Farrell and R. L. Mauck (2014). "Functional properties of bone marrow-derived MSC-based engineered cartilage are unstable with very long-term in vitro culture." J Biomech **47**(9): 2173-2182.
- Fehrer, C., R. Brunauer, G. Laschober, H. Unterluggauer, S. Reitering, F. Kloss, C. Güllly, R. Gaßner and G. Lepperdinger (2007). "Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan." Aging cell **6**(6): 745-757.
- Felson, D. T., R. C. Lawrence, P. A. Dieppe, R. Hirsch, C. G. Helmick, J. M. Jordan, R. S. Kington, N. E. Lane, M. C. Nevitt, Y. Zhang, M. Sowers, T. McAlindon, T. D. Spector, A. R. Poole, S. Z. Yanovski, G. Ateshian, L. Sharma, J. A. Buckwalter, K. D. Brandt and J. F. Fries (2000). "Osteoarthritis: new insights. Part 1: the disease and its risk factors." Ann Intern Med **133**(8): 635-646.
- Fermor, B., S. E. Christensen, I. Youn, J. M. Cernanec, C. M. Davies and J. B. Weinberg (2007). "Oxygen, nitric oxide and articular cartilage." Eur Cell Mater **13**: 56-65; discussion 65.
- Fermor, B., A. Gurumurthy and B. O. Diekman (2010). "Hypoxia, RONS and energy metabolism in articular cartilage." Osteoarthritis Cartilage **18**(9): 1167-1173.
- Finger, A. R., C. Y. Sargent, K. O. Dulaney, S. H. Bernacki and E. G. Loba (2007). "Differential effects on messenger ribonucleic acid expression by bone marrow-derived human mesenchymal stem cells seeded in agarose constructs due to ramped and steady applications of cyclic hydrostatic pressure." Tissue engineering **13**(6): 1151-1158.
- Foolen, J., C. van Donkelaar, N. Nowlan, P. Murphy, R. Huiskes and K. Ito (2008). "Collagen orientation in periosteum and perichondrium is aligned with preferential directions of tissue growth." Journal of Orthopaedic Research **26**(9): 1263-1268.
- Fox, A. J. S., A. Bedi and S. A. Rodeo (2009). "The basic science of articular cartilage: structure, composition, and function." Sports Health: A Multidisciplinary Approach **1**(6): 461-468.
- Friedl, G., H. Schmidt, I. Rehak, G. Kostner, K. Schauenstein and R. Windhager (2007). "Undifferentiated human mesenchymal stem cells (hMSCs) are highly sensitive to mechanical strain: transcriptionally controlled early osteo-chondrogenic response in vitro." Osteoarthritis Cartilage **15**(11): 1293-1300.
- Frohlich, M., W. L. Grayson, L. Q. Wan, D. Marolt, M. Drobic and G. Vunjak-Novakovic (2008). "Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance." Curr Stem Cell Res Ther **3**(4): 254-264.
- Fukuhara, S., S. Tomita, S. Yamashiro, T. Morisaki, C. Yutani, S. Kitamura and T. Nakatani (2003). "Direct cell-cell interaction of cardiomyocytes is key for bone marrow stromal cells to go into cardiac lineage in vitro." The Journal of thoracic and cardiovascular surgery **125**(6): 1470-1479.
- Gannon, A. R., T. Nagel and D. J. Kelly (2012). "The role of the superficial region in determining the dynamic properties of articular cartilage." Osteoarthritis Cartilage **20**(11): 1417-1425.
- Gao, L., R. McBeath and C. S. Chen (2010). "Stem cell shape regulates a chondrogenic versus myogenic fate through Rac1 and N-Cadherin." Stem Cells **28**(3): 564-572.
- Ghazanfari, S., M. Tafazzoli-Shadpour and M. A. Shokrgozar (2009). "Effects of cyclic stretch on proliferation of mesenchymal stem cells and their differentiation to smooth muscle cells." Biochemical and biophysical research communications **388**(3): 601-605.
- Gillette, B. M., J. A. Jensen, M. Wang, J. Tchao and S. K. Sia (2010). "Dynamic Hydrogels: Switching of 3D Microenvironments Using Two-Component Naturally Derived Extracellular Matrices." Advanced Materials **22**(6): 686-691.

Govoni, M., F. Lotti, L. Biagiotti, M. Lannocca, G. Pasquinelli, S. Valente, C. Muscari, F. Bonafe, C. M. Caldarera, C. Guarnieri, S. Cavalcanti and E. Giordano (2014). "An innovative stand-alone bioreactor for the highly reproducible transfer of cyclic mechanical stretch to stem cells cultured in a 3D scaffold." J Tissue Eng Regen Med **8**(10): 787-793.

Grant, J. L. and B. Smith (1963). "Bone marrow gas tensions, bone marrow blood flow, and erythropoiesis in man." Ann Intern Med **58**(5): 801-809.

Griffin, T. M. and F. Guilak (2005). "The role of mechanical loading in the onset and progression of osteoarthritis." Exerc Sport Sci Rev **33**(4): 195-200.

Grimaud, E., D. Heymann and F. Redini (2002). "Recent advances in TGF-beta effects on chondrocyte metabolism. Potential therapeutic roles of TGF-beta in cartilage disorders." Cytokine Growth Factor Rev **13**(3): 241-257.

Grimshaw, M. and R. Mason (2000). "Bovine articular chondrocyte function< i> in vitro</i> depends upon oxygen tension." Osteoarthritis and Cartilage **8**(5): 386-392.

Gu, W. Y., H. Yao, C. Y. Huang and H. S. Cheung (2003). "New insight into deformation-dependent hydraulic permeability of gels and cartilage, and dynamic behavior of agarose gels in confined compression." Journal of biomechanics **36**(4): 593-598.

Guilak, F., D. M. Cohen, B. T. Estes, J. M. Gimple, W. Liedtke and C. S. Chen (2009). "Control of stem cell fate by physical interactions with the extracellular matrix." Cell stem cell **5**(1): 17-26.

Guilak, F. and V. C. Mow (2000). "The mechanical environment of the chondrocyte: a biphasic finite element model of cell-matrix interactions in articular cartilage." J Biomech **33**(12): 1663-1673.

