LEABHARLANN CHOLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH | TRINITY COLLEGE LIBRARY DUBLIN
Ollscoil Átha Cliath | The University of Dublin

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.

A study of the potential of adult mesenchymal stem cells in bone and cartilage tissue engineering

by

Eric Farrell

Thesis submitted for the degree of Doctor in Philosophy
at the University of Dublin, Trinity College

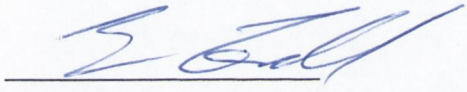
Thesis submitted September 2005

Department of Physiology
Trinity College Dublin

TRINITY COLLEGE
15 MAY 2006
LIBRARY DUBLIN
THESIS
7896

Declaration

This thesis is submitted by the undersigned for the degree of Doctor in Philosophy at the University of Dublin, Trinity College and has not been submitted to any other university as an exercise for a degree. I declare that this thesis is entirely my own work and I give permission to the library to lend or copy this thesis upon request



Eric Farrell B.A. Mod. Physiol

I Table of Contents

I	Table of contents	i
II	List of figures and tables	x
III	Acknowledgements	xiv
IV	Abstract	xv
V	List of abbreviations	xvii

Chapter 1 Introduction

1.1	Tissue engineering	1
1.2	Current bone replacement therapies	2
1.3	The tissue engineering triad	3
1.3.1	Stimulus	3
1.3.2	Cells	4
1.3.3	Scaffolds	5
1.4	Stem cells	6
1.5.1	Mesenchymal stem cells	7
1.5.2	Mesenchymal stem cell characterisation	8
1.5.3	Mesenchymal stem cell isolation	
1.6	Mesenchymal stem cell differentiation	12
1.6.1	Osteogenic differentiation	12
1.6.2	Chondrogenic differentiation	13
1.6.3	Factors affecting MSc differentiation	14
1.7	Culture of cells in three dimensions	16
1.7.1	Scaffolds	16
1.7.2	Collagen glycosaminoglycan scaffolds	18

1.8 Bioreactors 18

1.8.1 Cell seeding 21

1.8.2 Nutrient delivery and metabolite removal 22

1.8.3 Mechanical stimulation 23

1.9.1 MAP Kinases 24

1.9.2 ERK 1/2 25

1.9.3 JNK 26

1.9.4 p38 27

1.10 Phosphoisositide-3-kinase 28

1.11 Summary and aims 29

Chapter 2 Materials and Methods

2.1 Cell Culture

2.1.1 Aseptic Technique 31

2.1.2 Sterilisation of glassware, plastics and dissection instruments 31

2.1.3 Sterility of the work environment 31

2.1.4 Sterilisation of media and reagents 31

2.2 Mesenchymal stem cell isolation and culture

2.2.1 Animals 32

2.2.2 Dissection 32

2.2.3 Cell isolation and culture 33

2.3 Cell treatment

2.3.1 Cell culture in osteoinductive factors 34

2.3.2 Cell culture in chondroinductive factors 35

2.3.3 Treatment with U1026 35

2.3.4 Treatment with SB203580 **35**

2.3.5 Treatment with LY294002 **36**

2.4 Cell passaging **36**

2.5 Culture of cortical neurons **36**

2.6 Evaluation of stem cell viability

2.6.1 Immunofluorescent staining for CD105 (Endoglin) **38**

2.6.2 5-Bromo-2'-deoxy-uridine (BrdU) labelling and detection **39**

2.6.3 Aqueous One proliferation assay **39**

2.7 Sodium dodecyl sulphate polyacrylamide gel Electrophoresis (SDS-PAGE) **40**

2.7.1 Preparation of samples **40**

2.7.2 Protein quantification **40**

2.7.3 Preparation of polyacrylamide gels **41**

2.7.4 Semi-dry electrophoretic blotting of proteins **41**

2.7.5 Western immunoblotting **42**

2.7.6 Osteocalcin expression **42**

2.7.7 ERK expression **42**

2.7.8 Total actin expression **43**

2.7.9 Densitometry **44**

2.8 Scaffold seeding **44**

2.9 Paraffin wax embedding of scaffold samples **45**

2.10 Sectioning of wax embedded scaffolds **45**

2.11 Toluidine Blue staining **45**

2.12 Alizarin Red staining for calcium **46**

2.12.1 Alizarin Red S staining on scaffolds **46**

2.12.2 Alizarin Red S staining on coverslips 46

2.13 Von Kossa staining for calcium phosphate 46

2.14 Immunofluorescent staining for collagen type II 47

2.15 Immunofluorescent staining for osteocalcin 48

2.16 Immunofluorescent staining for collagen type I

2.17 Phosphorylated ERK analysis 49

2.17.1 Immunofluorescent staining for phosphorylated ERK expression in MSCs and ERK and JNK expression in neurons 49

2.17.2 Immunocytochemistry for phosphorylated ERK expression in MSCs and ERK and JNK expression in neurons 50

2.18 Immunocytochemistry for phosphorylated P38 expression in MSCs in 2-D 51

2.19 Quantification of mineralisation by stereology in collagen GAG scaffolds 52

2.20 Unconfined compression testing of scaffolds 52

2.21 Statistical Analysis 52

2.22 Collagen glycosaminoglycan scaffold manufacture 53

Chapter 3 Evaluation of mesenchymal stem cell viability and potential

3.1 Introduction 55

3.2 Results 59

3.2.1 Positive endoglin staining verifies the presence of MSCs in culture **59**

3.2.2 Cells proliferate for up to ten days in culture **59**

3.2.3 Cell proliferation begins after approximately 3 days and continues for up to 15 days **59**

3.2.4 Cells cultured in the presence of osteoinductive factors for a period of 2 weeks stop proliferating **59**

3.2.5 Collagen type I is expressed in 2-D from day three onwards **60**

3.2.6 Osteocalcin is expressed weakly in 2-D from 3 days onwards **60**

3.2.7 Cells form a mineralised matrix at day 10 of culture with osteoinductive factors **61**

3.2.8 Phospho-p42 and p44 expression are not altered significantly following osteoinductive treatment for 2 weeks **61**

3.2.9 Total ERK is not significantly different from control levels after 14 days treatment with osteoinductive factors **62**

3.2.10 Induction of Osteocalcin expression in MSCs is ERK dependant **62**

3.2.11 ERK activity is significantly increased at day 10 of osteogenic treatment **63**

3.2.12 p38 activity is significantly increased at day 7 of osteogenic treatment **64**

3.3 Discussion **65**

Chapter 4 Evaluation of the osteogenic differentiation potential of adult mesenchymal stem cells in 3-D

4.1 Introduction **72**

4.2 Results

4.2.1 In MSC-seeded scaffolds no cellular migration was observed after 7 days culture in collagen GAG

scaffolds 76

4.2.2 In MSC-seeded scaffolds no cellular migration was observed after 14 days with scaffolds experiencing complete penetration after 21 days **76**

4.2.3 Collagen I expression is evident after 3 days culture in collagen GAG scaffolds **77**

4.2.4 Collagen type I expression was sustained in collagen GAG scaffolds **77**

4.2.5 Osteocalcin expression is absent after 3 and 7 days in collagen GAG scaffolds **77**

4.2.6 Osteocalcin expression is evident after 14 and 21 days in collagen GAG scaffolds **78**

4.2.7 Matrix mineralisation is absent after 3 and 7 days in collagen GAG scaffolds **78**

4.2.8 Extensive mineralisation occurred following 21 days osteoinductive treatment **78**

4.2.9 Incomplete mineralisation is time related **79**

4.2.10 The ERK inhibitor U0126 reduced the level of matrix mineralisation in osteoinductive factor treated scaffolds **79**

4.2.11 The ERK inhibitor U0126 prevented the expression of osteocalcin in osteoinductive factor treated scaffolds **80**

4.2.12 In MSC seeded scaffolds the p38 inhibitor SB203580 did not appear to inhibit osteogenesis in osteoinductive factor treated samples **80**

4.2.13 SB203580 inhibited osteocalcin expression in osteoinductive factor treated scaffolds **81**

4.2.14 The Young's Modulus of osteoinductive factor treated scaffolds is significantly greater than controls **81**

4.3 Discussion **83**

Chapter 5 Evaluation of the chondrogenic differentiation potential of adult mesenchymal stem cells in 3-D

5.1 Introduction **89**

5.2 Results

5.2.1 In MSC seeded scaffolds, cellular migration was observed following 21 days culture in collagen GAG scaffolds **92**

5.2.2 In MSC-seeded scaffolds, 14 days treatment with chondroinductive factors led to type II collagen expression **92**

5.2.3 In MSC-seeded scaffolds, 21 days treatment with chondroinductive factors led to extensive type II collagen expression **92**

5.2.4 ERK is not involved in chondrogenic differentiation of MSCs **93**

5.2.5 p38 is not involved in chondrogenic differentiation of MSCs **93**

5.2.6 Phosphoisositide-3-kinase reduces collagen type II expression in MSC seeded scaffolds **93**

5.3 Discussion **94**

Chapter 6 Investigation of the effects of uniaxial tension on osteogenic differentiation capacity of MSCs

6.1 Introduction **101**

6.2 Results

6.2.1 A uniaxial tension rig is used for mechanical stimulation of MSCs **104**

6.2.2 Cells adhere to silicone strips following two days in culture **104**

6.2.3 2.5% strain at 0.17 Hz for 3 days does not induce MSC apoptosis **104**

6.2.4 5% strain at 0.17 Hz for 3 days does induce MSC apoptosis **105**

6.2.5 Cyclic strain at 10% and 0.17Hz for 3 days

led to significant cellular apoptosis 105

6.2.6 Collagen I but not osteocalcin was evident in strained cells following 2.5% strain at a frequency of 0.17Hz for 3 days **105**

6.2.7 Collagen I but not osteocalcin was evident in strained cells following 5% strain at a frequency of 0.17Hz for 3 days **106**

6.2.8 Collagen I but not osteocalcin was evident in strained cells following 10% strain at a frequency of 0.17Hz for 3 days **106**

6.2.9 ERK activity was not observed following 3 days stretching at 2.5% and 0.17Hz **106**

6.3 Discussion **107**

Chapter 7 Analysis of static magnetic field mediated ERK and JNK activation

7.1 Introduction **111**

7.2 Results

7.2.1 Apparatus used for culturing neurons in static magnetic field **113**

7.2.2 Static magnetic fields affect ERK activity in cultured cortical neurons **113**

7.2.3 Static magnetic fields affect JNK activity in cultured cortical neurons **114**

7.3 Discussion **115**

Chapter 8 Final discussion **118**

8.2 Future work **126**

References **127**

Appendix I Suppliers addresses **176**
Appendix II Solutions used **180**

II List of figures and tables

Figure 1.1 Age related increase in fracture incidences

Table 1.1. Phenotypic characterization of human mesenchymal stromal cultures

Figure 1.2 The tissue engineering triad

Table 1.2 Natural and synthetic polymers used for bone tissue engineering applications

Figure 1.3 The three step kinase pathway leading to MAP kinase activation

Table 1.3 Mammalian MAP Kinases

Figure 2.1 MSC cell culture 24 and 96 hours post plating

Table 2.1 Primary and secondary antibodies used for immunostaining procedures

Figure 2.2 Cultured cortical neurons (a) immediately following culture and (b) following neurite extension

Figure 3.1 Positive endoglin staining verifies the presence of MSCs in culture

Figure 3.2 Isolated cells have proliferative capacity *in vitro*

Figure 3.3 Cell proliferation begins after approximately 3 days and continues for up to 15 days

Figure 3.4 Cells cultured in the presence of osteoinductive factors for a period of 2 weeks stop proliferating

Figure 3.5 Collagen type I expression at day 0, 3 and 7 culture with osteoinductive factors

Figure 3.6 Collagen type I expression at day 10, 14 and 21 culture with osteoinductive factors

Figure 3.7 Osteocalcin expression at day 0, 3 and 7 culture with osteoinductive factors

Figure 3.8 Collagen type I expression at day 10, 14 and 21 culture with osteoinductive factors

Figure 3.9 Mineralisation at day 0, 3 and 7 culture with osteoinductive factors

Figure 3.10 Mineralisation at day 10, 14 and 21 culture with osteoinductive factors

Figure 3.11 Phospho p42 expression is not altered significantly following osteoinductive treatment for 2 weeks

Figure 3.12 Phospho p44 expression is not altered significantly following osteoinductive treatment for 2 weeks

Figure 3.13 Total ERK expression is not altered significantly following osteoinductive treatment for 2 weeks

Figure 3.14 Induction of osteocalcin expression in MSCs is ERK dependant

Figure 3.15 ERK activity is significantly increased following osteogenic treatment for 10 days.

Figure 3.16 p38 activity is significantly increased following osteogenic treatment for 7 days

Figure 4.1 In MSC-seeded scaffolds no cellular migration was observed after 3 or 7 days

Figure 4.2 In MSC-seeded scaffolds no cellular migration was observed after 14 days with scaffolds experiencing complete penetration after 21 days

Figure 4.3 Collagen I expression is evident after 3 and 7 days in collagen GAG scaffolds

Figure 4.4 Collagen I expression is evident after 14 and 21 days in collagen GAG scaffolds

Figure 4.5 Osteocalcin expression is absent after 3 and 7 days in collagen GAG scaffolds

Figure 4.6 Osteocalcin expression is evident after 14 and 21 days in collagen GAG scaffolds

Figure 4.7 Matrix mineralisation is absent after 3 and 7 days in collagen GAG scaffolds

Figure 4.8 Extensive mineralisation occurred following 21 days osteoinductive treatment

Figure 4.9 Extensive mineralisation occurred following 21 days osteoinductive treatment

Figure 4.10 In MSC-seeded scaffolds, 21 days treatment with osteoinductive factors led to matrix mineralization

Figure 4.11 Matrix mineralisation is evident after 4 weeks culture in 3-D in the presence of osteoinductive factors

Figure 4.12 Matrix mineralisation is evident after 5 weeks culture in 3-D in the presence of osteoinductive factors

Figure 4.13 Matrix mineralisation is evident after 6 weeks culture in 3-D in the presence of osteoinductive factors

Figure 4.14 Matrix mineralisation is evident after 7 weeks culture in 3-D in the presence of osteoinductive factors

Figure 4.15 The ERK inhibitor U0126 reduced the level of matrix mineralisation in osteoinductive factor treated scaffolds

Figure 4.16 The ERK inhibitor U0126 reduced the level of matrix mineralisation in osteoinductive factor treated scaffolds

Figure 4.17 The ERK inhibitor U0126 prevented the expression of osteocalcin in osteoinductive factor treated scaffolds

Figure 4.18 In MSC seeded scaffolds the p38 inhibitor SB203580 did not appear to inhibit osteogenesis in osteoinductive factor treated samples

Figure 4.19 In MSC seeded scaffolds the p38 inhibitor SB203580 did not appear to inhibit osteogenesis in osteoinductive factor treated samples

Figure 4.20 SB203580 inhibited osteocalcin expression in osteoinductive factor treated scaffolds

Figure 4.21 The stiffness of osteoinductive factor treated scaffolds is not significantly greater than controls

Figure 4.22 The stiffness of osteoinductive factor treated scaffolds is significantly greater than controls in repeated samples

Figure 5.1 In MSC-seeded scaffolds no cellular migration was observed until 21 days

Figure 5.2 In MSC-seeded scaffolds, 14 days treatment with chondroinductive factors led to type II collagen expression

Figure 5.3 In MSC-seeded scaffolds, 21 days treatment with chondroinductive factors led to extensive type II collagen expression

Figure 5.4 Inhibition of ERK activity did not have any effect on chondrogenesis

Figure 5.5 Inhibition of p38 activity did not have any effect on chondrogenesis

Figure 5.6 Spontaneous chondrogenesis was inhibited by the PI3 kinase inhibitor LY294002

Figure 5.7 Chondrogenesis was inhibited by the PI3 kinase inhibitor LY294002 in 3-D

Figure 6.1 Uniaxial tension rig

Figure 6.2 Cells adhere to silicone strips following two days in culture

Figure 6.3 2.5% strain at 0.17 Hz for 3 days does not induce MSC apoptosis

Figure 6.4 5% strain at 0.17 Hz for 3 days does not induce MSC apoptosis

Figure 6.5 Cyclic strain at 10% and 0.17Hz for 3 days led to MSC apoptosis

Figure 6.6 Collagen I but not osteocalcin was evident in strained cells following 2.5% strain at a frequency of 0.17Hz for 3 days

Figure 6.7 Collagen I but not osteocalcin was evident in strained cells following 5% strain at a frequency of 0.17Hz for 3 days

Figure 6.8 Collagen I but not osteocalcin was evident in strained cells following 10% strain at a frequency of 0.17Hz for 3 days

Figure 6.9 Following 3 days at 2.5% strain and 0.17Hz, no ERK activity was observed

Figure 7.1 Apparatus used for culturing neurons in static magnetic field

Figure 7.2 Static magnetic fields affect ERK activity in cultured cortical neurons

Figure 7.3 Static magnetic fields affect JNK activity in cultured cortical neurons

III Acknowledgements

I would like to take this opportunity to thank a long list of people. Without their help and support over the last three years, this thesis could not have been written.