Hamilton, D. W., T. M. Maul and D. A. Vorp (2004). "Characterization of the response of bone marrow-derived progenitor cells to cyclic strain: implications for vascular tissue-engineering applications." Tissue Eng **10**(3-4): 361-369.

Harrison, J. S., P. Rameshwar, V. Chang and P. Bandari (2002). "Oxygen saturation in the bone marrow of healthy volunteers." Blood **99**(1): 394.

Haselgrove, J. C., I. M. Shapiro and S. F. Silverton (1993). "Computer modeling of the oxygen supply and demand of cells of the avian growth cartilage." Am J Physiol **265**(2 Pt 1): C497-506.

Hattori, T., C. Müller, S. Gebhard, E. Bauer, F. Pausch, B. Schlund, M. R. Bösl, A. Hess, C. Surmann-Schmitt and H. von der Mark (2010). "SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification." Development **137**(6): 901-911.

Haudenschild, A. K., A. H. Hsieh, S. Kapila and J. C. Lotz (2009). "Pressure and distortion regulate human mesenchymal stem cell gene expression." Ann Biomed Eng **37**(3): 492-502.

Hennig, T., H. Lorenz, A. Thiel, K. Goetzke, A. Dickhut, F. Geiger and W. Richter (2007). "Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6." J Cell Physiol **211**(3): 682-691.

Heo, S. J., N. L. Nerurkar, B. M. Baker, J. W. Shin, D. M. Elliott and R. L. Mauck (2011). "Fiber stretch and reorientation modulates mesenchymal stem cell morphology and fibrous gene expression on oriented nanofibrous microenvironments." Ann Biomed Eng **39**(11): 2780-2790.

Heywood, H. K., D. L. Bader and D. A. Lee (2006). "Rate of oxygen consumption by isolated articular chondrocytes is sensitive to medium glucose concentration." Journal of cellular physiology **206**(2): 402-410.

- Heywood, H. K., M. M. Knight and D. A. Lee (2010). "Both superficial and deep zone articular chondrocyte subpopulations exhibit the crabtree effect but have different basal oxygen consumption rates." Journal of cellular physiology **223**(3): 630-639.
- Heywood, H. K. and D. A. Lee (2008). "Monolayer expansion induces an oxidative metabolism and ROS in chondrocytes." Biochem Biophys Res Commun **373**(2): 224-229.
- Heywood, H. K. and D. A. Lee (2010). "Low oxygen reduces the modulation to an oxidative phenotype in monolayer-expanded chondrocytes." J Cell Physiol **222**(1): 248-253.
- Holle, A. W. and A. J. Engler (2011). "More than a feeling: discovering, understanding, and influencing mechanosensing pathways." Current opinion in biotechnology **22**(5): 648-654.
- Horowitz, S. B. and Y. T. Lau (1988). "A function that relates protein synthetic rates to potassium activity in vivo." Journal of cellular physiology **135**(3): 425-434.
- Hu, J. C. and K. A. Athanasiou (2006). "The effects of intermittent hydrostatic pressure on self-assembled articular cartilage constructs." Tissue Eng **12**(5): 1337-1344.
- Huang, A. H., M. J. Farrell, M. Kim and R. L. Mauck (2010). "Long-term dynamic loading improves the mechanical properties of chondrogenic mesenchymal stem cell-laden hydrogels." European Cells and Materials **19**: 72-85.
- Huang, C. Y., K. L. Hagar, L. E. Frost, Y. Sun and H. S. Cheung (2004). "Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells." Stem Cells **22**(3): 313-323.
- Huang, C. Y., P. M. Reuben and H. S. Cheung (2005). "Temporal expression patterns and corresponding protein inductions of early responsive genes in rabbit bone marrow-derived mesenchymal stem cells under cyclic compressive loading." Stem Cells **23**(8): 1113-1121.
- Huang, Y., L. Zheng, X. Gong, X. Jia, W. Song, M. Liu and Y. Fan (2012). "Effect of cyclic strain on cardiomyogenic differentiation of rat bone marrow derived mesenchymal stem cells." PLoS One **7**(4): e34960.
- Huebsch, N., P. R. Arany, A. S. Mao, D. Shvartsman, O. A. Ali, S. A. Bencherif, J. Rivera-Feliciano and D. J. Mooney (2010). "Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate." Nat Mater **9**(6): 518-526.
- Hunziker, E. B. (1994). "Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes." Microsc Res Tech **28**(6): 505-519.
- Ignat'eva, N. Y., N. A. Danilov, S. V. Averkiev, M. V. Obrezkova, V. V. Lunin and E. N. Sobol' (2007). "Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues." Journal of Analytical Chemistry **62**(1): 51-57.
- Ikenoue, T., M. C. Trindade, M. S. Lee, E. Y. Lin, D. J. Schurman, S. B. Goodman and R. L. Smith (2003). "Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro." J Orthop Res **21**(1): 110-116.
- Inerot, S., D. Heinegard, L. Audell and S. E. Olsson (1978). "Articular-cartilage proteoglycans in aging and osteoarthritis." Biochem J **169**(1): 143-156.
- Jackson, D. W. and T. M. Simon (1999). "Tissue engineering principles in orthopaedic surgery." Clin Orthop Relat Res(367 Suppl): S31-45.
- James, A. W. (2013). "Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation." Scientifica (Cairo) **2013**: 684736.

- Jang, J.-Y., S. W. Lee, S. H. Park, J. W. Shin, C. Mun, S.-H. Kim, D. H. Kim and J.-W. Shin (2011). "Combined effects of surface morphology and mechanical straining magnitudes on the differentiation of mesenchymal stem cells without using biochemical reagents." BioMed Research International **2011**.
- Jeon, E. S., H. J. Moon, M. J. Lee, H. Y. Song, Y. M. Kim, Y. C. Bae, J. S. Jung and J. H. Kim (2006). "Sphingosylphosphorylcholine induces differentiation of human mesenchymal stem cells into smooth-muscle-like cells through a TGF- β -dependent mechanism." Journal of cell science **119**(23): 4994-5005.
- Johnstone, B., T. M. Hering, A. I. Caplan, V. M. Goldberg and J. U. Yoo (1998). "In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells." Exp Cell Res **238**(1): 265-272.
- Jortikka, M. O., J. J. Parkkinen, R. I. Inkinen, J. Karner, H. T. Jarvelainen, L. O. Nelimarkka, M. I. Tammi and M. J. Lammi (2000). "The role of microtubules in the regulation of proteoglycan synthesis in chondrocytes under hydrostatic pressure." Arch Biochem Biophys **374**(2): 172-180.
- Julkunen, P., T. Harjula, J. Iivarinen, J. Marjanen, K. Seppanen, T. Narhi, J. Arokoski, M. J. Lammi, P. A. Brama, J. S. Jurvelin and H. J. Helminen (2009). "Biomechanical, biochemical and structural correlations in immature and mature rabbit articular cartilage." Osteoarthritis Cartilage **17**(12): 1628-1638.
- Juncosa-Melvin, N., K. S. Matlin, R. W. Holdcraft, V. S. Nirmalanandhan and D. L. Butler (2007). "Mechanical stimulation increases collagen type I and collagen type III gene expression of stem cell-collagen sponge constructs for patellar tendon repair." Tissue Eng **13**(6): 1219-1226.
- Jurvelin, J., I. Kiviranta, M. Tammi and H. J. Helminen (1986). "Effect of physical exercise on indentation stiffness of articular cartilage in the canine knee." Int J Sports Med **7**(2): 106-110.
- Kadiyala, S., R. G. Young, M. A. Thiede and S. P. Bruder (1997). "Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro." Cell Transplant **6**(2): 125-134.
- Kafienah, W. and T. J. Sims (2004). "Biochemical methods for the analysis of tissue-engineered cartilage." Methods Mol Biol **238**: 217-230.
- Kelly, D. and P. J. Prendergast (2004). "Effect of a degraded core on the mechanical behaviour of tissueengineered cartilage constructs: A poro-elastic finite element analysis." Medical and Biological Engineering and Computing **42**(1): 9-13.
- Kelly, D. J., A. Crawford, S. C. Dickinson, T. J. Sims, J. Mundy, A. P. Hollander, P. J. Prendergast and P. V. Hatton (2007). "Biochemical markers of the mechanical quality of engineered hyaline cartilage." J Mater Sci Mater Med **18**(2): 273-281.
- Kelly, D. J. and C. R. Jacobs (2010). "The role of mechanical signals in regulating chondrogenesis and osteogenesis of mesenchymal stem cells." Birth Defects Res C Embryo Today **90**(1): 75-85.
- Khatiwala, C. B., P. D. Kim, S. R. Peyton and A. J. Putnam (2009). "ECM compliance regulates osteogenesis by influencing MAPK signaling downstream of RhoA and ROCK." J Bone Miner Res **24**(5): 886-898.
- Khoshgoftar, M., W. Wilson, K. Ito and C. C. van Donkelaar (2013). "Influence of tissue- and cell-scale extracellular matrix distribution on the mechanical properties of tissue-engineered cartilage." Biomech Model Mechanobiol **12**(5): 901-913.
- Kim, Y.-J., R. L. Y. Sah, J.-Y. H. Doong and A. J. Grodzinsky (1988). "Fluorometric assay of DNA in cartilage explants using Hoechst 33258." Analytical Biochemistry **174**(1): 168-176.
- Kim, Y. and G. Kim (2013). "Collagen/alginate scaffolds comprising core (PCL)–shell (collagen/alginate) struts for hard tissue regeneration: fabrication, characterisation, and cellular activities." Journal of Materials Chemistry B **1**(25): 3185-3194.

- Kinner, B., J. M. Zaleskas and M. Spector (2002). "Regulation of smooth muscle actin expression and contraction in adult human mesenchymal stem cells." Exp Cell Res **278**(1): 72-83.
- Kisiday, J. D., D. D. Frisbie, C. W. McIlwraith and A. J. Grodzinsky (2009). "Dynamic compression stimulates proteoglycan synthesis by mesenchymal stem cells in the absence of chondrogenic cytokines." Tissue Eng Part A **15**(10): 2817-2824.
- Kiviranta, I., M. Tammi, J. Jurvelin, A. M. Saamanen and H. J. Helminen (1988). "Moderate running exercise augments glycosaminoglycans and thickness of articular cartilage in the knee joint of young beagle dogs." J Orthop Res **6**(2): 188-195.
- Kofoed, H., E. Sjøtoft, S. O. Siemssen and H. P. Olesen (1985). "Bone marrow circulation after osteotomy. Blood flow, pO₂, pCO₂, and pressure studied in dogs." Acta Orthop Scand **56**(5): 400-403.
- Kornblatt, J. A. and M. J. Kornblatt (2002). "The effects of osmotic and hydrostatic pressures on macromolecular systems." Biochim Biophys Acta **1595**(1-2): 30-47.
- Kreja, L., A. Liedert, H. Schlenker, R. E. Brenner, J. Fiedler, B. Friemert, L. Durselen and A. Ignatius (2012). "Effects of mechanical strain on human mesenchymal stem cells and ligament fibroblasts in a textured poly(L-lactide) scaffold for ligament tissue engineering." J Mater Sci Mater Med **23**(10): 2575-2582.
- Kreke, M. R., L. A. Sharp, Y. W. Lee and A. S. Goldstein (2008). "Effect of intermittent shear stress on mechanotransductive signaling and osteoblastic differentiation of bone marrow stromal cells." Tissue Eng Part A **14**(4): 529-537.
- Kulterer, B., G. Friedl, A. Jandrositz, F. Sanchez-Cabo, A. Prokesch, C. Paar, M. Scheideler, R. Windhager, K. H. Preisegger and Z. Trajanoski (2007). "Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation." BMC genomics **8**(1): 70.
- Kuo, C. K. and R. S. Tuan (2008). "Mechanoactive tenogenic differentiation of human mesenchymal stem cells." Tissue Eng Part A **14**(10): 1615-1627.
- Kurpinski, K., J. Chu, C. Hashi and S. Li (2006). "Anisotropic mechanosensing by mesenchymal stem cells." Proc Natl Acad Sci U S A **103**(44): 16095-16100.
- Kyburz, K. A. and K. S. Anseth (2013). "Three-dimensional hMSC motility within peptide-functionalized PEG-based hydrogels of varying adhesivity and crosslinking density." Acta Biomaterialia **9**(5): 6381-6392.
- Lai, W. M. and V. C. Mow (1980). "Drag-induced compression of articular cartilage during a permeation experiment." Biorheology **17**(1-2): 111-123.
- Lane Smith, R., K. Thomas, D. Schurman, D. Carter, M. Wong and M. Van Der Meulen (1992). "Rabbit knee immobilization: bone remodeling precedes cartilage degradation." Journal of Orthopaedic Research **10**(1): 88-95.
- Lee, C. H., A. Singla and Y. Lee (2001). "Biomedical applications of collagen." Int J Pharm **221**(1-2): 1-22.
- Lee, J. S., P. Panorchan, C. M. Hale, S. B. Khatau, T. P. Kole, Y. Tseng and D. Wirtz (2006). "Ballistic intracellular nanorheology reveals ROCK-hard cytoplasmic stiffening response to fluid flow." J Cell Sci **119**(Pt 9): 1760-1768.
- Lee, R. B. and J. P. Urban (1997). "Evidence for a negative Pasteur effect in articular cartilage." Biochem J **321** (Pt 1): 95-102.
- Li, R., L. Liang, Y. Dou, Z. Huang, H. Mo, Y. Wang and B. Yu (2015). "Mechanical stretch inhibits mesenchymal stem cell adipogenic differentiation through TGFβ1/Smad2 signaling." Journal of biomechanics **48**(13): 3656-3662.