Firstly I would like to extend the warmest thanks to Dr. Veronica Campbell for supervising me through my PhD. Her door was always open, both literally and figuratively. Dr. Campbell always showed great interest in my work and I consider myself extremely lucky to have benefited from her knowledge and experience during the course of my PhD.

Thank you to Prof. Chris Bell and Prof. Patrick Prendergast for permitting me to carry out this work in the Physiology department and the Trinity Centre for Bioengineering.

I would like to thank my lab companions in Physiology and Bioengineering, Marie Fogarty, Eric Downer, Roisin McCormack, Aoife Gowran, Elaine Byrne, Louise Mc Mahon, Emma Kearney, Conor Buckley it was great working with each of you. I would also like to acknowledge all those in the lab and office with whom I had the pleasure of working, Frank ,Darren, Michelle Walsh, Michelle Moore, Anne Marie, Thady, Claire, Aedin, Aileen, Yvonne, Peter, Rachel, Ceire, Amy, Joan, Noreen, Eoin and Charlotte. Thanks also to all those who I worked with on this project; Adriele Prina-Mello, Paul Doyle, Emma Kearney, Jan Fischer and Peter McColgan.

I would also like to thank all of the technical staff in Physiology and Bioengineering who helped in many ways; Kieran, Alice, Doreen, Aidan, Leslie, Quentin, Siobhan, David, Ann, Peter, Tom, Joan and Sheena. A special thank you to Leslie for all of her advice and help on all things histological. Thank you to all of the cleaning staff in Physiology and Engineering particularly to Ann Devoy for our morning chats.

I am grateful to the Programme for Research in Third Level Institutions for their financial support, enabling me to carry out this work. This work would also not have been possible without the generous donation of the collagen GAG scaffold manufactured in MIT by Brendan Harley and brought here by Dr Fergal O'Brien.

I would like to thank my parents for their constant love and support and my brothers Andrew and Carl and my sister Michelle.

Thank you to all of my friends, too numerous to be named. It was great to have all of you as welcome distractions during this work.

I would finally like to say a huge thanks Elaine for being there for me from the beginning of this journey and particularly for feeding me while I wrote up. I couldn't have done it without her.

IV Abstract

Adult mesenchymal stem cells (MSCs) have the potential to self-renew and differentiate into bone, cartilage, fat and muscle cells. This cell population offers significant potential for engineering of musculoskeletal tissue. The aim of my PhD was to generate viable bone and cartilage constructs from MSCs. Successful tissue engineering requires a suitable 3-dimensional (3-D) environment, which was provided by a novel collagen glycosaminoglycan (Collagen-GAG) scaffold. Bone and cartilage formation from adult MSCs seeded onto collagen GAG scaffolds was induced and some of the intracellular signalling mechanisms behind this process were characterised.

My work optimised the techniques required to culture MSCs for an extended period of time and verified the presence of MSCs in culture and their proliferation capacity. My first study examined the potential for MSCs to be directed along the osteogenic pathway in a 2-D environment. To that end, cells were treated with osteoinductive factors for three weeks. This led to significant osteocalcin expression, a late stage protein marker of osteogenesis. The induction of osteocalcin was abolished by the inhibition of ERK using the selective inhibitor U0126, suggestive of a role for ERK in osteogenesis. Also, a timecourse study showed that ERK activity was induced at day 10 of treatment with osteoinductive factors. Activity of p38, was shown to be upregulated at day 7.

To induce cellular differentiation in a 3-D environment, which is more applicable to tissue engineering applications, MSCs were seeded onto collagen-GAG scaffolds and treated with osteoinductive factors for 3 weeks. By this timepoint, the seeded scaffolds exhibited bone formation as evidenced by production of bone specific osteocalcin, and mineralization. Also, the seeded scaffolds when cultured with chondroinductive factors for 3 weeks showed evidence of chondrogenesis as assessed by expression of cartilage-specific collagen type II. This demonstrates the ability of MSCs to differentiate along both the osteogenic and chondrogenic lineages within collagen-GAG scaffolds. In studies where ERK and p38 activities were blocked, mineralisation and osteocalcin expression were abrogated, suggestive of roles for both ERK and p38 in the production of bone constructs. No role for ERK or p38 in chondrogenesis was observed. However, when PI3 kinase activity was inhibited, collage type II expression was reduced. When these scaffolds underwent unconfined compression testing, to measure their stiffness, it

was observed that osteoinductive factor treated scaffolds were significantly stiffer than those scaffolds cultured in control medium.

MSCs were also seeded onto 2-D silicon strips and were mechanically stimulated in a uniaxial tension rig. Cells were assessed for their ability to produce bone specific proteins. It was shown that 2.5% and 5% strain led to collagen I production an early marker of osteogenesis, in the absence of osteoinductive factors, suggesting that osteogenic differentiation may be stimulated solely by a mechanical stimulus. While 2.5% and 5% strains were not detrimental to cell survival, a strain rate of 10% led to over 50% apoptosis of cells on the strips. Osteocalcin expression was not observed in any samples following three days of cyclic stretching.

The effect of static magnetic fields on the activity of ERK and JNK in cultured cortical neurons was also assessed. ERK activity was elevated in magnetically stimulated cells at 0.75T compared to unstimulated controls and JNK activity was significantly increased at the higher field intensities of 2T and 5T. This suggests that lower field intensities might have protective or mitogenic effects on neurons, whereas higher intensity magnetic fields will lead to activation of stress related signalling pathways.

In summary, my PhD demonstrated the suitability of collagen-GAG scaffolds for the induction of bone and cartilage from adult stem cells. The protein kinases ERK and p38 were shown to be involved in this process. Also a possible role for mechanical and magnetic stimulation on the induction cell differentiation was demonstrated. This work demonstrates the potential of adult MSCs for use in tissue engineering applications for the treatment of bone and cartilage related diseases and injuries.

List of Abbreviations

AP	Alkaline phosphatase
AP-1	Activating protein-1
APS	Ammonium persulphate
ASC	Adult stem cell
ATP	Adenosine tri-phosphate
BMP	Bone morphogenetic protein
BrdU	Bromo-deoxy-uridine
BSA	Bovine serum albumin
Cbfα-1	Core binding factor alpha 1
CFU-F	colony forming unit fibroblast
Collagen GAG	Collagen glycosaminoglycan
Dex	Dexamethasone
DMEM	Dulbecco's modified Eagles medium
DMSO	Di-methyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine-tetraacetic acid
ERK	Extracellular signal regulated kinase
ESC	Embryonic stem cell
FBS	Foetal bovine serum
FGF	Fibriblast growth factor
FITC	Fluoroisothiocyanate
GDF-5	Growth and differenatiation factor 5
HCl	Hydrochloric acid
HEPA	High efficiency particle air
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid])
HRP	Horseradish peroxidase
HSC	Haemopoietic stem cell
Hz	Hertz
IGF	Insulin-like growth factor
IgG	Immunoglubulin G
JNK	c-jun-N-terminal kinase

LIF	Leukaemia inhibitory factor
mM	Millimolar
mL	Millilitre
µL	Microlitre
µM	MicroMolar
µε	Microstrain
M	Molar
MAPC	Multipotent adult progenitor cell
MAP kinase	Mitogen activated protein kinase
MEK	MAP/ERK kinase
MEKK	MAP/ERK kinase kinase
$MgCl_2$	Magnesium chloride
Mnk	MAP kinase interacting kinase
MSC	Mesenchymal stem cell
Msk	Mitogen and stress-activated protein kinase
NASA	National aeronautics and space administration
NSC	Neural stem cell
nM	Nanomolar
PAK	p21 activated kinase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Poly(L-lactide-co-ε-caprolactone)
PLGA	Poly(L-lactic acid-co-glycolic acid)
PLLA	Poly(L-lactide)
PMSF	Phenylmethylsolphonyl fluoride
PPARγ	Peroxisome proliferator activated receptor gamma
RT	Room temperature
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide gel Electrophoresis
SEM	Standard error of the mean

TBS	Tris-buffered saline
TBS-T	TBS-tween
TEMED	N, N, N,-N tetramethylenediamine
TGFβ	Transforming growth factor β
2-D	Two dimension/dimensional
3-D	Three dimension/dimensional
TUNEL	TdT-mediated-UTP-nick end labelling
U	Unit
VEGF	Vascular endothelial growth factor

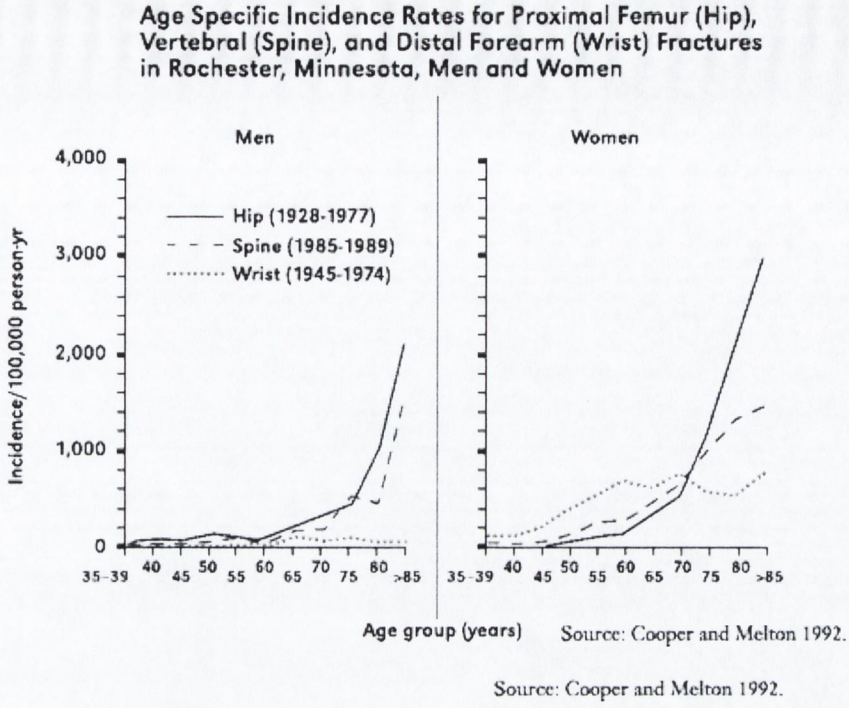
Chapter 1

Introduction

1.1 Tissue Engineering

Jenkins *et al.* (2003) described tissue engineering as "the *in vivo* or *ex vivo* manipulation of biologic tissues with organic, inorganic or synthetic material for the purpose of restoring function". Rose *et al.,* (2002) define it as "the application of scientific principles to the design, construction, modification and growth of living tissues using biomaterials, cells and factors, alone or in combination" There are many variations of this definition available, but all encompass the general methods and aims of tissue engineering. These are, to use the "tissue engineering triad" of cells, scaffolds and stimuli (Chemical/physical) to generate a fully functioning tissue replacement, be it bone, cartilage, liver, nerve or other tissue.

Why is there a need for tissue engineering? In the year 2000 approximately 72,000 people were on the waiting list for an organ transplant due to end-stage organ failure in the USA alone, and only 23,000 transplants were performed (Port, 2002). Also in the USA, the population over the age of 65 is expected to double and the number of people over the age of 85 is expected to quadruple over the next 20 years (Perry 2000). This will lead to huge increases in organ failures, neurodegenerative diseases and diseases of the musculoskeletal system. The problem is not confined to the USA alone, however. In Germany 1.5 million people with degenerative joint diseases are under medical treatment (Ringe *et al.,* 2002) and there are over 150,000 osteoporosis related fractures in the UK every year (Rose and Oreffo, 2002). In the coming years, with an increasing demographic trend towards an older population in both Europe and the USA, there is a serious socioeconomic need for the development of new treatments for these ailments. Figure 1.1 illustrates the sharp rise in fracture risk observed in individuals aged above 60, which rises even more sharply above the age of 70.



Source: Cooper and Melton 1992.

Figure 1.1 Age related increase in fracture incidences

1.2 Current bone replacement therapies

Not only are current treatments expensive, the success rate is far from optimal. For example, the gold standard method of bone replacement for the treatment of diseases or non-union fractures is the autologous bone graft, where a piece of bone is taken from another body site, usually the iliac crest, and transplanted into the defect (Salgado *et al.*, 2004). While the success rate of this procedure is quite high, the number of cases in which it can be used are small, due to the limited amount of available tissue, and there is also a risk of donor site morbidity (Rose *et al.*, 2002, Spitzer *et al.*, 2002). The second most common treatment is allografting, using tissue from another person. This treatment, however leads to a lower rate of graft incorporation with the host tissue (Salgado *et al.*, 2004) and leads to the risk of immune rejection and pathogen transmission in the recipient (Vacanti *et al.*, 2000). An alternative to bone grafting involves the use of ceramic or metal implants. There are several disadvantages to this approach also. In the case of metals, while there is good structural support, integration with the host tissue is poor and can lead to infection or failure of the bone at the implant site (Yaszemski *et al.*, 1994). Ceramics are very brittle materials and as such cannot be used in many load bearing situations

(Yaszemski *et al.,* 1994). From these examples, it can clearly be seen that there is a need for a more suitable bone replacement in the treatment of large bone defects. The same problems arise with treatment of cartilage defects, with insufficient amounts of available autologous tissue and replacement materials with poor material properties and long term success rates. The statistics and examples provided here are the very reasons for the development of tissue engineering as a rapidly emerging field of research that, it is hoped, will successfully tackle the problem of tissue regeneration and replacement.

1.3 The tissue engineering triad

1.3.1 Stimulus

As stated, successful tissue engineering requires cells, scaffolds and a stimulus, with several possibilities for each aspect available. The stimulus is usually chemical in nature, with various cytokines being used to induce phenotypical changes in a cell or to induce matrix synthesis. *In vivo*, growth factors (cytokines) are released by cells and bind to the receptors of other cells or the cell from which they were released. Once bound they cause a cascade of signalling events within the cell causing some type of response, for example, differentiation or matrix production. As a result these factors have a very important role to play in tissue engineering. A very important family of factors in bone and cartilage generation are the transforming growth factor β (TGFβ) factors, which include the bone morphogenetic proteins (BMPs) discovered in 1983 (Urist *et al.,* 1983). BMPs play an integral role in osteogenic differentiation and bone formation and the TGFβs are very important in the chondrogenic pathway. Other important cytokines in bone and cartilage maintenance and repair include fibroblast growth factors (FGFs) insulin-like growth factors (IGFs) platelet derived growth factors (PDGFs) and vascular endothelial growth factor (VEGFs, Salgado *et al.,* 2004). In combination with other chemicals, essential for cellular differentiation, or matrix generation in the case of bone and cartilage tissue engineering, these growth factors make up the stimulus part of the tissue engineering triad. Increasingly however, bioreactors are being employed to expose the cells to a mechanical stimulus in order to evoke a response. This is usually in combination with growth factors (Huang *et al.,* 2004, Kobayashi *et al.,* 2004, Tang *et al.,* 2004, Simmons *et al.,* 2003,). There is some interest in stimulating cells with mechanical strain alone also (Ignatius *et al.,* 2005, Jessop *et al.,* 2002). There are two reasons for this. In the field of

bone and cartilage tissue engineering, it is known that mechanical stimulation is essential for healthy bone and cartilage homeostasis. Clearly mechanical strain is important and is sensed by cells and transduced to their nucleus in order to elicit a response. Tissue engineers consider this an important phenomenon to simulate in the in vitro setting. Also, the growth factor combinations that are in use sometimes consist of non-endogenous chemicals and are always used at supra-physiological levels. Their use is far from ideal when the end goal is implantation into a human patient. If the desired cellular response can be achieved by mechanical strain, it is the preferred mode of stimulation. To date however, this has not been the case. At best, some modes of mechanical stimulation have been shown to augment the response elicited by the growth factor combination (Kaspar *et al.*, 2002).