- Li, Z., L. Kupcsik, S. J. Yao, M. Alini and M. J. Stoddart (2010). "Mechanical load modulates chondrogenesis of human mesenchymal stem cells through the TGF- β pathway." Journal of cellular and molecular medicine **14**(6a): 1338-1346.
- Li, Z., S.-J. Yao, M. Alini and M. J. Stoddart (2009). "Chondrogenesis of human bone marrow mesenchymal stem cells in fibrin-polyurethane composites is modulated by frequency and amplitude of dynamic compression and shear stress." Tissue Engineering Part A **16**(2): 575-584.
- Lightfoot, E. N. and K. A. Duca (1999). The Roles of Mass Transfer in Tissue Function. The Biomedical Engineering Handbook, Second Edition. 2 Volume Set, CRC Press.
- Lima, E. G., L. Bian, K. W. Ng, R. L. Mauck, B. A. Byers, R. S. Tuan, G. A. Ateshian and C. T. Hung (2007). "The beneficial effect of delayed compressive loading on tissue-engineered cartilage constructs cultured with TGF- β 3." Osteoarthritis and Cartilage **15**(9): 1025-1033.
- Liu, Y., C. T. Buckley, R. Downey, K. J. Mulhall and D. J. Kelly (2012). "The Role of Environmental Factors in Regulating the Development of Cartilaginous Grafts Engineered Using Osteoarthritic Human Infrapatellar Fat Pad-Derived Stem Cells." Tissue Engineering Part A.
- Liu, Y., C. T. Buckley, K. J. Mulhall and D. J. Kelly (2013). "Combining BMP-6, TGF- β 3 and hydrostatic pressure stimulation enhances the functional development of cartilage tissues engineered using human infrapatellar fat pad derived stem cells." Biomaterials Science.
- Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method." methods **25**(4): 402-408.
- Loebel, C., E. M. Czekanska, M. Bruderer, G. Salzmann, M. Alini and M. J. Stoddart (2014). "In vitro osteogenic potential of human mesenchymal stem cells is predicted by runx2/sox9 ratio." Tissue Engineering Part A **21**(1-2): 115-123.
- Lu, X. L. and V. C. Mow (2008). "Biomechanics of articular cartilage and determination of material properties." Med Sci Sports Exerc **40**(2): 193-199.
- Luo, L., S. D. Thorpe, C. T. Buckley and D. J. Kelly (2015). "The effects of dynamic compression on the development of cartilage grafts engineered using bone marrow and infrapatellar fat pad derived stem cells." Biomedical Materials **10**(5): 055011.
- Macirowski, T., S. Tepic and R. W. Mann (1994). "Cartilage stresses in the human hip joint." J Biomech Eng **116**(1): 10-18.
- Mackay, A. M., S. C. Beck, J. M. Murphy, F. P. Barry, C. O. Chichester and M. F. Pittenger (1998). "Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow." Tissue Eng **4**(4): 415-428.
- Malda, J., D. E. Martens, J. Tramper, C. A. van Blitterswijk and J. Riesle (2003). "Cartilage tissue engineering: controversy in the effect of oxygen." Crit Rev Biotechnol **23**(3): 175-194.
- Malda, J., J. Rouwkema, D. E. Martens, E. P. Le Comte, F. K. Kooy, J. Tramper, C. A. van Blitterswijk and J. Riesle (2004). "Oxygen gradients in tissue-engineered PEGT/PBT cartilaginous constructs: measurement and modeling." Biotechnol Bioeng **86**(1): 9-18.
- Malladi, P., Y. Xu, M. Chiou, A. J. Giaccia and M. T. Longaker (2006). "Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells." Am J Physiol Cell Physiol **290**(4): C1139-1146.
- Marlovits, S., P. Zeller, P. Singer, C. Resinger and V. Vecsei (2006). "Cartilage repair: generations of autologous chondrocyte transplantation." Eur J Radiol **57**(1): 24-31.

Maroudas, A., M. Bayliss and M. Venn (1980). "Further studies on the composition of human femoral head cartilage." Annals of the rheumatic diseases **39**(5): 514-523.

Maroudas, A., P. Bullough, S. Swanson and M. Freeman (1968). "The permeability of articular cartilage." Journal of Bone & Joint Surgery, British Volume **50**(1): 166-177.

Maroudas, A., H. Muir and J. Wingham (1969). "The correlation of fixed negative charge with glycosaminoglycan content of human articular cartilage." Biochim Biophys Acta **177**(3): 492-500.

Martin, I., B. Obradovic, L. E. Freed and G. Vunjak-Novakovic (1999). "Method for quantitative analysis of glycosaminoglycan distribution in cultured natural and engineered cartilage." Ann Biomed Eng **27**(5): 656-662.