1.3.2 Cells

The second requirement of tissue engineering is a cell source. The options available depend on the desired tissue to be generated. In the case of bone or cartilage tissue engineering, there are several available options. The obvious choice would always be autologous cells, since there would be no immunity or infection issues. There are problems associated with this approach, however. These cells are generally not readily available and a long time period for increasing cell numbers *in vitro* would be required. Allogeneic cells would obviously overcome this issue, being stored and ready for use. The issue of infection transmission and rejection by the recipient calls for serious concern though (Nerem, 2000). Another option is the use of stem cells. Both embryonic and adult stem cells have the potential for tissue engineering. The use of embryonic stem cells offers huge promise but has been very slow to deliver and is also mired in ethical and legislative problems (Langer and Vacanti, 1999, Pederson, 1999). Adult stem cells or progenitor cells offer a more likely short term stem cell option and are proving to be much more plastic than was originally thought (Sanchez-Ramos *et al.*, 2002, Toma *et al.*, 2001, Galli *et al.*, 2000, Bjornson *et al.*, 1999). Not only are these cells showing promising results in a number of tissue engineering areas, it also appears that, rather than cause an immune response when implanted *in vivo*, they actually act in an immunosuppressive manner (Augello, 2005, Devine, 2002).

1.3.3 Scaffolds

The final requirement for tissue engineering is the scaffold in which to culture cells. This is usually comprised of either a ceramic such as hydroxyapatite or tricalcium phosphate (le Geros, 2002) or a polymer. The polymers are further subdivided into natural and synthetic. All of the scaffold materials in use have one important criterion to be fulfilled before they are used to make scaffolds; they must be biodegradable, or at the very least, biocompatible. After this has been met, there are a huge number of factors to be taken into account in order to generate the most suitable scaffold for its intended tissue engineering purpose. Pore size, surface properties, mechanical properties, composition and porosity all have to be considered in order to best achieve the desired cellular response. The main role of a tissue engineering scaffold is to provide a 3-D environment for cells *in vitro* because they will not form one spontaneously. The scaffold provides a suitable structure in which cells can make 3-D connections, more akin to the *in vivo* setting. A suitable scaffold will provide ample anchorage sites for cells, mechanical stability and structural guidance to whatever cells are seeded onto it (Rose and Oreffo, 2002). The combination of a cell source, a 3-D scaffold and a stimulus, usually chemical but also mechanical, form the basis of all research being carried out under the heading of "tissue engineering". The following sections will discuss each of these aspects in more detail.

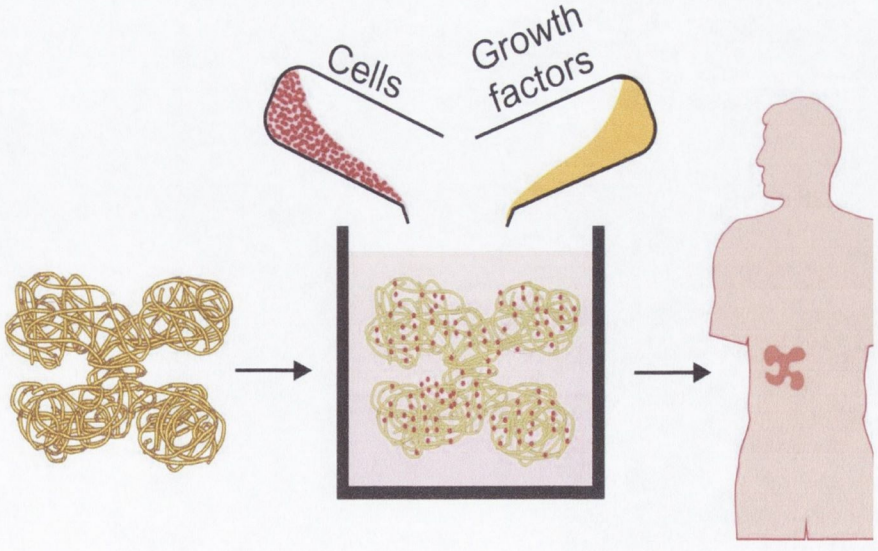


Figure 1.2: The tissue engineering triad
(Taken from http://www.clemson.edu/agbioeng/bio/images/sumint/jackson1.jpg)

1.4 Stem cells

A stem cell is a cell from the embryo, foetus or adult that has, under certain conditions, the ability to reproduce itself for long periods or, in the case of adult stem cells, throughout the life of the organism. It can also give rise to specialised cells that make up the tissues and organs of the body (Kirchstein *et al.,* 2001). This occurs through several intermediate cell stages including progenitor cells, precursor cells and the "pro" form of the final cell type. Embryonic stem cells (ESCs) were first obtained from mouse embryos over twenty years ago (Martin, 1981, Evans *et al.,* 1981) and more recently from humans (Bongso *et al.,* 1994). Embryonic stem cell lines have been shown repeatedly to survive up to as many 300 to 400 cell doublings or longer when cultured under the right conditions in the presence of leukaemia inhibitory factor (LIF). The enzyme telomerase and the transcription factors Oct4 and STAT3 maintain the ESC in a proliferative state as long as LIF is added to the culture medium (Keller, 2002). ESCs can give rise to all somatic cell types when injected into a blastocyst and form mature progeny from all three germ layers. Adult stem cells (ASCs) are found in mature tissues. To date they have been identified in a host of organs and tissues including the bone marrow (Pittenger *et al.,* 1999), periosteum (Hanada *et al.,* 2001), muscle (Williams *et al.,* 1999), fat (Zuk *et al.,* 2001), and the brain (McKay 1997). ASCs show a limited capacity with regard to both proliferation capacity and pluripotentiality. To date no-one has demonstrated the ability of adult stem cells to differentiate into cells derived from all three germ layers nor has plasticity (the ability of stem cells to differentiate into cells along a different lineage) been demonstrated *in vivo*. For this reason adult stem cells are often referred to as being multipotential, (capable of differentiating along several lineages) as opposed to pluripotential (giving rise to cells from all three germ layers). However, despite this lack of evidence, several adult stem cell types, including haemopoietic stem cells (HSCs), neural stem cells (NSCs) mesenchymal stem cells (MSCs) and epidermal stem cells have been shown to be capable of both self renewal and differentiation, merely on a more limited scale, (for review see Verfaille, 2002). As research in this area continues, there is increasing evidence for high levels of plasticity in these cells (Jiang *et al.,* 2002), suggestive of pluripotentiality.

As cells proceed toward the more differentiated cell type their proliferative capacity is reduced and cells are thought to become committed to differentiation along that specific

lineage (Caplan and Bruder, 2001). This lineage commitment has recently been called into question however, as evidence for dedifferentiation and transdifferentiation capabilities of cells mounts. Nuttall *et al.* (1998) found that osteoblasts that had differentiated to the point of expressing osteocalcin, a late stage marker of osteogenesis, could rapidly transdifferentiate to adipogenic cells when transfected with the nuclear receptor family member peroxisome proliferator activated receptor γ2 (PPARγ2). Another study has proposed that hypertrophic chondrocytes can further differentiate into osteoblasts in chicks (Cancedda *et al.,* 1995). It is not known if this is a further step in the differentiation process, a transdifferentiation step or a dedifferentiation. Others have also demonstrated the "transdifferentiation" of cells from one germ line to another (Sanchez-Ramos *et al.,* 2000, Bjornson *et al.,* 1999). Despite these findings, it has been suggested that transdifferentation or dedifferentiation and redifferentiation along another route are not taking place, but rather stem cells are fusing with existing cells (Ying *et al.,* 2002). Perhaps cells are not as committed as was once believed, or cell fusion is the cause. Either way, much more work is needed to determine the transdifferentiation potential of ASCs and their committed progeny.

1.5.1 Mesenchymal stem cells

The stromal cell system as first described by Owen in 1985 includes the marrow derived stromal cell that supports haematopoiesis, the mesenchymal stem cell and its progeny and cells of the connective tissues (osteocytes, chondrocytes, tenocytes, adipocytes, and smooth muscle cells) and their precursors (Deans *et al.,* 2000). Endothelial precursors have also been proposed to be present in the marrow stroma (for review see Kirschstein *et al.,* 2001). Within the marrow system these cells are loosely grouped together into three subsets, the haemopoietic cells (all blood cells and their precursors and haematopoietic stem cells themselves), endothelial cells and stromal cells, into the latter of which cells of the mesenchymal lineage fall. The stromal system is believed to support the haematopoietic system as well as maintaining the various connective tissue lineages through a process of self renewal and differentiation. This maintenance of the connective tissues as well as the numbers of mesenchymal stem cells is negatively correlated with age and disease states such as osteoarthritis and osteoporosis.

As stated, within the marrow there are at least three distinct types of stem cell, the HSC the MSC and the endothelial stem cell. The HSC is by far the most active of the three since it must replace the most short-lived of all cells of the body, the blood cells. The endothelial stem cells receive very little attention due to the recentness of the evidence of their bone marrow derived origins and the lack of knowledge as to whether they are indeed a distinct subset of stem cells. It is estimated that 1 in 100,000 cells in the bone marrow is a mesenchymal stem cell. The discovery that bone marrow contained precursor cells that would give rise to bone began in 1963 when Petrakova *et al.* demonstrated osseus tissue formation in the renal capsule when pieces of bone marrow were implanted into it. The same group (Friedenstein *et al.,* 1966, 1968) subsequently went on to demonstrate the presence of osteogenic stem cells *in vitro* and developed the colony forming unit-fibroblast (CFU-F) assay to phenotypically describe these precursor cells. Over twenty years later, these cells were given the name that is most commonly in use today; Mesenchymal stem cells (MSCs, Caplan, 1991). Since then, these cells have been shown to differentiate along several lineages including bone (Pittenger *et al.,* 1999, Jaiswal *et al.,* 1997, Kadiyala *et al.,* 1997), cartilage (Pittenger *et al.,* 1999, Mackay *et al.,* 1998, Johnstone *et al.,* 1998), fat (Pittenger *et al.,* 1999) and tendon (Awad *et al.,* 1999, Young *et al.,* 1998). These mesenchymal stem cells are also referred to as bone marrow stromal cells or multipotent adult progenitor cells, however it is generally accepted that all three terms refer to the same subset of cells. MSCs and HSCs are easily separated from one another *in vitro*, as the MSCs adhere to the surface of the plate or flask in which they are cultured while the HSCs are non-adherent. Despite this it has not yet been possible to isolate a totally pure population of MSCs in culture, and though there are a host of markers now available and under development, monospecific and unique molecular probes do not exist to unequivocally identify these cells *in situ* (Caplan and Bruder, 2001, Herzog *et al.,* 2003).

1.5.2 Mesenchymal stem cell characterisation

Recent efforts have focused on investigating the plasticity of MSCs and their exact origins and defining characteristics. A review by Catherine Verfaillie (2002) was cynical towards research in the area that has attempted to illustrate pluripotential properties of these cells. She cites possible reasons why the appearance of pluripotentiality might be inaccurate, such as the possibility of cell fusion between implanted and native cells, or

different colonies of cells within a culture giving rise to cells from different germ layers as opposed to one single cell type. The problem of cell fusion has arisen recently, whereby researchers have found that after stem cell transplantation studies, donor cells were found to have fused with those of the recipient and as such didn't display absolute pluripotentiality or the ability to regenerate tissue alone. Despite these shortcomings, several interesting papers have been published in an attempt to shed light on the issue of pluripotentiality. In 1999 Pittenger *et al.* demonstrated the ability of single colonies of MSCs to give rise to adipogenic, chondrogenic and osteogenic cells and more recently Jiang *et al.* (2002) carried out some very elegant experiments demonstrating the ability of highly purified "multipotent adult progenitor cells" (MAPCs) to give rise to cells from all three germ layers. This is a very important development in the field of adult stem cell research as it is the first paper to definitively describe the pluripotentiality of marrow derived stem cells. This paper further develops the use of surface markers to classify MAPCs: the human cells (hMAPCs) were found to be negitive for CD34, CD44, CD45, c-kit and major histocompatibility complex class I and II, to express low levels of Flk-1, Sca-1 and Thy-1 and high levels of CD13 and stage specific antigen I. As can be seen this was a very specific phenotype, the characterisation of which is becoming increasingly important.

MSCs are easily separated from the majority of HSCs due to their adherent properties, but not all of these cells are removed, and as already mentioned there is a large variety of other cells also present in the marrow. As a result, much work has focused on the classification of MSCs and the other stem cells present in the marrow. It is now generally accepted that CD34 positive cells (cells expressing the CD34 antigen on their surface) will, under the right conditions give rise to all cellular elements of the blood (Caplan and Bruder, 2001). However the isolation of specific stromal cell cultures and haematopoietic cell cultures can be made much more complicated not only by the fact that some cells of mesenchymal origin express the CD34 antigen. In a review by Deans *et al.* (2000) some of the more common cell surface markers and other lineage specific markers are of mesenchymal stem cells are discussed. The expression or lack of specific markers includes $CD34^{-}$ $CD50^{-}$,Thy-1 positive $CD45^{-}$, Stro-1 positive and SH2 positive. Stro-1 is a monoclonal antibody developed against stromal precursors by Simmons *et al.* (1991). SH2 was also developed as a monoclonal antibody by Haynesworth *et al.* (1992), against marrow derived mesenchymal stem cells. This antibody was later shown to recognise an

epitope on CD105/Endoglin, a TGF(receptor III (Barry et al., 1999). This antigen is now often used as a marker of MSCs and has been referred to as a putative stem cell marker (Lodie et al., 2002). This list of mesenchymal stem cell markers is by no means extensive and a more complete list is provided in table 1.2. However, it does serve to illustrate the point that a single monoclonal antibody specifically against mesenchymal stem cells, that doesn't recognise other subsets of marrow derived cells is needed. Table 1.1 shows an extensive list of cell adhesion molecules, growth factors receptors and other cell surface markers that are either present or absent from the surfaces of MSCs.

Common name	CD locus	Detection
Adhesion molecules*		
ALCAM	CD 166	Pos
ICAM-1	CD54	Pos
ICAM-2	CD102	Pos
ICAM-3	CD50	Pos
E-selectin	CD62E	Neg
L-selectin	CD62L	Pos
P-selectin	CD62P	Neg
LFA-3	CD58	Pos
Cadherin 5	CD144	Neg
PECAM-1	CD31	Neg
NCAM	CD56	Pos
HCAM	CD44	Pos
VCAM	CD106	Pos
Hyaluronate receptor	CD44	Pos
Growth factors and cytokine receptors*		
IL-1R(c t and B)	CD121a,b	Pos
IL-2R	CD25	Neg
IL-3R	CD 123	Pos
IL-4R	CD 124	Pos
IL-6R	CD 126	Pos
IL-7R	CD 127	Pos
Inteferon -y R	CDwll9	Pos
TNF-a-lR	CD120a	Pos
TNF-a-2R	CD120b	Pos
FGFR		Pos
PDGFR	CD140a	Pos
Transferrin receptor	CD71	Pos
Integrins*		
VLA-al	CD49a	Pos
VLA-a2	CD49b	Pos
VLA-a3	CD49c	Pos
VLA-a4	CD49d	Neg
VLA-a5	CD49e	Pos
VLA-a6	CD49f	Pos
VLA-3 chain	CD29	Pos
(3_4 integrin	CD 104	Pos
LFA-1 a chain	CDlla	Neg
LFA-1 B chain	CD18	Neg
Vitronectin R *a* chain	CD51	Neg
Vitronectin R (3 chain	CD61	Pos
CR4 a chain	CDllc	Neg
Macl	CDllb	Neg
Additional markers*		
T6	CDla	Neg
CDS complex	CD3	Neg
T4,T8	CD4, CDS	Neg
Tetraspan	CD9	Pos
LPS receptor	CD14	Neg
LewisX	CD15	Neg
—	CD34	Neg
Leukocyte common antigen	CD45	Neg
5' terminal nucleotidase	CD73	Pos
B7-1	CD80	Neg
HB-15	CD83	Neg
B7-2	CD86	Neg
Thy-1	CD90	Pos
Endoglin	CD105	Pos
MUC18	CD 146	Pos
BST-1	CD 157	Pos

*Data are from Pittenger et al. (1999) and Azizi et al. , (1998) or are previously unreported communication.

Table 1.1. Phenotypic characterization of human mesenchymal stromal cultures (Taken from Deans *et al.*, 2000)

1.5.3 Mesenchymal stem cell isolation

The problem of characterisation is compounded by the fact that there are several different methods for the extraction and culture of mesenchymal stem cells in vitro; some acquire MSCs from larger animals such as canines (Kadiyala et al., 1997). The standard procedure for the isolation and culture of animal MSCs is from young adult rats, where the femurs and tibia are removed, the marrow cavity flushed out, the cells are pelleted and resuspended and then left for 24 hours before the non-adherent cells are removed. However this procedure has many variations (Tsuchida et al., 2003, Herbertson and Aubin, 1997, Cassiede et al., 1996) and is not the only method used in the study of stromal progenitor cells. Foetal rat calvaria are often used instead (Ishida et al., 1995, Gerber and Gwynn, 2002). Tibia and Femurs from chick embryos also yield these cultures (Martin et al., 1998) as do bone marrow aspirates from humans from both the iliac crest (Schecroun and Delloye, 2003) and the femur (Locklin et al., 1999). Currently, human MSC studies make up the majority of the research field. Several progenitor cell lines are also used, the most common of which is the MC3T3-E1 mouse osteoblastic cell line (Ueno et al., 2001). MSCs have also been found in several other tissues within the body including adipose tissue, periosteum, synovial membrane, muscle and blood (for review see Tuan *et al.,* 2003)

1.6 Mesenchymal stem cell differentiation

1.6.1 Osteogenic differentiation

Osteoblasts can be characterised morphologically and also by their ability to produce certain proteins. For stem cells to become an osteocyte they must follow several differentiation steps and become several precursor cell types before differentiation is complete. In osteogenesis, the multipotential stem cell gives rise to an osteoprogenitor that still has the capability to self-replicate, which declines as this cell matures. The ability of these cells to self renew can also indicate the level of differentiation, as the more differentiated a cell becomes, the lesser its ability to replicate (Banfi *et al.,* 2000). Under the right conditions the osteoprogenitor will differentiate into a preosteoblast and then a mature osteoblast. *In vivo*, depending on the circumstances (location, intrinsic molecular cues, tissue state), osteoblasts will differentiate further into a lining cell or an

osteocyte, or will remain as an osteoblast and begin to lay down osteoid. During this differentiation process, a huge number of markers of osteogenesis are expressed. Osteoblasts are known to produce type I collagen, osteonectin, osteopontin, bone sialoprotein and osteocalcin. They also have high alkaline phosphatase (AP) activity and are capable of synthesising a mineralised matrix (for review see Aubin *et al.,* 1995). The transcription factor, core binding factor α-1 (cbfα-1 also knows as Runx 2) has also been recently been shown to be essential for the differentiation process to take place (Ducy *et al.,* 1997). The final marker of osteogenesis is nodule formation and mineralisation. These markers are not all simultaneously or sequentially expressed. Instead, a combination of these markers and the level of AP activity and mineralisation are an indicator of the degree of differentiation from MSC to osteoblast. For example, osteonectin is usually expressed quite early in the process of differentiation and remains throughout, osteopontin peaks twice during proliferation and again later, while osteocalcin is a later stage marker of osteogenesis and therefore a more reliable one. This is supported by the fact that hypertrophic chondrocytes are also known to express some of these earlier markers, including bone sialoprotein, osteopontin and osteonectin (Aubin, 1999). Osteocalcin is a calcium binding protein located abundantly in the organic matrix of bone. It contains 47-51 amino acid residues with a molecular weight of 5.2-5.8 kilodaltons depending on the species. Osteocalcin is believed to be involved in matrix mineralisation, hence its calcium binding properties and the calcium induced transition of the protein to an α-helical conformation, permitting tight binding to hydroxyapatite (Hauschka *et al.,* 1981). Its late role in the osteogenic process makes it an ideal marker. Collagen type I is expressed much earlier in the differentiation of MSCs along the osteogenic route but it is a very important component of the process. Not only does it make up 95% of the organic component of bone, it is also required for the expression of later stage osteogenic markers, including osteocalcin via a mechanism that involves α2-integrin-collagen interactions leading to cbfα-1 activation (Xiao *et al.,* 1998Xiao *et al.,* 1997).

1.6.2 Chondrogenic differentiation

Chondrogenesis occurs in a similar manner to osteogenesis, with the multipotent stem cell differentiating into a chondroprogenitor, chondroblast and finally a chondrocyte. During the differentiation process, synthesis of glycosaminoglycan takes place and a trend

towards higher levels of 6-sulphated chondroitin sulphate versus 4-sulphated chondroitin sulphate indicate later stages in the differentiation process, as occurs in human articular cartilage (Bayliss, 1990). The expression of fibromodulin, cartilage oligomeric matrix protein, decorin, aggrecan and types II and X collagen are also indicators of chondrogenic activity. Mature chondrocytes also express type IX and type XI collagen and Matrix Gla protein (Corvol, 2000). After a period of time the chondrocyte hypertrophies and secretes proteins that are important in calcification of the matrix. In this way they play a vital role in endochondral ossification. Hypertrophic chondrocytes can also give rise to osteoblast like cells, though the mechanism by which this takes place is currently unknown (Cancedda *et al.,* 1995). If chondrocytes are located within articular cartilage, hypertrophy does not normally occur and the chondrocytes play a role in cartilage maintenance once sufficient matrix has been formed. The production of collagen type II and X and the expression of the transcription factor sox 9 are the most commonly used definitive markers of the chondrogenic lineage.

1.6.3 Factors affecting MSC differentiation

The ability of MSCs to differentiate depends on several factors including, but not exclusively, age, cell density and most importantly the presence of soluble differentiating factors. These factors include some endogenous signalling molecules known to induce differentiation along a particular lineage *in vivo*. The number of factors involved in the induction of osteogenesis and chondrogenesis is still not known, but glucocorticoids and members of the TGF-β superfamily, which includes the family of bone morphogenetic proteins (BMPs), are known to play important regulatory roles (Aubin *et al.,* 1995). The use of such inductive factors is necessary to induce complete differentiation; however there is no set group of factors, or specific concentration that is proven to best result in osteogenesis or chondrogenesis. Under standard *in vitro* conditions, to induce osteogenesis, the glucocorticoid dexamethasone is used in conjunction with β-glycerophosphate and ascorbic acid-2-phosphate, (Jaiswal *et al.,* 1997, Simmons *et al.,* 2003, Wang *et al.,* 2003) though concentrations may vary. Dexamethasone is responsible for the upregulation of bone specific proteins and enzymes, β-glycerophosphate is an organic phosphate which acts as a substrate for the bone forming enzyme alkaline phosphatase and ascorbic acid or vitamin C is involved in the conversion of pro-collagen

to collagen, an important early step in osteoblast differentiation. The induction of chondrogenesis is also dependent upon a number of important factors.

The conditions under which MSCs must be cultured to generate such a phenotype are somewhat different, however. The standard accepted method of culturing MSCs to induce chondrogenesis is in a 3-D format, in the absence of serum and with some member of the TGFβ superfamily, usually TGFβ-1, 2 or 3 (Barry and Murphy, 2004). Johnstone *et al.* (1998) were the first to develop a reproducible system for the differentiation of MSCs into cartilage and found the combined effects of both dexamethasone and TGF-β1 and a 3D environment in the form of a micromass pellet culture provided the optimal conditions for cartilage formation. Chondrogenesis was indicated by an increase in AP and an up-regulation of the cartilage-specific type IIα and IIβ collagen. Recently, TGFβ-3 has been shown to be the most efficacious of these cytokines at inducing chondrogenesis *in vitro* (Barry *et al.,* 2001). These culture conditions will lead to morphological changes, and the expression of cartilage-specific extracellular matrix components. The earliest protein expressed is cartilage oligomeric protein, followed by an increase in aggrecan expression. The final marker indicative of complete chondrogenesis is the appearance of type II collagen (Barry *et al.,* 2001).

There is large variation in the concentrations of soluble factors used in the induction of osteogenesis and chondrogenesis. Recently, several articles have also reported the use of novel factors to induce osteogenesis. In 2002, Liu *et al.* experimented with culturing osteogenic precursors in the presence of leukaemia inhibitory factor (LIF) also known as interleukin-6 suggesting a role for it in osteogenesis, independent of dexamethasone. Cassiede *et al.* (1996) suggested a possible role for platelet derived growth factor BB (PDGF-BB) in osteogenesis, while Ishida *et al.* (1995) showed that depleted levels of Tri-iodothyronine increased nodule formation and osteoprogenitor cell differentiation. These papers studying novel factors are rare, with the majority of research groups using slightly different combinations of common inductive factors to induce osteogenesis and chondrogenesis. (Pri Chen *et al.,* 1998, Stevens and Williams 1999, Gerber and Gwynn, 2002, Herbertson and Aubin 1997). This leads to the same problems associated with the isolation and culture of these cells, where there is no gold standard of growth factor use and concentration, thus making comparisons between studies difficult.

1.7 Culture of cells in three dimensions

1.7.1 Scaffolds

While it is very important to understand the mechanisms leading to osteogenesis and chondrogenesis, and a certain degree of osteogenesis can occur on the 2 dimensional surface of a tissue culture flask, it is important that 3 dimensional constructs be developed for use in the clinical setting. In recent years, several scaffolds have been developed with great variety in surface properties, strength, degradability, and structure. Regardless of what the scaffold is made of, it must fulfil certain criteria in order to successfully promote cell adhesion, growth, proliferation and differentiation. These criteria as described by O' Brien *et al.* (2004) are as follows; an ideal scaffold must be composed of a biocompatible substance that will degrade in the body at a rate that allows the scaffold to remain insoluble just for the duration of the critical cellular processes. The chemical composition must incorporate ligands appropriate for the binding of cells specific to each application. It should have an average pore size which is large enough to permit adequate cell migration but small enough to retain critical total surface area for cellular attachment. The scaffold should also have a large pore volume fraction to allow transport of nutrients and metabolites.

Most of the scaffolds currently being developed satisfy at least some of these criteria and are also developed with their end use in mind. For example, scaffolds developed for use in bone implants are much harder and tougher than those made for cartilage implants. Sintered dicalcium pyrophosphate is one example of a scaffold material used for bone tissue. It has very recently been shown to be biocompatible with osteoblasts, even when in powder form in culture medium (Sun *et al.,* 2004) and gives rise to elevated calcium concentration and activation of bone cell genes. A more common bone tissue scaffold material is hydroxyapatite, a brittle substance found in bone that can be infiltrated with other chemicals to improve strength and rigidity while remaining biocompatible. A lot of work has been carried out on the seeding of hydroxyapatite scaffolds with osteoblasts, with varying success (Bilezikian *et al.,* 2001).

Other scaffolds used for the induction of bone formation include the use of lactic acid composites. Yang *et al.,* (2001) investigated the effects of culturing osteoprogenitor cells on poly(L-lactic acid-co-glycolic acid; PLGA) when surface properties had been altered by incorporating the Arg-Gly-Asp (RGD) sequence into the surface of the scaffold. This led to increased alkaline phosphatase activity and increased collagen type I and Cbfα-1 expression. Alteration of the surface appears to confer useful properties to a scaffold. In 2002 Yang *et al.* coated poly(L-lactide; PLLA) scaffolds with BAY K8644, a calcium channel agonist, and seeded them with primary human bone cells. They had previously shown that voltage operated calcium channels play an important role in mechano-transduction in bone cells. Scaffolds were mechanically stimulated and those with the agonist showed elevated responses to the mechanical stimulus.

The variety of scaffolds used in tissue engineered cartilage constructs is equally broad though the field is more recent. It was only in 1991 that Vacanti *et al.* reported the first example of tissue-engineered cartilage using chondrocytes and biodegradable polymers. In 2003 Honda *et al.* demonstrated the ability of rat chondrocytes to adhere to and proliferate on poly(L-lactide-co-ε-caprolactone; PLC) scaffolds. Cells were shown to adhere much better when these scaffolds were coated with Type I collagen. When compared to standard collagen scaffolds the PLC scaffolds were shown to retain their original shape better as a result of reduced degradation.

Sodium alginate is often used for bone marrow cell tissue engineering particularly for chondrocytes. The alginate is a copolymer derived from brown sea algae and forms a stable gel at room temperature. The method of seeding alginate gels is different to that of other scaffolds. The gel is combined with a cell suspension while it is still liquid and the gel sets with the cells evenly dispersed through the scaffold. The cells are also easily dissociated from the gel by uncrosslinking the gel using mild chelating agents (Wang *et al.,* 2003). Wang *et al.* demonstrated that proliferation levels of rat bone marrow cells were comparable to those seen on tissue culture flasks, and for the first time that alginate gels could support their differentiation along the osteoblast lineage. There are, however, problems associated with alginate. In 2001, Wong *et al.* stated that the stiffening potential of alginate is inherently limited by the fact that alginate cannot be remodelled by chondrocytes. Alginate is not very strong either and wouldn't be able to withstand any

compressive force imposed upon it. If the chondrocytes are unable to remodel a structure that is too weak to withstand compression, then its use as a biocompatible scaffold is called into question.

1.7.2 Collagen glycosaminoglycan scaffolds

The use of pure collagen constructs as reviewed by Honda *et al.* (2003) is common, however more interest has focused on glycosaminoglycans (GAGs) used in conjunction with collagen to form copolymers. Burke and Yannas (1981) first described the use of collagen GAG copolymers as an artificial skin substitute. Since then it has received FDA approval as treatment in skin regeneration in burn victims, has been shown to induce regeneration of the conjunctiva and has enhanced peripheral nerve regeneration (O'Brien et al., 2004). It is only more recently that thought has been given to using collagen GAG copolymers as bioactive scaffolds for the culture of bone and cartilage derived cells (Zaleskas *et al.,* 2003). To date nobody has reported the use of collagen GAG scaffolds in the culture of mesenchymal stem cells or any other stem cell from any animal or humans. GAGs are common constituents of cell surfaces and extracellular matrices and they are involved in binding of enzymes, protease inhibitors and cytokines (Pieper *et al.,* 2000). In 2000, Pieper *et al.* showed that the attachment of chondroitin sulphate or heparan sulphate, two endogenous glycosaminoglycans, to porous type I collagen matrices led to increased preservation of matrix integrity, promotion of angiogenesis in *in vivo* models and reduced foreign body reactions when compared to pure collagen matrices. It was observed that non-crosslinked collagen was resorbed over time *in vivo* and replaced by collagenous connective tissue, while over the ten week study the GAG crosslinked matrix maintained its integrity and supported the interstitial deposition of types I and III collagen. More recently the same group demonstrated the ability to generate a variety of different scaffolds composed of collagen-elastin-glycosaminoglycan with varying proportions of the three ingredients (Daamen *et al.,* 2003). This article was more focused on the manufacture of the scaffold and merely showed that they were not cytotoxic with regard to cell culture. It did however illustrate the ability to change the structural properties of a scaffold by altering the ratios of the main components.

As already shown type I collagen is often used as a substrate on other scaffolds to promote cell adherence. Indeed, the presence of type I collagen has been shown to be

essential for the induction of osteocalcin expression (Xiao *et al.,* 1998). In the case of collagen GAG scaffolds this coating is not necessary and the presence of type I collagen should promote osteogenesis. This fulfils one of the criteria set out at the beginning of this section. O'Brien *et al.* described several other criteria a scaffold should fulfil in order to be suitable for the culture of cells. Pore size should be bound within an upper and lower limit, with an optimal diameter of approximately 96μm (O'Brien *et al.,* 2004, O'Brien *et al.,* 2005). The collagen GAG scaffold developed by O'Brien *et al.* has a homogeneous structure with an average pore size of 96μm. Scaffolds should be biocompatible as should the products of their degradation. Both type I collagen and glycosaminoglycans are found endogenously and are therefore not toxic. These facts demonstrate the potential benefits afforded by collagen GAG scaffolds in the culture of mesenchymal stem cells, in 3 dimensions, in the formation of viable constructs of bone and cartilage. Table 1.2 lists some of the natural and synthetic polymer scaffolds in use in tissue engineering today.