Matthews, B. D., D. R. Overby, R. Mannix and D. E. Ingber (2006). "Cellular adaptation to mechanical stress: role of integrins, Rho, cytoskeletal tension and mechanosensitive ion channels." J Cell Sci **119**(Pt 3): 508-518.

Mauck, R. L., B. A. Byers, X. Yuan and R. S. Tuan (2007). "Regulation of cartilaginous ECM gene transcription by chondrocytes and MSCs in 3D culture in response to dynamic loading." Biomech Model Mechanobiol **6**(1-2): 113-125.

Mauck, R. L., X. Yuan and R. S. Tuan (2006). "Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture." Osteoarthritis Cartilage **14**(2): 179-189.

Maul, T. M., D. W. Chew, A. Nieponice and D. A. Vorp (2011). "Mechanical stimuli differentially control stem cell behavior: morphology, proliferation, and differentiation." Biomech Model Mechanobiol **10**(6): 939-953.

Maxson, S. and K. J. Burg (2012). "Synergistic Effects of Conditioned Media and Hydrostatic Pressure on the Differentiation of Mesenchymal Stem Cells." Cellular and Molecular Bioengineering **5**(4): 414-426.

McBeath, R., D. M. Pirone, C. M. Nelson, K. Bhadriraju and C. S. Chen (2004). "Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment." Dev Cell **6**(4): 483-495.

McMahon, L. A., V. A. Campbell and P. J. Prendergast (2008). "Involvement of stretch-activated ion channels in strain-regulated glycosaminoglycan synthesis in mesenchymal stem cell-seeded 3D scaffolds." J Biomech **41**(9): 2055-2059.

Mesallati, T., E. J. Sheehy, T. Vinardell, C. T. Buckley and D. J. Kelly (2015). "Tissue engineering scaled-up, anatomically shaped osteochondral constructs for joint resurfacing." Eur Cell Mater **30**: 163-185; discussion 185-166.

Meyer, E. G., C. T. Buckley, A. J. Steward and D. J. Kelly (2011). "The effect of cyclic hydrostatic pressure on the functional development of cartilaginous tissues engineered using bone marrow derived mesenchymal stem cells." J Mech Behav Biomed Mater **4**(7): 1257-1265.

Meyer, E. G., C. T. Buckley, S. D. Thorpe and D. J. Kelly (2010). "Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression." Journal of biomechanics **43**(13): 2516-2523.

Miyamishi, K., M. C. Trindade, D. P. Lindsey, G. S. Beaupre, D. R. Carter, S. B. Goodman, D. J. Schurman and R. L. Smith (2006). "Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis in vitro." Tissue Eng **12**(6): 1419-1428.

Miyamishi, K., M. C. Trindade, D. P. Lindsey, G. S. Beaupré, D. R. Carter, S. B. Goodman, D. J. Schurman and R. L. Smith (2006). "Dose-and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor- β 3-induced chondrogenesis by adult human mesenchymal stem cells in vitro." Tissue engineering **12**(8): 2253-2262.

Mizuno, S. (2005). "A novel method for assessing effects of hydrostatic fluid pressure on intracellular calcium: a study with bovine articular chondrocytes." Am J Physiol Cell Physiol **288**(2): C329-337.

Mouw, J. K., J. T. Connelly, C. G. Wilson, K. E. Michael and M. E. Levenston (2007). "Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells." Stem Cells **25**(3): 655-663.

Mow, V. C., M. H. Holmes and W. M. Lai (1984). "Fluid transport and mechanical properties of articular cartilage: a review." J Biomech **17**(5): 377-394.

Mow, V. C., C. S. Proctor and M. A. Kelly (1989). Biomechanics of articular cartilage. Basic biomechanics of the musculoskeletal system. M. Nordin and V. H. Frankel. Philadelphia, PA ; London, Lea & Febiger: 31-57.

Mow, V. C., A. Ratcliffe and A. Robin Poole (1992). "Cartilage and diarthrodial joints as paradigms for hierarchical materials and structures." Biomaterials **13**(2): 67-97.

Muir, H., P. Bullough and A. Maroudas (1970). "The distribution of collagen in human articular cartilage with some of its physiological implications." Journal of Bone & Joint Surgery, British Volume **52**(3): 554-563.

Nagel, T. and D. J. Kelly (2013). "The composition of engineered cartilage at the time of implantation determines the likelihood of regenerating tissue with a normal collagen architecture." Tissue Engineering - Part A **19**(7-8): 824-833.

Naqvi, S. M. and C. T. Buckley (2015). "Extracellular matrix production by nucleus pulposus and bone marrow stem cells in response to altered oxygen and glucose microenvironments." J Anat **227**(6): 757-766.

Nieponice, A., T. M. Maul, J. M. Cumer, L. Soletti and D. A. Vorp (2007). "Mechanical stimulation induces morphological and phenotypic changes in bone marrow-derived progenitor cells within a three-dimensional fibrin matrix." Journal of Biomedical Materials Research Part A **81**(3): 523-530.

O'Connor, K. M. (1997). "Unweighting accelerates tidemark advancement in articular cartilage at the knee joint of rats." Journal of Bone and Mineral Research **12**(4): 580-589.

Ogawa, R., S. Mizuno, G. F. Murphy and D. P. Orgill (2009). "The effect of hydrostatic pressure on three-dimensional chondroinduction of human adipose-derived stem cells." Tissue Eng Part A **15**(10): 2937-2945.

Parekh, S. H., K. Chatterjee, S. Lin-Gibson, N. M. Moore, M. T. Cicerone, M. F. Young and C. G. Simon, Jr. (2011). "Modulus-driven differentiation of marrow stromal cells in 3D scaffolds that is independent of myosin-based cytoskeletal tension." Biomaterials **32**(9): 2256-2264.

Park, J. S., J. S. Chu, C. Cheng, F. Chen, D. Chen and S. Li (2004). "Differential effects of equiaxial and uniaxial strain on mesenchymal stem cells." Biotechnol Bioeng **88**(3): 359-368.

Park, J. S., J. S. Chu, A. D. Tsou, R. Diop, Z. Tang, A. Wang and S. Li (2011). "The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta." Biomaterials **32**(16): 3921-3930.

Park, S. H., W. Y. Sim, S. W. Park, S. S. Yang, B. H. Choi, S. R. Park, K. Park and B. H. Min (2006). "An electromagnetic compressive force by cell exciter stimulates chondrogenic differentiation of bone marrow-derived mesenchymal stem cells." Tissue Eng **12**(11): 3107-3117.

Parkkinen, J. J., J. Ikonen, M. J. Lammi, J. Laakkonen, M. Tammi and H. J. Helminen (1993). "Effects of cyclic hydrostatic pressure on proteoglycan synthesis in cultured chondrocytes and articular cartilage explants." Arch Biochem Biophys **300**(1): 458-465.

Pattappa, G., H. K. Heywood, J. D. de Bruijn and D. A. Lee (2011). "The metabolism of human mesenchymal stem cells during proliferation and differentiation." J Cell Physiol **226**(10): 2562-2570.

- Pattappa, G., S. D. Thorpe, N. C. Jegard, H. K. Heywood, J. D. de Bruijn and D. A. Lee (2013). "Continuous and uninterrupted oxygen tension influences the colony formation and oxidative metabolism of human mesenchymal stem cells." Tissue Eng Part C Methods **19**(1): 68-79.
- Paukkonen, K., H. Helminen, M. Tammi, J. Jurvelin, I. Kiviranta and A. Säämänen (1983). "Quantitative morphological and biochemical investigations on the effects of physical exercise and immobilization on the articular cartilage of young rabbits." Acta Biologica Hungarica **35**(2-4): 293-304.
- Pelttari, K., A. Winter, E. Steck, K. Goetzke, T. Hennig, B. G. Ochs, T. Aigner and W. Richter (2006). "Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice." Arthritis and Rheumatism **54**(10): 3254-3266.
- Peterson, L., M. Brittberg, I. Kiviranta, E. L. Akerlund and A. Lindahl (2002). "Autologous chondrocyte transplantation. Biomechanics and long-term durability." Am J Sports Med **30**(1): 2-12.
- Peyton, S. R., C. M. Ghajar, C. B. Khatriwala and A. J. Putnam (2007). "The emergence of ECM mechanics and cytoskeletal tension as important regulators of cell function." Cell biochemistry and biophysics **47**(2): 300-320.
- Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig and D. R. Marshak (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-147.
- Puetzer, J., J. Williams, A. Gillies, S. Bernacki and E. G. Lobo (2012). "The Effects of Cyclic Hydrostatic Pressure on Chondrogenesis and Viability of Human Adipose- and Bone Marrow-Derived Mesenchymal Stem Cells in Three-Dimensional Agarose Constructs." Tissue Engineering Part A **19**(1-2): 299-306.
- Puetzer, J. L., J. N. Petite and E. G. Lobo (2010). "Comparative review of growth factors for induction of three-dimensional in vitro chondrogenesis in human mesenchymal stem cells isolated from bone marrow and adipose tissue." Tissue Eng Part B Rev **16**(4): 435-444.
- Qi, M. C., J. Hu, S. J. Zou, H. Q. Chen, H. X. Zhou and L. C. Han (2008). "Mechanical strain induces osteogenic differentiation: Cbfa1 and Ets-1 expression in stretched rat mesenchymal stem cells." Int J Oral Maxillofac Surg **37**(5): 453-458.
- Radosevich, M., H. Goubran and T. Burnouf (1997). "Fibrin sealant: scientific rationale, production methods, properties, and current clinical use." Vox sanguinis **72**(3): 133-143.
- Rahfoth, B., J. Weisser, F. Sternkopf, T. Aigner, K. Von Der Mark and R. Bräuer (1998). "Transplantation of allograft chondrocytes embedded in agarose gel into cartilage defects of rabbits." Osteoarthritis and Cartilage **6**(1): 50-65.
- Rajpurohit, R., C. J. Koch, Z. Tao, C. M. Teixeira and I. M. Shapiro (1996). "Adaptation of chondrocytes to low oxygen tension: relationship between hypoxia and cellular metabolism." Journal of cellular physiology **168**(2): 424-432.
- Rathbone, S. R., J. R. Glossop, J. E. Gough and S. H. Cartmell (2012). "Cyclic tensile strain upon human mesenchymal stem cells in 2D and 3D culture differentially influences CCNL2, WDR61 and BAHCC1 gene expression levels." Journal of the mechanical behavior of biomedical materials **11**: 82-91.
- Redman, S. N., S. F. Oldfield and C. W. Archer (2005). "Current strategies for articular cartilage repair." Eur Cell Mater **9**: 23-32; discussion 23-32.
- Rieppo, J., M. M. Hyttinen, E. Halmesmaki, H. Ruotsalainen, A. Vasara, I. Kiviranta, J. S. Jurvelin and H. J. Helminen (2009). "Changes in spatial collagen content and collagen network architecture in porcine articular cartilage during growth and maturation." Osteoarthritis Cartilage **17**(4): 448-455.