Material	*Origin*	*Characteristics*
Collagen	Natural	Low immune response
		Good substrate for cell adhesion
		Chemotactic
		Scaffolds with low mechanical properties
Fibrin	Natural	Promotes cell migration and vascularization
		Promotes Osteoconduction
		Usually is used as a cell carrier for cell seeding on scaffolds
Chitosan	Natural	Hemostatic
		Promotes osteoconduction and wound healing
Starch	Natural	Thermoplastic behavior
		Good substrates for cell adhesion
		Non-cytotoxic and biocompatible
		Bone bonding behavior when reinforced with hydroxylapatite
		Scaffolds based on these materials have good mechanical properties
Hyaluronic acid (HA)	Natural	Minimal immunogenicity
		Chemotactic when combined with appropriate agents
		Scaffolds with low mechanical properties
Poly(hydroxybutyrate)	Natural	Natural occurring β-hydroxyacid
		Adequate substrate for bone growth
		Usefullness is limited due to brittle nature
Poly(α-hydroxy acids)	Synthetic	Extensively studied aliphatic polyesters
		Degradation by hydrolysis
		Already approved for other health related applications
		Acidic by products (e.g. lactic acid, glycolic acid), that enter the tricarboxylic acid cycle or in alternative (e.g. glycolic acid) are excreted in the urine
		It can present problems regarding biocompatibility and cytotoxicity in the surrounding area of the implantation site
Poly(ε-caprolactone)	Synthetic	Aliphatic polyester
		Degraded by hydrolysis or bulk erosion
		Slow degrading
		Degradation products incorporated in the tricarboxylic acid cycle
		Low chemical versatility
		Some problems related with withstanding mechanical loads
Poly(propylene fumarates)	Synthetic	Unsaturated polyester consisting on alternating propylene glycol and fumaric acids.
		Main degradation products are fumaric acid and propylene glycol
		Satisfactory biological results
Poly(BPA iminocarbonates)	Synthetic	Good biocompatibility when implanted in a bone canine chamber model
Poly(phosphazenes)	Synthetic	Contain alternating nitrogen and phosphorous with no carbon athoms in the backbone structure
		Degradation through hydrolysis
Poly(anhydrides)	Synthetic	Mainly developed as drug delivery carriers
		Biocompatible
		Support both endosteal and cortical bone regeneration

Table 1.2:Natural and synthetic polymers used for bone tissue engineering applications (Adapted from Salgado *et al.*, 2004)

1.8 Bioreactors

1.8.1 Cell seeding

The use of chemicals to induce osteogenesis and chondrogenesis has been discussed in the previous sections. This section will deal with the increasing use of bioreactors to aid or induce the chondrogenic and osteogenic differentiation of MSCs and the generation of viable tissue engineered constructs. The use of bioreactors predates their arrival into the tissue engineering field. Before the development of the multitude of rigs now in use in tissue engineering labs around the world, bioreactors were being used in industrial fermentation processing, wastewater treatment, food processing and in the pharmaceutical industry (Martin *et al.,* 2004). The use of bioreactors has three roles in the tissue engineering field. Firstly, the seeding of the different scaffold types mentioned above is a difficult process that to date has been sub-optimal in most labs in terms of homogeneous cell dispersion, dispersion rates and cell survival. For example Freed *et al.,* (1998) observed that it took up to 40 days for chondrocytes to migrate to the centre of a scaffold 5mm thick. In this instance, cells were observed in the centre even though it took almost seven weeks. Efficient seeding is vital for the successful generation of any tissue engineered construct (Vunjak-Novakovic *et al.,* 1998) and while static seeding is the standard method in use at the moment, several other groups have reported low seeding efficiencies (Bruinink *et al.,* 2001, Li *et al.,* 2001, Kim *et al.,* 1998) and non-uniform cell distributions within scaffolds (Wendt *et al.,* 2003, Burg *et al.,* 2002, Nehrer *et al.,* 1997,). Indeed, the initial cellular distribution within a scaffold has been related to subsequent tissue distribution following culture (Freed *et al.,* 1998, Ishaug-Riley *et al.,* 1998, Kim *et al.,* 1998). This fact would suggest that optimum and uniform cell seeding will lead to more successful tissue engineered constructs. One technique to overcome the problem of poor cell seeding has been to seed scaffolds at very high cell densities, which has led to higher rates of cartilage production (Freed *et al.,* 1997), matrix mineralisation (Holy *et al.,* 2000) and enhanced cardiac tissue structure (Carrier *et al.,* 1999). This however requires high cell numbers and it is therefore essential to achieve maximum cell seeding efficiency to minimise the required number of cells to be harvested. The use of bioreactors has become a popular technique to achieved this high cell seeding efficiency. Spinner flask bioreactors are the preferred bioreactor currently in use. In these systems, scaffolds are hung from the lid of the flask, submerged in medium, while a magnetic stir

bar continuously mixes the medium (Martin *et al.,* 2004). This stirring, transports cells into the scaffolds by convection and has been shown to result in more efficient and homogenous seeding (Vunjak-Novakovic *et al.,* 1996). This cell seeding technique has also yielded somewhat unreliable results, however. Carrier *et al.* (1999) and Kim *et al.* (1998) observed low seeding efficiencies using the spinner flask method. Compared with spinner flasks and static seeding, higher seeding efficiencies have been observed using direct perfusion bioreactors (Wendt *et al.,* 2003), which will possibly be the next standard seeding technique used in the coming years.

1.8.2 Nutrient delivery and metabolite removal

The second role of the bioreactor in tissue engineering is the delivery of nutrients and oxygen and removal of metabolic wastes to and from the cells within a scaffold. Mass transport within a scaffold is of critical importance and the lack of it usually causes the formation of a necrotic core surrounded by viable cells (Kelly *et al.,* 2004, Pei *et al.,* 2002). Distances as little as 240-400μm from the surface of a scaffold will lead to poor cellular responses (Martin *et al.,* 1999, Ishaug *et al.,* 1997). As with the seeding approaches, the spinner flask is often used to improve mass transfer through scaffolds preferentially over other bioreactor types because of its simple design and use (Martin *et al.,* 2004). This however, does not make it the most suitable design available. Gooch *et al.* (2001) observed reduced net average GAG fractions throughout constructs cultured in spinner flasks and Martin *et al.* (1999) observed the formation of a fibrous capsule at the scaffold surface possibly due to turbulent eddies formed within the bioreactor. The development of the rotating wall vessel, designed by NASA to simulate the effects of microgravity, solved this issue by reducing diffusional limitations of nutrients and wastes without leading to an increase in the shear strains imposed on the scaffolds. In this system, the outer wall of the vessel rotates while the inner wall remains stationary. This system has been shown to generate superior cartilaginous constructs, biochemically and biomechanically, when compared to constructs generated in either static culture or in a spinner flask (Vunjak Novakovic *et al.,* 1999). The perfusion flow system has also been used to reduce nutrient limitations in scaffolds. This is another simple design, using a peristaltic pump to constantly deliver medium to the culture on one side of the chamber, while medium is removed on the other side. Good matrix formation and a mature chondrocytic phenotype has also been demonstrated with this setup also (Sittinger *et al.,*

1994). Raimondi *et al.,* (2002) have modelled direct perfusion through a 3-D mesh and found that the random architecture of the scaffold would yield highly variable shear stresses within the scaffold structure, possibly leading to varying responses in different scaffold regions and making it difficult to characterise the effects of strain on cells in 3-D. Thie problem could be avoided by the use of a scaffold with a uniform pore structure.

1.8.3 Mechanical stimulation

Since the development of Wolff's Law in 1892, mechanobiology has played an important role in the areas of biomechanics and bioengineering. Over the intervening period, several models have been used to describe the role of mechanical forces in musculoskeletal development (Prendergast *et al.,* 1997, Carter *et al.,* 1991, Pauwels *et al.,* 1980). As research in the areas of physiology, mechanobiology and tissue engineering progresses, it is becoming increasingly clear that a variety of cell types are not only responsive to mechanical stimuli, but that these stimuli are necessary for healthy tissue development and homeostasis (Butler *et al.,* 2000). The third and most recently developed role of the bioreactor has become the mechanical stimulation of cells to induce matrix production increase/improve cellular activity. The bioreactors used for this purpose range widely in design and function, depending on the strain type to be imposed. For example, as discussed with regard to spinner flasks and perfusion systems, fluid shear stresses can be imposed on cells and scaffolds. Other forces employed include compression, tension and hydrostatic pressure. Cyclic mechanical strain has been demonstrated to be effective at inducing positive cellular responses in muscle cells (Akhyari *et al.,* 2002, Powell *et al.,* 2002), as has pulsatile radial stress (Niklason *et al.,* 1999). Mechanical strain has also been shown to affect osteoblast cells *in vitro* (Ignatius *et al.,* 2005) and is now being looked at as a mechanism of inducing osteogenic differentiation of osteoblast precursors (Jogodzinski *et al.,* 2004). GAG synthesis by chondrocytes seeded in 3-D scaffolds has been stimulated by static and dynamic deformational loading and shear stress (Waldman *et al.,* 2003, Davisson *et al.,* 2002) and mechanical strength has also been improved over static cultures by as much as sixfold (Waldman *et al.,* 2003, Mauck *et al.,* 2000). There is an obvious benefit of the use of mechanical stimulation when generating tissue engineered constructs. However, despite these findings and many others demonstrating the efficacy of mechanical strain in improving the biochemical and biomechanical properties of tissue engineered constructs, very little is known about the specific strain

magnitudes and frequencies needed to induce an optimal response in a given cell type (Martin *et al.,* 2004). The mechanisms behind these mechanotransduction events also remain largely unknown though some have been proposed, such as alteration of cell shape which influences the cellular interaction with the biochemical environment (Stein and Bronner, 1989). Much more research is needed in this area to optimise what is a promising new mode of cellular stimulation, that might replace the use of chemicals that can have wide ranging effects when used in the *in vivo* setting.

1.9.1 MAP Kinases

The mitogen activated protein kinases are a family of signalling molecules that are involved in many of the most important cell signalling processes including, embryogenesis, differentiation, proliferation, survival and death (Pearson *et al.,* 2001). The first of these molecules to be discovered was extracellular signal regulated kinase (ERK) (Ray and Sturgill, 1988). This molecule was originally termed microtubule associated protein kinase (MAP kinase) because of the substrate on which it acted. While the acronym was retained, it now means mitogen activated protein kinase because these kinases were first detected as mitogen-stimulated tyrosine phosphoproteins (Rossomando *et al.,* 1989). Since then, several MAP kinases have been identified with varying roles and signalling pathways. Some of the defining characteristics of the MAP kinase family are similar to the cyclin-dependent kinases. MAP kinases have a preference for serine/threonine residues followed by proline on their substrates. MAP kinases are activated directly by phosphorylation in the absence of a regulatory subunit and usually at two phosphorylation sites in what is known as the activation loop, one a tyrosine and one a threonine separated by a single variable amino acid residue (Pearson *et al.,* 2001). They are also activated by phosphorylation on the same residues. The MAP kinases consist mainly of ERKs, c-Jun-N-terminal kinases (JNK) and p38s, though other MAP kinases have also been discovered recently.

Upstream, the MAP kinases are regulated by phosphorylation cascades. The MAP kinases are activated by two protein kinases that are activated in series. Directly upstream of all MAP kinase members is a MAP/ERK kinase (MEK) family member. These are dual specificity enzymes that can phosphorylate both the serine/threonine and tyrosine residues in the activation loop on the MAP kinase (Seger *et al.,* 1992, Crews *et al.,* 1992, Kosako

et al., 1992). While there is some crosstalk between MEKs and their designated substrates, most MEKs phosphorylate only one or a few of the MAP kinases (Pearson *et al.,* 2001). This 3 kinase cascade provides scope for signal amplification and input from other signalling pathways. For example several of the MEK family members contain phosphorylation sites that are activated or inactivated by kinases in other pathways, thereby affecting its activity (Frost *et al.,* 1997, Mansour *et al.,* 1994, Rossomando *et al.,* 1994).

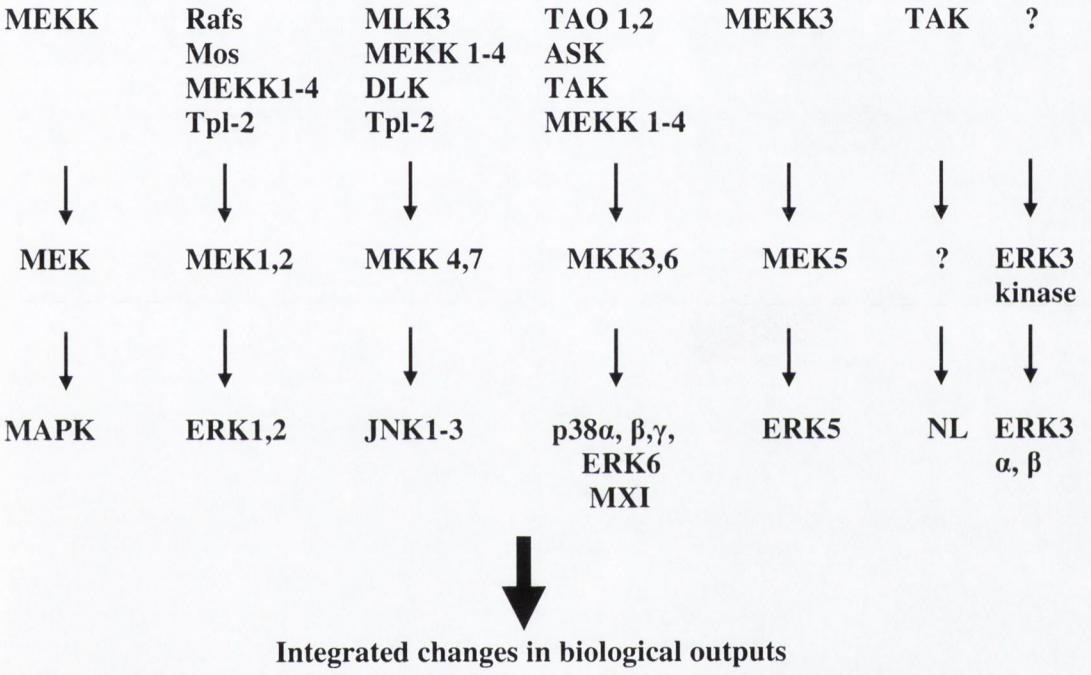


Figure 1.3: The three step kinase pathway leading to MAP kinase activation (Adapted from Pearson *et al.,* 2001)

1.9.2 ERK 1/2

As stated, ERK was the first of the MAP kinases to be discovered. ERK1 is 44kDa and ERK2 is 42kDa in weight and they are nearly 85% identical (Boulton *et al.,* 1991, Boulton *et al.,* 1990). Other ERKs have also been identified and will be discussed later. The tyrosine and threonine phosphoacceptor sites in the activation loop are separated by a glutamate residue in both ERK1 and 2 and both ERKs are expressed ubiquitously

(Pearson *et al.,* 2001). The ERK cascade is involved in cell growth, differentiation and survival (Schaeffer and Weber, 1999). ERKs are activated by serum, growth factors, cytokines, certain stresses, ligands for G protein-coupled receptors and transforming agents (Pearson *et al.,* 2001). The direct upstream activators of ERK1 and 2 are MEK1 and 2 (Crews *et al.,* 1992, Kosako *et al.,* 1992). Both MEKs will activate both ERKs *in vitro* (Robinson *et al.,* 1996, Zheng *et al.,* 1993). According to Pearson *et al.* (2001) MEK1/2 are assumed to have no other substrates. However, this is only because no other substrate has as yet been identified. Upstream of the MEKs 1and 2, the MEK kinases (MEKKs) are responsible for their activation. The MEKKs upstream of MEK1 and 2 are the most widely studied. Raf and Mos are two MEKKs that are known to exclusively phosphorylate MEKs 1and 2, forming the first step in the three kinase pathway (Dent *et al.,* 1992, Kyriakis *et al.,* 1992). Raf is activated by the small G-protein coupled receptor Ras (Schaeffer and Weber, 1999). Raf itself can be influenced by many other pathways, including protein kinase C (PKC), protein kinase B (PKB) or Akt and p21 activated protein kinase (PAK, Pearson *et al.,* 2001). Once again this demonstrates the scope for crosstalk and influencing of the ERK pathway by many other signalling kinases. ERK1 and 2 have many potential downstream targets, including kinases, p90 and MAPKAP (Blenis *et al.,* 1993, Stockoe *et al.,* 1992). ERK can also translocate directly to the nucleus and activate transcription factors such as AP-1 (activating protein-1) family members, c-jun, c-fos as well as Elk-1 (Pearson *et al.,* 2001), to name but a few.

1.9.3 JNK

JNK, also known as stress activated protein kinase (SAPK) was first discovered in 1990, purified from the livers of rats exposed to cycloheximide (Kriakis and Avruch, 1990). There are 3 isoforms of JNK, 1-3, and they are more than 85% identical to one another. Like ERKs they are activated by phosphorylation on a tyrosine and threonine residue. In the activation loop of JNKs 1-3, these residues are separated by a proline to give a TPY motif. The JNKs are activated by cytokines, agents that interfere with DNA and protein synthesis and as their alternate name suggests several other environmental stresses, such as ultraviolet radiation (Kyriakis *et al.,* 2001, Cano *et al.,* 1995). JNK has also been implicated in the apoptotic pathway (Herr and Debatin, 2001). This is achieved by JNK mediated phosphorylation of the anti-apoptotic proteins Bcl-xl and Bcl-2, causing their inactivation (Park *et al.,* 1997). Two MEK family members are believed to be responsible

for activation of the JNKs. These are MKK4 and MKK7. Both of these MEKs are also capable of activating p38 *in vitro*, though preferential phosphorylation of JNKs occurs (Meier *et al.,* 1996). As mentioned JNK will phosphorylate Bcl family members. It can also directly interact with transcription factors c-jun and Spi-B, the physiological relevance of which has not yet been determined (Derijard *et al.,* 1994, Pearson *et al.,* 2001).

1.9.4 p38

Like the other MAP kinases, there are several members of the p38 family, p38α, β, γ and δ. p38α and β are sensitive to pyridinyl imidazole inhibitors while the γ and δ isoforms are resistant to them (Goedert *et al.,* 1997, Kumar *et al.,* 1997). P38 was first discovered in 1994 in response to physiological stress (Han *et al.,* 1994). All of the p38 family members contain the motif TGY in their activation loop. The MEK members MEK3 and MEK 6 have a high activity towards the p38 MAP kinases, with MEK3 preferentially phosphorylating the α and β isoforms and MEK6 having equal affinity for all p38 members. Both MEK3 and MEK6 will phosphorylate JNK also, again demonstrating the immense interplay in the MAP kinase cascades (Pearson *et al.,* 2001). p38 is also a member of the SAPK group of MAP kinases, due to its responsiveness to stress. Downstream, it has some of the same substrates as ERK, however. Mnk1 (MAP kinase interacting kinase) and Mnk2, serine/threonine kinases, are activated by both ERK and p38, suggesting that p38 might not only play a role in stress responses. MAPKAP kinase and Msk (Mitogen and stress-activated protein kinase), other protein kinases are also downstream targets of both ERK and p38 (Stockoe *et al.,* 1992, Deak *et al.,* 1998), though in the case of Msk, by different stimuli. p38 also has possible nuclear targets but according to Pearson *et al.* (2001) findings are contradictory to date. The MAP kinase family are obviously a very important group of signalling molecules with a wide range of activities in the cell. Understanding their role in critical cellular processes is vital for the understanding of that process itself. Table 1.3 illustrates some other MAP kinases that have been discovered to date and not discussed in this introduction.

MAP Kinase	Other names	Comments	P Site motif
ERK1	p44 MAPK	>80% identical to ERK2; abundant and ubiquitous	TEY
ERK2	p42 MAPK	Abundant and ubiquitous	TEY
ERK3α	p63, rat ERK3	Immunoblotting detects 63K and full-length 95–100K species; αis present in many species including human	SEG
ERK3ß	Human ERK3	~75% identical to ERK3α	SEG
ERK1b	(ERK4)	46K splice form of ERK1; comigrates with band originally named ERK4	TEY
JNK1	SAPKγ	Multiple spliced forms	TPY
JNK2	SAPKα	Multiple spliced forms	TPY
JNK3	SAPKß	Multiple spliced forms	TPY
p38α	p38, CSBP, SAPK2	Sensitive to SB203580	TGY
p38ß	p38-2	Partially sensitive to SB203580	TGY
p38ß2		Sensitive to SB203580; lacks the 8-amino acid insertion unique to p38ß	TGY
p38γ	ERK6, SAPK3	Insensitive to SB203580	TGY
p38δ	SAPK4	Insensitive to SB203580	TGY
Mxi		p38α splice form lacking 80 C-t residues and containing 17 novel ones	TGY
ERK5		Involved in proliferation	TEY
ERK7		May have a role in cell proliferation	TEY
NLK	Nemo-like kinase	Regulation of Wnt pathway; ortholog of *C. elegans* LIT-1; relative of *Drosophila* nemo	TQE
MAK	Male germ cell associated kinase	Expressed in cells undergoing meiosis in the testis but not ovary	TDY
MRK	MAK-related kinase	Expressed in embryonic myocardium; ubiquitous in adult tissues	TDY
MOK		Phorbol ester sensitive	TEY
KKIALRE		Cdc2-related kinase	TDY
KKIAMRE		T, Y mutants still activated in cells	TDY

Table 1.3: Mammalian MAP Kinases (adapted from Pearson *et al.*, 2001)

1.10 Phosphatidylinositol-3-kinase

Phosphatidylinositol-3-kinases (PI3Ks) are a group of enzymes that phosphorylate the various phosphatidylinositols (PIP, PIP_2, PIP_3) leading to further downstream signalling events including the activation of second messengers. Like the MAP kinases, the PI3Ks are very complex, comprising 3 distinct groups, classes I-III, with class I further subdivided into Ia and Ib. The structure of the adaptor and catalytic subunits and the specific PI substrates of these enzymes dictates the class into which they fall (Golden *et*

al., 2004). The activity of PI3K is increased by growth factors and hormones, such as colony-stimulating factor, IGF, PDGF and EGF (Kapellar *et al.,* 1994). Direct activation occurs by growth factor receptors, such as protein-tyrosine receptors in response to these hormones and growth factors (Kapellar *et al.,* 1994). PI3Ks are also regulated internally by phosphatases such as PTEN and SHIP (Golden *et al.,* 2004). Dephosphorylation of PI3Ks by these phosphatases cause a downregulation in PI3K mediated signalling. In the case of the class I PI3Ks direct regulation of the catalytic subunit by the regulatory subunit and vice versa occurs. An important downstream target of PI3K is Akt also known as protein kinase B (PKB). This signalling molecule plays an important role in cell proliferation and survival (Datta *et al.,* 1999). PI3K activation causes Akt translocation to the plasma membrane leading to its eventual activation (Golden *et al.,* 2004). The roles of PI3Ks are numerous but include control of differentiation, cytoskeletal alterations and cell survival (Golden *et al.,* 2004, Kapellar *et al.,* 1994) mediated through Akt and GTPases.

1.11 Summary and aims

This introduction has discussed the various facets of tissue engineering and described how each is important in the successful generation of any 3 dimensional tissue. Tissue engineering is a rapidly emerging field with much hope and enthusiasm for the projected outcomes. The time and money invested in tissue engineering has seen these outcomes come to fruition slower than was expected. This thesis aims to address some of the issues in the field in the hope of contributing to the development of replacement tissues for use in the clinical setting. The aims of this thesis are listed below

- The first aim of this thesis is to set up the necessary protocols and procedures for the isolation and culture of MSCs *in vitro*. Following this, it will be necessary to verify the presence of MSCs in culture and to determine their proliferative capacity under the growth conditions established.

- Culture of MSCs in 2-D under osteoinductive conditions will be the next step in the launching of this project. It will be very important to demonstrate the osteogenic potential of the MSCs *in vitro* in the easily managed 2-D environment.

• While culture in 2-D is instructive, the main aim of this thesis is to develop 3-D tissue engineered constructs for eventual use in bone and cartilage tissue engineering. A novel collagen glycosaminoglycan (Collagen GAG) scaffold will be seeded with culture expanded MSCs and cultured under osteoinductive and chondroinductive conditions. Cell attachment and penetration through the scaffolds as well as osteogenic and chondrogenic potential will be assessed.

• Upon successful osteogenesis in 2-D and 3-D and chondrogenesis in 3-D, the role of ERK and p38 in these processes will be evaluated. In understanding the mechanisms behind these differentiation processes, we can better tailor the next treatment to generate more suitable constructs in less time.

• As discussed in the introduction, mechanical strain is proving to be a potent stimulus in matrix production in bone cells. I intend to investigate the effects of uniaxial tension on the differentiation capacity of MSCs using a custom designed rig developed at the Trinity Centre for Bioengineering.

• In a preliminary study, the effects of static magnetic fields on ERK and JNK activity in cultured cortical neurons will be assessed. The purpose of this study is to determine if magnetic fields can have a protective or mitogenic effect, by activating the ERK pathway, in a readily available cell source, with the aim of potentially using this information to develop a method of priming MSCs before seeding into scaffolds or exposure to strain.

Chapter 2

Materials and Methods

2.1 Cell Culture

2.1.1 Aseptic technique

To maintain cell cultures *in vitro,* it is important to carry out all work under aseptic conditions. This involves keeping all surfaces, especially those in contact with cells, completely sterile. In order to ensure this sterility, the following procedures were followed during all cell culture work.

2.1.2 Sterilisation of glassware, plastics and dissection instruments

All glassware, pipette tips (Sarstedt, Leicester, England) and ultra-pure water and microfuge tubes (Sarstedt, Leicester, England) were wrapped in aluminium foil and autoclave tape and autoclaved at 121°C for 20min (Priorclave Ltd., Model #EH150, London, England). Dissection instruments were baked at 200°C overnight in a hotbox oven (Sanyo-Gallenkamp Hotbox Oven, Model #OHG050, Loughborough, England). Following this all equipment was sprayed with 70% ethanol and placed in the laminar flow hood (Astec-Microflow laminar flow workstation, Florida, U.S.A.). All culture flasks and falcon tubes (Sarstedt, Liecester, England) were purchased sterile and only opened within the confines of the laminar flow hood.

2.1.3 Sterility of the work environment

All work involving the culture of cells, including the preparation of media and other reagents was carried out in a laminar flow hood. Air is drawn into the top of the hood and passed through a pre-filter (AGB, Dublin, Ireland; Model #PNC05818F) and then a HEPA (high efficiency particle air) filter (AGB, Dublin, Ireland; Model #M50069/2A29). The downflow of sterile air creates a positive pressure within the hood, thereby keeping airborne pathogens and contaminants out of the hood. The laminar flow hood is equipped with an ultraviolet light to sterilise the hoods as necessary and all equipment placed into the hood was sprayed liberally with 70% ethanol. Any work in the hood was carried out using disposable latex gloves that were also sprayed with ethanol. Gloves were changed regularly to avoid infection of cultures and the hood was also wiped down with ethanol on a regular basis.

2.1.4 Sterilisation of media and reagents

All solutions and media were filtered through a 0.2µm cellulose acetate membrane syringe filter (Pall Gellman, Michigan, U.S.A.) into autoclaved glass bottles or sterile 15/50ml centrifuge tubes (Sarstedt, Liecester, England). All reagents were aliquoted under sterile conditions and were sterile filtered before use.

2.2 Mesenchymal stem cell isolation and culture

2.2.1 Animals

Young adult Wistar rats (Approximately 200-250g), bred at the BioResources unit of Trinity College Dublin, were used in this study. Animals were maintained in a 12hr light/dark cycle at an ambient temperature of 22-23°C. Food (laboratory chow) and water was available *ad libitum*. Animals were sacrificed by CO_2 asphyxiation. They were placed in a CO_2 chamber and the gas was turned on. Animals were left for five minutes, then retrieved for dissection.

2.2.2 Dissection

Fur around the hind limbs was sprayed liberally with 70% alcohol and the fur was removed. The hip joint was dislocated and the leg was cut free from the body. The femur was dislocated from the tibia at the knee joint and was pulled free from any muscle. Any remaining muscle was carefully dissected from the femur. The femur was placed in sterile pre-warmed Dulbecco's Modified Eagles Medium (DMEM; Sigma-Aldrich, England), supplemented with 2% penicillin/streptomycin (Gibco BRL, Dublin), 10% Foetal Bovine Serum (FBS; Gibco BRL, Dublin), 0.5% l-Glutamine (Gibco BRL, Dublin), 0.5% Glutamax (Gibco BRL, Dublin) and 1% non essential amino acids (Gibco BRL, Dublin). To obtain the tibia, the Achilles tendon was severed as were several other tendons on the near side of the foot. The foot was levered away from the experimenter and pushed towards the knee joint. The remaining muscles were pulled free from their insertions by their tendons towards the kneecap. The kneecap was then removed and with it all

adherent muscle tissue. Once all bones were removed and freed of muscle tissue, they were transferred to a laminar flow hood to maintain sterile conditions.

2.2.3 Cell isolation and culture

Bones were cut at both epiphyses and the marrow was flushed into a 50ml falcon tube using pre-warmed (37°C) supplemented DMEM and a 25gauge needle (Beckton Dickinson Labware Europe, France) and syringe (Beckton Dickinson Labware Europe, France). Approximately 5ml of medium was used for each bone. Once all bones were flushed through, the suspension was centrifuged at 2000g (Sigma Aldrich, Model #2K15C, St. Louis, USA) for 5 minutes at 22°C. The supernatant was discarded and the pellet was resuspended in 10ml of complete medium. This suspension was serially passed through 16 gauge, 18 gauge and 20 gauge needles three times each to obtain a single cell suspension. The suspension was passed through 40μm sterile nylon mesh to remove any cell clumps. The suspension was plated onto a petri dish for a half an hour after which time the supernatant was removed and the adherent cells discarded. The cell suspension was counted using a haemacytometer, and diluted accordingly with supplemented DMEM, to give a cell suspension of approximately $5x10^7$ nucleated cells per $75cm^3$ tissue culture flask. After 24 hours the flask culture medium was replaced to remove non-adherent cells. The flask was rinsed once with supplemented DMEM and then 10 ml supplemented DMEM was added to the flask. Culture medium was replaced every three to four days. Cells were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air.

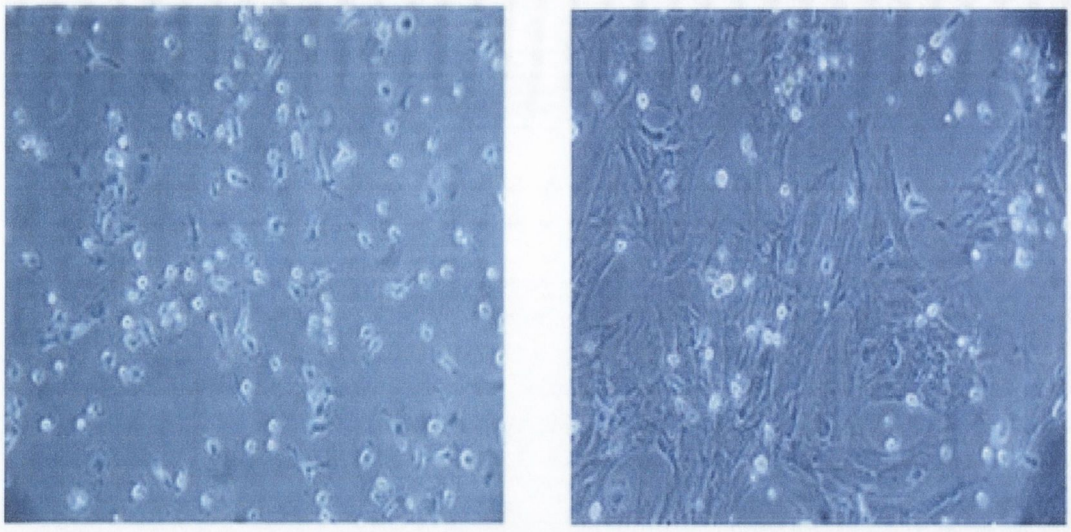


Figure 2.1: MSC cell culture 24 and 96 hours post plating

2.3 Cell treatment

2.3.1 Cell culture in osteoinductive factors

In order to induce osteogenesis, normal cell culture medium (supplemented DMEM) needs to be supplemented with certain factors that will provoke differentiation in MSCs along a defined pathway. Initial treatment of MSC cultures involved supplementing the standard culture medium with bone morphogenetic protein-2 (BMP; Sigma Aldrich, England) at a concentration of 60ng/ml and dexamethasone (Dex; Sigma Aldrich, England) at a concentration of 10nM. Medium was changed every three to four days as usual.