- Rosenthal, O., M. A. Bowie and G. Wagoner (1941). "Studies in the metabolism of articular cartilage. I. Respiration and glycolysis of cartilage in relation to its age." Journal of Cellular and Comparative Physiology **17**(2): 221-233.
- Rowlands, A. S., P. A. George and J. J. Cooper-White (2008). "Directing osteogenic and myogenic differentiation of MSCs: interplay of stiffness and adhesive ligand presentation." Am J Physiol Cell Physiol **295**(4): C1037-1044.
- Russell, K. C., D. G. Phinney, M. R. Lacey, B. L. Barrilleaux, K. E. Meyertholen and K. C. O'Connor (2010). "In vitro high-capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment." Stem Cells **28**(4): 788-798.
- Säämänen, A., M. Tammi, I. Kiviranta and H. Helminen (1988). "Running exercise as a modulatory of proteoglycan matrix in the articular cartilage of young rabbits." International journal of sports medicine **9**(2): 127-133.
- Safshekan, F., M. Tafazzoli-Shadpour, M. A. Shokrgozar, N. Haghighipour, R. Mahdian and A. Hemmati (2012). "Intermittent hydrostatic pressure enhances growth factor-induced chondroinduction of human adipose-derived mesenchymal stem cells." Artif Organs **36**(12): 1065-1071.
- Sakaguchi, Y., I. Sekiya, K. Yagishita and T. Muneta (2005). "Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source." Arthritis and Rheumatism **52**(8): 2521-2529.
- Sandell, L. J. and T. Aigner (2001). "Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis." Arthritis Res **3**(2): 107-113.
- Sato, M., T. Ochi, T. Nakase, S. Hirota, Y. Kitamura, S. Nomura and N. Yasui (1999). "Mechanical Tension-Stress Induces Expression of Bone Morphogenetic Protein (BMP)-2 and BMP-4, but Not BMP-6, BMP-7, and GDF-5 mRNA, During Distraction Osteogenesis." Journal of Bone and Mineral Research **14**(7): 1084-1095.
- Segawa, Y., T. Muneta, H. Makino, A. Nimura, T. Mochizuki, Y. J. Ju, Y. Ezura, A. Umezawa and I. Sekiya (2009). "Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles." J Orthop Res **27**(4): 435-441.
- Sengers, B. G., H. K. Heywood, D. A. Lee, C. W. Oomens and D. L. Bader (2005). "Nutrient utilization by bovine articular chondrocytes: a combined experimental and theoretical approach." J Biomech Eng **127**(5): 758-766.
- Sheehy, E. J., C. T. Buckley and D. J. Kelly (2012). "Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells." Biochem Biophys Res Commun **417**(1): 305-310.
- Sheehy, E. J., T. Mesallati, L. Kelly, T. Vinardell, C. T. Buckley and D. J. Kelly (2015). "Tissue Engineering Whole Bones Through Endochondral Ossification: Regenerating the Distal Phalanx." BioResearch open access **4**(1): 229-241.
- Sheehy, E. J., T. Vinardell, C. T. Buckley and D. J. Kelly (2012). "Engineering osteochondral constructs through spatial regulation of endochondral ossification." Acta Biomaterialia.
- Simmons, C. A., S. Matlis, A. J. Thornton, S. Chen, C. Y. Wang and D. J. Mooney (2003). "Cyclic strain enhances matrix mineralization by adult human mesenchymal stem cells via the extracellular signal-regulated kinase (ERK1/2) signaling pathway." J Biomech **36**(8): 1087-1096.

Smith, R. L., S. Goodman, D. Schurman and D. Carter (2000). "Time-dependent effects of intermittent hydrostatic pressure on articular chondrocyte type II collagen and aggrecan mRNA expression." Journal of Rehabilitation Research and Development **37**(2): 153-161.

Smith, R. L., S. Rusk, B. Ellison, P. Wessells, K. Tsuchiya, D. Carter, W. Caler, L. Sandell and D. Schurman (2005). "In vitro stimulation of articular chondrocyte mRNA and extracellular matrix synthesis by hydrostatic pressure." Journal of Orthopaedic Research **14**(1): 53-60.

Soltz, M. A. and G. A. Ateshian (1998). "Experimental verification and theoretical prediction of cartilage interstitial fluid pressurization at an impermeable contact interface in confined compression." J Biomech **31**(10): 927-934.

Soltz, M. A. and G. A. Ateshian (2000). "Interstitial fluid pressurization during confined compression cyclical loading of articular cartilage." Ann Biomed Eng **28**(2): 150-159.

Steck, E., J. Fischer, H. Lorenz, T. Gotterbarm, M. Jung and W. Richter (2009). "Mesenchymal stem cell differentiation in an experimental cartilage defect: restriction of hypertrophy to bone-close neocartilage." Stem Cells Dev **18**(7): 969-978.

Steward, A., D. Wagner and D. Kelly (2013). "The pericellular environment regulates cytoskeletal development and the differentiation of mesenchymal stem cells and determines their response to hydrostatic pressure." European cells & materials **25**: 167.

Steward, A. J., S. D. Thorpe, T. Vinardell, C. T. Buckley, D. R. Wagner and D. J. Kelly (2012). "Cell-matrix interactions regulate mesenchymal stem cell response to hydrostatic pressure." Acta Biomater **8**(6): 2153-2159.

Steward, A. J., D. R. Wagner and D. J. Kelly (2014). "Exploring the roles of integrin binding and cytoskeletal reorganization during mesenchymal stem cell mechanotransduction in soft and stiff hydrogels subjected to dynamic compression." Journal of the mechanical behavior of biomedical materials **38**: 174-182.

Subramony, S. D., B. R. Dargis, M. Castillo, E. U. Azeloglu, M. S. Tracey, A. Su and H. H. Lu (2013). "The guidance of stem cell differentiation by substrate alignment and mechanical stimulation." Biomaterials **34**(8): 1942-1953.

Suh, J. K., G. H. Baek, A. Aroen, C. M. Malin, C. Niyibizi, C. H. Evans and A. Westerhausen-Larson (1999). "Intermittent sub-ambient interstitial hydrostatic pressure as a potential mechanical stimulator for chondrocyte metabolism." Osteoarthritis Cartilage **7**(1): 71-80.

Suh, J. K., Z. Li and S. L. Woo (1995). "Dynamic behavior of a biphasic cartilage model under cyclic compressive loading." J Biomech **28**(4): 357-364.

Sumanasinghe, R. D., S. H. Bernacki and E. G. Lobo (2006). "Osteogenic differentiation of human mesenchymal stem cells in collagen matrices: effect of uniaxial cyclic tensile strain on bone morphogenetic protein (BMP-2) mRNA expression." Tissue Eng **12**(12): 3459-3465.

Temenoff, J. S. and A. G. Mikos (2000). "Review: tissue engineering for regeneration of articular cartilage." Biomaterials **21**(5): 431-440.

Terraciano, V., N. Hwang, L. Moroni, H. B. Park, Z. Zhang, J. Mizrahi, D. Seliktar and J. Elisseeff (2007). "Differential response of adult and embryonic mesenchymal progenitor cells to mechanical compression in hydrogels." Stem Cells **25**(11): 2730-2738.

Thorpe, S., C. Buckley, T. Vinardell, F. J. O'Brien, V. Campbell and D. J. Kelly (2008). "Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells." Biochemical and biophysical research communications **377**(2): 458-462.