In studies involving the seeding of scaffolds standard culture medium was supplemented with 10nM dexamethasone (Sigma Aldrich, England), 10mM β-glycerophosphate (Sigma Aldrich, England) and 0.05mM ascorbic acid-2-phosphate (Sigma Aldrich, England). Under these conditions, only half the medium was replaced every three to four days in order to make use of any soluble factors that might be releasd by the cells. Cells were initially cultured in 1ml of medium. Every three to four days 500µl was removed and replaced with fresh culture medium supplemented with osteoinductive factors or control medium as appropriate.

2.3.2 Cell culture in chondroinductive factors

In order to induce chondrogenesis, normal culture medium (supplemented DMEM) was supplemented with 100nM dexamethasone and 10ng/ml transforming growth factor-β (Sigma Aldrich, England). Under these conditions, only half the medium was replaced every three to four days. Cells were initially cultured in 1ml of medium and every three to four days, 500µl was removed and replaced with fresh culture medium supplemented with chondroinductive factors or control medium.

2.3.3 Treatment with U0126

To block the activation of the mitogen activated protein kinase (MAPK) ERK, cells were cultured as usual in the presence or absence of osteoinductive or chondroinductive factors and incubated with the MAPK inhibitor U0126 (Calbiochem; 2µM). U0126 binds selectively to only MEK 1 and MEK 2 with IC_{50}s = 0.07 µM ± 0.02 and 0.06µM ± 0.02, respectively. It also has no effect on any downstream targets of MEK 1 and 2 (Duncia *et al.,* 1998). U0126 was reconstituted at 1mg/ml in dimethyl-sulfoxide (DMSO, Sigma-Aldrich, England). Since U0126 is light sensitive, cells were treated in the dark for the duration of the study. Medium was changed every three to four days.

2.3.4 Treatment with SB203580

To block the activation of the mitogen activated protein kinase (MAPK) p38, cells were cultured as usual in the presence or absence of osteoinductive or chondroinductive factors and incubated with the p38 inhibitor SB203580 (Calbiochem; 10µM). SB203580 was reconstituted at 2mg/ml in dimethyl-sulfoxide (DMSO, Sigma-Aldrich, England). Since SB203580 is light sensitive, cells were treated in the dark for the duration of the study. Medium was changed every three to four days.

2.3.5 Treatment with LY294002

To block the activation of PI3 kinase, cells were cultured as usual in the presence or absence of chondroinductive factors and incubated with the PI3K inhibitor L294002 (Calbiochem; 50μM). L294002 was reconstituted at .75mg/ml in dimethyl-sulfoxide (DMSO, Sigma-Aldrich, England). Since L294002 is light sensitive, cells were treated in the dark for the duration of the study. Medium was changed every three to four days.

2.4 Cell Passaging

Upon reaching 80-90% confluency, as assessed by visual inspection under and inverted microscope (Nikon Labophot, Nikon Instech Co. Ltd, Kanagawa, Japan) cells were passaged at lower densities onto new culture flasks. Flasks were rinsed twice in prewarmed sterile phosphate buffered saline (PBS; 100mM NaCl, 80mM Na_2HPO_4, 20mM $Na_2H_2PO_4$; Sigma-Aldrich, England). 5ml of Trypsin EDTA (Sigma-Aldrich, England) was placed onto each flask to detach the cells from the flask surface and flasks were incubated for 5 mins at 37°C in a CO_2 incubator. The cultures were examined under an inverted light microscope (Nikon Labophot, Nikon Instech Co., Ltd, Kanagawa, Japan) to determine that they had sufficiently detached from the surface and the flask was knocked once on the side to release any remaining attached cells. The suspension was poured into a 50ml tube (Sarstedt, Leicester, England) and the flask was rinsed twice with 5ml of supplemented DMEM and this solutuion was also added to the 50ml tube. The cell suspension was spun down at 2000g for 5 min at 22°C. The supernatant was discarded and the pellet was resuspended in 10ml of supplemented DMEM. The suspension was aspirated through a 20 gauge needle three times to obtain a single cell suspension and the cells were replated onto T75 culture flasks (Sarstedt, Leicester, England) at half their original density. Cultures were maintained at 37°C in a 95% air 5% CO_2 incubator. Supplemented DMEM was changed every 3 to 4 days.

2.5 Culture of cortical neurons

Primary cortical neurons were prepared from 1-day old Wistar rats and maintained in neurobasal medium (Gibco BRL, Paisley, United Kingdom). Rats were decapitated, the cerebral cortices dissected and the meninges removed. The cortices were incubated in

phosphate-buffered saline (PBS) (Sigma-Aldrich, England) with trypsin (0.25 mg ml^{-1}) (Sigma-Aldrich, England), for 25 min at 37^0C. The cortical tissue was then triturated (x5) in PBS containing soyabean trypsin inhibitor (Sigma-Aldrich, England, 0.2 mg ml^{-1}) and DNAse (Sigma-Aldrich, England, 0.2 mg ml^{-1}) and gently filtered through a sterile mesh filter (40 μm, Beckton Dickinson Labware Europe, France). The suspension was centrifuged at 2000 x *g* for 3 min at 20^0C and the pellet resuspended in warm neurobasal medium, supplemented with heat inactivated horse serum (10%), penicillin (100U/ml), streptomycin (100U/ml) and glutamax (2mM). Suspended cells were plated at a density of 0.25 x 10^6 cells on circular 10mm diameter coverslips, coated with poly-L-lysine (60 mg/ml), and incubated in a humidified atmosphere containing 5% CO_2: 95% air at 37^0C for 2hr prior to being flooded with pre-warmed neurobasal medium. After 48hr 5ng/ml cytosine-arabino-furanoside was added to the culture medium to suppress the proliferation of non-neuronal cells and maintain the purity of the cortical culture. This ensures that microglia and astrocyte contamination is less than 6% in all culture preparations. Media was exchanged for fresh media every 3 days and cells were grown in culture for up to 14 days.

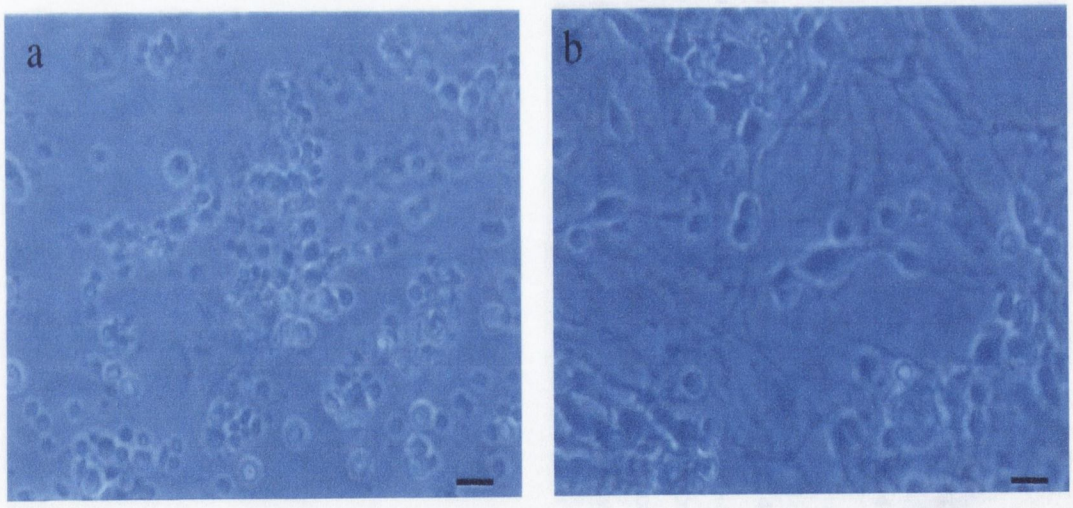


Figure 2.2: Cultured cortical neurons (a) immediately following culture and (b) following neurite extension

2.6 Evaluation of Stem cell viability

2.6.1 Immunofluorescent staining for CD105 (Endoglin)

Day 1

MSCs cultured on glass coverslips (Chance Propper, West Midlands, England) in 24 well plates (Sarstedt, Leicester, England) for 3 days, were fixed in 4% paraformaldehyde (Sigma Aldrich, England) for 30 min at room temperature and then rinsed three times in tris buffered saline (TBS; 20mM Tris-HCl, 150mM NaCl; pH 7.4; Sigma Aldrich, England). Cells were permeabilised by placing in TBS containing 0.1% Triton-X100 for 10 minutes at room temperature (Sigma Aldrich, England) and cells were then refixed in 4% paraformaldehyde for 30min. Following this, cells were washed three times (5min each) in TBS. Non-reactive sites were blocked for 2hr at room temperature using blocking buffer (TBS with 0.1% Triton-X100 and 10% heat-inactivated horse serum; Gibco BRL, Dublin). Coverslips were placed in a humidified chamber and the primary CD105/Endoglin antibody (1:250 in blocking buffer; Santa Cruz Biotechnology, California, USA) was added overnight at 4°C. The antibody is a rabbit polyclonal IgG raised against a recombinant protein corresponding to amino acids 27-326 mapping near the amino terminus of endoglin of human origin.

Day 2

Cells were washed three times (5min each) in TBS and incubated with a biotinylated secondary anti-rabbit IgG (1:50 in blocking buffer; Vector, California, USA) for 1hr at RT. Cells were washed again three times with TBS. Extravadin FITC (1:50 in TBS; Vector, California, USA) was added for 1hr at RT in a darkened room. Cells were washed six times in distilled water. Coverslips were mounted cell side down onto glass slides in vectashield (Vector, California, USA) and the edges of the coverslip were sealed with nail varnish. Cells were visualised under x40 magnification using fluorescence microscopy (Leitz Orthoplan Microscope, Leica Microsystems AG, Wetzlar, Germany) and Openlab software.

2.6.2 5-Bromo-2'-deoxy-uridine (BrdU) labelling and detection

Cell proliferation was assayed using the 5-Bromo-2'-deoxy-uridine (BrdU) labelling and detection kit 1 (Roche Diagnostics GmbH, Germany) according to the manufacturers' instructions. 5-Bromo-2'-deoxy-uridine (BrdU) is a chemical, that when co-cultured with cells, is incorporated into the DNA of dividing cells. The 5-Bromo-2'-deoxy-uridine (BrdU) labelling and detection kit 1 uses this to label proliferating cells with BrdU. Cells are then fixed and those labelled with BrdU are fluorescently tagged so that mitotic cells can be visualised to determine the rate of cell proliferation. Cells were cultured on glass coverslips in 24 well plates and were maintained in culture until the desired timepoint had been reached. Cell culture medium was removed and BrdU labelling reagent, diluted 1:1000 in culture medium, was added to the culture wells. Cells were incubated for 60 min. The labelling reagent was removed and the cells were washed three times in washing buffer (PBS). Cells were fixed in ethanol fixative (50mM glycine (Sigma Aldrich, England) in 70ml absolute alcohol to give 100ml fixative, pH 2.0) for 30min at -20°C. Cells were again washed three times in washing buffer. Cells were incubated with anti-BrdU working solution for 30 min. at 37°C. Coverslips were washed three times in washing buffer. Cells were incubated at 37°C for 30 min in anti-mouse-Ig-fluorescein. Coverslips were washed three times in washing buffer. Coverslips were mounted cell side down onto glass slides in vectashield (Vector, California, USA) and the edges of the coverslip were sealed with nail varnish and examined under a fluorescent microscope (Leitz Orthoplan Microscope, Leica Microsystems AG, Wetzlar, Germany) at an excitation wavelength of 491nm, using Openlab software.

2.6.3 Aqueous one proliferation assay

The CellTiter 96® AQueous One Solution Reagent contains a novel tetrazolium compound[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. Assays are performed by adding a small amount of the CellTiter 96® AQueous One Solution Reagent directly to culture wells, incubating for 1–4 hours and then recording absorbance at 490nm with a 96-well plate reader. CellTiter 96® AQueous One Solution Reagent was thawed at room

temperature. 20µl of CellTiter 96® AQueous One Solution Reagent was pipetted into each well of a 96-well assay plate containing the cell samples in 100µl of culture medium. The reagent was incubated for 4 hours at 37°C in a humidified, 5% CO_2, 95% air atmosphere. Absorbance was read at 490nm using a 96-well plate reader. Relative absorbance related to cell numbers in each well, thereby giving a proliferation rate over a period of days.

2.7 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.7.1 Preparation of samples

MSC cultures were harvested following incubation in lysis buffer (25mM HEPES, 5mM $MgCl_2$, 5mM dithiothreitol, 5mM EDTA, 2mM PMSF, 5µg/ml aprotinin, pH 7.4; 1ml for T25 flasks, 2ml for T75 flasks; Sigma Aldrich, England) on ice. Lysates were homogenised in lysis buffer using a 1ml glass homogeniser (Jencons, Bedfordshire, England). Protein concentration was assessed (see section 2.6.2) and the samples were equalised. The lysates were diluted 1:2 with sodium dodecyl sulphate (SDS) sample buffer (50mM tris HCl pH 7.4, 10%w/v glycerol, 4% w/v SDS, 5% v/v β-mercaptoethanol, 0.002% w/v Bromophenol Blue). The samples were heated to 100°C for 10 minutes to denature the proteins. All samples were stored at -20°C until required.

2.7.2 Protein Quantification

Calculation of protein concentration in cell samples was carried out according to the method of Bradford (1976). Standards were prepared from stock solution of 1000µg/ml BSA (in dH_2O) and ranged from 0µg/ml to 1000µg/ml. 10µl of sample was added to a well of a 96 well plate (Sarstedt, Leicester, England) and 200µl of filtered BioRad dye reagent (diluted 1:5 in dH_2O; BioRad Laboratories, Munich, Germany) was added to each well. All samples were prepared in triplicate. Samples were incubated for 5 min at room temperature. Absorbance readings were made at a wavelength of 595nm using a 96 well plate reader (Labsystems Multiskan RC). A regression line was plotted (GraphPad Instat) and the concentration of protein was calculated and converted to mg protein/ml.

2.7.3 Preparation of polyacrylamide gels

Polyacrylamide separating gels (1mm thick) with a monomer concentration of either 10% (Acrylamide/bis-acrylamide (30% stock, 33% (v/v), dH_2O 40% (v/v), Tris-HCl, (0.05M pH 8.8, 25% (v/v), SDS (10% w/v stock, 1% (v/v), APS (10% w/v stock, 0.5% (v/v), TEMED, 0.05% (v/v); Sigma Aldrich, England) or 12% (Same ingredients as 10% gels with 40% (v/v) dH_2O and 33% (v/v) Tris-HCl), overlaid with 4% stacking gels (Acrylamide/bis-acrylamide (30% stock, 13% (v/v)), dH_2O 60% (v/v), Tris-HCl, (0.05M pH 6.8, 25% (v/v), SDS (10% w/v stock, 1% (v/v), APS (10% w/v stock, 0.5% (v/v), TEMED, 0.05% (v/v)) were cast by setting them between two 10cm wide glass plates (Sigma Aldrich, England), which were mounted on an electrophoresis unit (Sigma Aldrich, England) using spring clamps. The upper and lower reservoirs of the unit were flooded with electrode running buffer (25mM Tris-base, 200mM glycine, 17mM SDS). Samples (10µl) were loaded into wells and proteins were separated by the application of a 32mA current to the gel apparatus. Pre-stained molecular standards (5µl; Sigma Aldrich, England and Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) were used to confirm the molecular weight of protein bands. The migration of the bromophenol blue was monitored and the current switched off when the dye reached the bottom of the gel.