Thorpe, S. D., C. T. Buckley, A. J. Steward and D. J. Kelly (2012). "European Society of Biomechanics S.M. Perren Award 2012: the external mechanical environment can override the influence of local substrate in determining stem cell fate." J Biomech **45**(15): 2483-2492.

Thorpe, S. D., C. T. Buckley, T. Vinardell, F. J. O'Brien, V. A. Campbell and D. J. Kelly (2010). "The response of bone marrow-derived mesenchymal stem cells to dynamic compression following TGF- β 3 induced chondrogenic differentiation." Annals of biomedical engineering **38**(9): 2896-2909.

Thorpe, S. D., T. Nagel, S. F. Carroll and D. J. Kelly (2013). "Modulating gradients in regulatory signals within mesenchymal stem cell seeded hydrogels: a novel strategy to engineer zonal articular cartilage." PLoS One **8**(4): e60764.

Tuan, R. S., G. Boland and R. Tuli (2003). "Adult mesenchymal stem cells and cell-based tissue engineering." Arthritis Res Ther **5**(1): 32-45.

Vanwanseele, B., F. Eckstein, H. Knecht, E. Stussi and A. Spaepen (2002). "Knee cartilage of spinal cord-injured patients displays progressive thinning in the absence of normal joint loading and movement." Arthritis and Rheumatism **46**(8): 2073-2078.

Vinardell, T., C. T. Buckley, S. D. Thorpe and D. J. Kelly (2011). "Composition-function relations of cartilaginous tissues engineered from chondrocytes and mesenchymal stem cells isolated from bone marrow and infrapatellar fat pad." J Tissue Eng Regen Med **5**(9): 673-683.

Vinardell, T., R. A. Rolfe, C. T. Buckley, E. G. Meyer, M. Ahearne, P. Murphy and D. J. Kelly (2012). "Hydrostatic pressure acts to stabilise a chondrogenic phenotype in porcine joint tissue derived stem cells." Eur Cell Mater **23**: 121-132; discussion 133-124.

Vinardell, T., E. J. Sheehy, C. T. Buckley and D. J. Kelly (2012). "A comparison of the functionality and in vivo phenotypic stability of cartilaginous tissues engineered from different stem cell sources." Tissue Eng Part A **18**(11-12): 1161-1170.

Wagner, D. R., D. P. Lindsey, K. W. Li, P. Tummala, S. E. Chandran, R. L. Smith, M. T. Longaker, D. R. Carter and G. S. Beaupre (2008). "Hydrostatic pressure enhances chondrogenic differentiation of human bone marrow stromal cells in osteochondrogenic medium." Ann Biomed Eng **36**(5): 813-820.

Wagner, W., R. E. Feldmann, A. Seckinger, M. H. Maurer, F. Wein, J. Blake, U. Krause, A. Kalenka, H. F. Bürgers and R. Saffrich (2006). "The heterogeneity of human mesenchymal stem cell preparations—evidence from simultaneous analysis of proteomes and transcriptomes." Experimental hematology **34**(4): 536-548.

Wakitani, S., T. Kimura, A. Hirooka, T. Ochi, M. Yoneda, N. Yasui, H. Owaki and K. Ono (1989). "Repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel." J Bone Joint Surg Br **71**(1): 74-80.

Wang, J. H. and E. S. Grood (2000). "The strain magnitude and contact guidance determine orientation response of fibroblasts to cyclic substrate strains." Connect Tissue Res **41**(1): 29-36.

Waterton, J. C., S. Solloway, J. E. Foster, M. C. Keen, S. Gandy, B. J. Middleton, R. A. Maciewicz, I. Watt, P. A. Dieppe and C. J. Taylor (2000). "Diurnal variation in the femoral articular cartilage of the knee in young adult humans." Magn Reson Med **43**(1): 126-132.

Weiss, S., R. Baumgart, M. Jochum, C. J. Strasburger and M. Bidlingmaier (2002). "Systemic regulation of distraction osteogenesis: a cascade of biochemical factors." J Bone Miner Res **17**(7): 1280-1289.

Winer, J. P., P. A. Janmey, M. E. McCormick and M. Funaki (2008). "Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli." Tissue Engineering Part A **15**(1): 147-154.

- Wong, M., M. Siegrist and K. Goodwin (2003). "Cyclic tensile strain and cyclic hydrostatic pressure differentially regulate expression of hypertrophic markers in primary chondrocytes." Bone **33**(4): 685-693.
- Yang, P. J., M. E. Levenston and J. S. Temenoff (2012). "Modulation of mesenchymal stem cell shape in enzyme-sensitive hydrogels is decoupled from upregulation of fibroblast markers under cyclic tension." Tissue Engineering Part A **18**(21-22): 2365-2375.
- Yeung, T., P. C. Georges, L. A. Flanagan, B. Marg, M. Ortiz, M. Funaki, N. Zahir, W. Ming, V. Weaver and P. A. Janmey (2005). "Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion." Cell Motil Cytoskeleton **60**(1): 24-34.
- Zhang, T., F. Wen, Y. Wu, G. S. H. Goh, Z. Ge, L. P. Tan, J. H. P. Hui and Z. Yang (2015). "Cross-talk between TGF-beta/SMAD and integrin signaling pathways in regulating hypertrophy of mesenchymal stem cell chondrogenesis under deferral dynamic compression." Biomaterials **38**: 72-85.
- Zhao, Q., H. Eberspaecher, V. Lefebvre and B. De Crombrughe (1997). "Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis." Dev Dyn **209**(4): 377-386.
- Zhou, G., Q. Zheng, F. Engin, E. Munivez, Y. Chen, E. Sebal, D. Krakow and B. Lee (2006). "Dominance of SOX9 function over RUNX2 during skeletogenesis." Proc Natl Acad Sci U S A **103**(50): 19004-19009.
- Zhou, S., Z. Cui and J. P. Urban (2004). "Factors influencing the oxygen concentration gradient from the synovial surface of articular cartilage to the cartilage-bone interface: a modeling study." Arthritis and Rheumatism **50**(12): 3915-3924.
- Zirn, J. R., D. J. Schurman and R. L. Smith (1984). "Keratan sulfate content and articular cartilage maturation during postnatal rabbit growth." J Orthop Res **2**(2): 143-150.