2.7.4 Semi-dry electrophoretic blotting of proteins

The gel was removed from the apparatus and washed in ice-cold transfer buffer (25mM Tris base, 192mM glycine, 20% methanol (v/v), 0.05% SDS (w/v). It was placed on top of a sheet of nitrocellulose blotting paper (0.45µm pore size ; Sigma Aldrich, England), which had been cut to the approximate size of the gel and soaked in transfer buffer. A sandwich was made by placing one piece of filter paper (Standard Grade No. 3, Whatman, Kent, England) on top of the gel and one piece beneath the nitrocellulose paper. This sandwich was soaked in transfer buffer and placed on the platinum coated titanium electrode (anode) of a semi-dry blotter (Sigma Aldrich, England). The lid of the blotter (stainless steel cathode) was moistened with transfer buffer, placed on top of the sandwich and sealed. The uncovered portion of the cathode was shielded with a mylar cut-out (Sigma Aldrich, England), ensuring that all applied current passed through the sandwich. A constant current of 225mA was applied for 75min.

2.7.5 Western Immunoblotting

The nitrocellulose blotting paper was blocked for non-specific binding with 7% bovine serum albumin (BSA; Sigma Aldrich, England) in TBS tween (TBS with 0.05% tween; TBS-T; Sigma Aldrich, England) and probed with an antibody raised against the protein sought. This was washed off and incubated with a secondary antibody, directed against the animal in which the primary was raised, that was horseradish peroxidase (HRP)-conjugated. A chemiluminescent detection chemical (SuperSignal Ultra; Pierce, Leiden, Netherlands) was added and the blotting paper exposed to 5 x 7 inch photographic film (Hyperfilm, Amersham, Buckinghamshire, England) and developed using a Fuji X-ray processor.

2.7.6 Osteocalcin expression

To assess osteocalcin expression, non-specific binding was blocked by incubating the nitrocellulose in TBS-T containing 7% BSA for 2 hours at room temperature. The membrane was rinsed in TBS-T. The primary antibody used was a goat anti-rat polyclonal osteocalcin antibody (Diagnostic Systems Labs, Webster, Texas, USA). This was incubated overnight at room temperature (10ml 1:1000 dilution in TBS-T containing 0.1% BSA). The nitrocellulose was washed three times for 15 minutes each with TBS-T and then incubated in the secondary antibody (Anti-goat IgG HRP, 1:2000 dilution in TBS-T with 0.1% BSA; Sigma Aldrich, England) for 2 hours at room temperature. The nitrocellulose was again rinsed in TBS-T five times for 15 minutes. A chemiluminescence detection chemical (2ml Supersignal Ultra; Pierce Biotechnology, Illinois, USA) was placed over the nitrocellulose for 3 min and the membranes were exposed to 5 x 7 inch photographic film for 5 sec in the dark before being developed using a Fuji X-ray processor (Model # RGII, FUJIFILM Medical systems, Stamford, USA).

2.7.7 ERK Expression

To assess both active (phosphorylated) isoforms of ERK (p42 and p44) expression and total ERK expression, non-specific binding was blocked by incubating the nitrocellulose in TBS-T containing 7% BSA for two hours at room temperature. The membrane was rinsed in TBS containing 0.05% Tween (TBS-T; Sigma Aldrich, England). The primary

antibody used was a mouse monoclonal pERK antibody raised against an epitope corresponding to a short amino acid sequence containing phosphorylated Tyr-204 of ERK 1 of human origin (identical to corresponding ERK 2 sequence) and a mouse monoclonal antibody for ERK 2 for total ERK expression (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). These were incubated overnight at 4°C (1:500 dilution in TBS-T containing 0.1% BSA). The nitrocellulose was washed three times for 15 minutes each and then incubated in a secondary antibody (Goat anti-mouse IgG HRP, 1:700 dilution in TBS-T with 0.1% BSA; Sigma Aldrich, England) for two hours at room temperature. The nitrocellulose was again washed in TBS-T five times for 15 minutes. Supersignal Ultra (2ml; Pierce Biotechnology, Illinois, USA) was placed over the nitrocellulose for 3min and the membranes were exposed to photographic film for 5 sec in the dark before being developed using a Fuji X-ray processor.

2.7.8 Total Actin Expression

Following western immunoblotting for pERK and total ERK, blots were stripped with an antibody stripping solution (Reblot plus strong solution; 1:10 in dH_2O) and reprobed for analysis of total actin expression in order to confirm equal loading of protein. Non-specific binding was blocked by incubating the nitrocellulose in TBS-T containing 7% BSA for 2 hours at room temperature. The membrane was rinsed in TBS-T. The primary antibody used was mouse monoclonal IgG_1 antibody corresponding to an amino acid sequence mapping at the carboxy terminus of actin of human origin (Santa Cruz Biotechnology, USA). This was incubated overnight at room temperature (10ml 1:200 dilution in TBS-T containing 0.1% BSA). The nitrocellulose was washed three times for 15 minutes each with TBS-T and then incubated in the secondary antibody (Anti-mouse IgG HRP, 1:500 dilution in TBS-T with 0.1% BSA; Sigma Aldrich, England) for 2 hours at room temperature. The nitrocellulose was again rinsed in TBS-T five times for 15 minutes. Supersignal Ultra (2ml; Pierce Biotechnology, Illinois, USA) was placed over the nitrocellulose for 3 min and the membranes were exposed to 5 x 7 inch photographic film for 5 sec in the dark before being developed using a Fuji X-ray processor.

2.7.9 Densitometry

In all cases quantification of protein bands was achieved by densitometric analysis using the Zero-Dscan Image Analysis System (Scanalytics Inc., Fairfax, USA). Values are expressed as arbitrary units.

2.8 Scaffold seeding

Upon reaching 80-90% confluency, as determined by light microscopy, cells were trypsinised and seeded onto collagen GAG scaffolds (1cm x 1cm; MIT Boston MA, USA). Flasks were rinsed twice in prewarmed PBS. 5ml of Trypsin EDTA (Sigma-Aldrich, England) was placed onto each flask to detach the cells from the flask surface and flasks were incubated for five minutes at 37°C. The cultures were examined under an inverted microscope to determine that they had sufficiently detached from the surface and the flask was knocked once on the side to release any remaining fixed cells. The suspension was poured into a 50ml falcon tube (Sarstedt, Leicester, England) and the flask was rinsed twice with 5ml of supplemented culture medium and this suspension was also added to the tube. The cell suspension was spun down at 2000g for five minutes at 22°C. The supernatant was discarded and the pellet was resuspended in 2ml of supplemented DMEM. The medium was aspirated through a 20 gauge needle three times to obtain a single cell suspension. Cell numbers were determined using a haemocytometer and suspensions were diluted or concentrated to give approx $1*10^6$ cells/ml. Each scaffold was placed in a single well of a six well plate (Sarstedt, Liecester, England), seeded with 150µl of cell suspension and incubated for 30 minutes. Scaffolds were overturned onto agar coated wells (2ml of 2% autoclaved agarose gel per well; Promega Corporation, Madison, USA) and another 150µl of suspension was placed onto the samples. After 30 minutes 500µl of supplemented DMEM was added to control wells. To induce osteogenesis, dexamethasone (10nM; Sigma-Aldrich, England), ascorbic acid-2-phosphate (0.05mM ; Sigma-Aldrich, England) and β-glycerophosphate (10mM; Sigma-Aldrich, England) was added to the normal supplemented DMEM. To induce chondrogenesis, dexamethasone (100nM) and transforming growth factor (10ng/ml TGF (; Sigma-Aldrich, England), was added to the normal supplemented DMEM. Medium was

replaced every 3 to 4 days. Samples were maintained in culture for either two or three weeks following seeding.

2.9 Paraffin wax embedding of scaffold samples

Scaffolds were removed from the incubator, placed into plastic cassettes and soaked in 4% paraformaldehyde (4% paraformaldehyde in dH2O; pH 7.4; Sigma-Aldrich, England) for 1 hr. Cassettes were placed in the histokinette (Type FE 7326; British American Optical Company, England) where they were sequentially soaked in 70% alcohol twice, spirit alcohol twice, absolute alcohol twice, xylene twice and finally molten paraffin wax for two hours each. Following this treatment, scaffolds were removed from the cassettes and placed into metal mouldings. Heated liquid paraffin wax was poured into each of the mouldings in the Tissue Tek II (British American Optical Company, England), and these were allowed to cool on a chilled surface until the wax solidified.

2.10 Sectioning of wax embedded sections

Wax embedded scaffolds were placed in the microtome (Ernst Leitz Ltd. Germany) and 10 μm thick sections were cut. Three sections were placed onto each subbed slide (0.5% Gelatine and 0.05% Chromalum in dH_2O ; Sigma-Aldrich, England) and placed in a hotbox oven (60°C; Sanyo-Gallenkamp Hotbox Oven, Model #OHG050, Loughborough, England) overnight in order to fix the wax sections to the glass slide.

2.11 Toluidine blue staining

Wax embedded sections were de-waxed in xylene for 5min and rehydrated for 30s in absolute alcohol, spirit and 70% alcohol and water. Slides were placed in 250ml toluidine blue (1% in dH_2O; Sigma-Aldrich, England) for 30 sec and then washed five times in distilled water and dehydrated through 70% alcohol, spirit, absolute alcohol and xylene. DPX (BDH Laboratory Supplied, Poole, England) was placed over each scaffold section and samples were mounted with a glass coverslip (Sigma-Aldrich, England). Slides were viewed under a light microscope (Olympus Model CH-2; Mason, Dublin, Ireland)

2.12 Alizarin Red S staining for calcium

2.12.1 Alizarin Red S staining on scaffolds

Wax embedded sections were de-waxed in xylene for 5min and rehydrated for 30s in absolute alcohol, spirit and 70% alcohol and water. Slides were placed in 250ml Alizarin red (2% in dH_2O; Sigma-Aldrich, England) for 2 min and then washed five times in distilled water and dehydrated through 70% alcohol, spirit, absolute alcohol and xylene. DPX (BDH Laboratory Supplied, Poole, England) was placed over each scaffold section and samples were mounted with a glass coverslip (Sigma-Aldrich, England). Slides were viewed under a light microscope (Olympus Model CH-2; Mason, Dublin, Ireland).

2.12.2 Alizarin Red staining for calcium on coverslips

Paraformaldehyde fixed MSCs were rinsed in distilled water. Coverslips were placed in 250ml Alizarin red (2% in dH_2O; Sigma-Aldrich, England) for 2 min and then washed five times in distilled water and dehydrated through 70% alcohol, spirit, absolute alcohol and xylene. DPX (BDH Laboratory Supplied, Poole, England) was placed onto each coverslip and samples were mounted, cell side down onto a glass slide (Sparks, England). Slides were viewed under a light microscope (Olympus Model CH-2; Mason, Dublin, Ireland).

2.13 Von Kossa Staining For Calcium Phosphate

Wax embedded sections were de-waxed by soaking slides in xylene for 5min and for 30s in absolute alcohol, spirit and 70% alcohol and cells were washed five times in distilled water. Cells were incubated in 5% silver nitrate (500µl per slide in dH_2O; Sigma-Aldrich, England) under an electric lamp for 1hr at room temperature. Cells were washed five times in distilled water and placed in 5% sodium thiosulphate (in dH_2O; Sigma-Aldrich, England) for 2min. Cells were washed again three times in distilled water and placed in toluidine blue (1% in dH_2O) for 10s. Cells were washed three times with distilled water and dehydrated through 70% alcohol, spirit, absolute alcohol and xylene. DPX (BDH Laboratory Supplied, Poole, England) was placed over each scaffold section and samples

were mounted with a glass coverslip (Sigma-Aldrich, England) and viewed under a light microscope (Olympus Model CH-2; Mason, Dublin, Ireland).

2.14 Immunofluorescent Staining for Collagen type II

Day 1

Wax embedded sections of collagen GAG scaffold were de-waxed by soaking slides in xylene for 5min and for 30s in absolute alcohol, spirit and 70% alcohol. Cells were permeabilised by placing in TBS containing 0.1% Triton-X100 for 10 minutes (Sigma Aldrich, England) and fixed in 4% paraformaldehyde for 30min at room temperature. Following this, cells were washed three times (5min each) in TBS. Non-reactive sites were blocked for 2hr at RT using blocking buffer (2% BSA in TBS with 0.1% Triton-X100 and 20% heat-inactivated horse serum, (Gibco BRL, Dublin)). Slides were placed in a humidified chamber and a goat anti-human collagen type II antibody (1:500 in blocking buffer) was added overnight.

Day 2

Cells were washed three times (5min each) in TBS and incubated with a biotinylated secondary anti-goat IgG (1:50 in blocking buffer; Vector, California, USA) for 1hr at room temperature. Cells were washed again three times with TBS. Extravadin FITC (1:50 in TBS; Sigma Aldrich, England) was added for 1hr at RT in a darkened room. Cells were washed six times in distilled water. coverslips were mounted cell side down onto glass slides in vectashield and the edges of the coverslip were sealed with nail varnish. Coverslips were mounted cell side down onto glass slides in vectashield (Vector, California, USA) and the edges of the coverslip were sealed with nail varnish and examined under a fluorescent microscope (Leitz Orthoplan Microscope, Leica Microsystems AG, Wetzlar, Germany) at an excitation wavelength of 491nm, using Openlab software.

2.15 Immunofluorescent Staining For Osteocalcin

Day 1

Wax embedded sections of collagen GAG scaffold were de-waxed by soaking slides in xylene for 5min and for 30s in absolute alcohol, spirit and 70% alcohol. Cells were permeabilised by placing in TBS containing 0.1% Triton-X100 for 10 minutes (Sigma Aldrich, England) and fixed in 4% paraformaldehyde for 30min at room temperature. Following this, cells were washed three times (5min each) in TBS. Non-reactive sites were blocked for 2hr at RT using blocking buffer (2% BSA in TBS with 0.1% Triton-X100 and 20% heat-inactivated horse serum, (Gibco BRL, Dublin)). Slides were placed in a humidified chamber and a goat anti-rat osteocalcin antibody (1:1000 in blocking buffer) was added overnight.

Day 2

Cells were washed three times (5min each) in TBS and incubated with a biotinylated secondary anti-goat IgG (1:50 in blocking buffer) for 1hr at RT. Cells were washed again three times with TBS. Extravadin FITC (1:50 in TBS) was added for 1hr at RT in a darkened room. Cells were washed six times in distilled water. Coverslips were mounted cell side down onto glass slides in vectashield and the edges of the coverslip were sealed with nail varnish. Coverslips were mounted cell side down onto glass slides in vectashield (Vector, California, USA) and the edges of the coverslip were sealed with nail varnish and examined under a fluorescent microscope (Leitz Orthoplan Microscope, Leica Microsystems AG, Wetzlar, Germany) at an excitation wavelength of 491nm, using Openlab software. Coverslips seeded with MSCs were stained using the same technique without the dewaxing step.

2.16 Immunofluorescent staining for collagen type I

Day 1

Wax embedded sections of collagen GAG scaffold were de-waxed by soaking slides in xylene for 5min and for 30s in absolute alcohol, spirit and 70% alcohol. Cells were

permeabilised by placing in TBS containing 0.1% Triton-X100 for 10 minutes (Sigma Aldrich, England) and fixed in 4% paraformaldehyde for 30min at room temperature. Following this, cells were washed three times (5min each) in TBS. Non-reactive sites were blocked for 2hr at RT using blocking buffer (2% BSA in TBS with 0.1% Triton-X100 and 20% heat-inactivated horse serum, (Gibco BRL, Dublin)). Slides were placed in a humidified chamber and a goat anti-rat collagen type I antibody (1:500 in blocking buffer) was added overnight.

Day 2

Scaffolds were washed three times (5min each) in TBS and incubated with a biotinylated secondary anti-goat IgG (1:50 in blocking buffer) for 1hr at RT. Scaffolds were washed again three times with TBS. Extravadin FITC (1:50 in TBS) was added for 1hr at RT in a darkened room. Scaffolds were washed six times in distilled water. Coverslips were mounted onto glass slides in vectashield and the edges of the coverslip were sealed with nail varnish. Coverslips were mounted cell side down onto glass slides in vectashield (Vector, California, USA) and the edges of the coverslip were sealed with nail varnish and examined under a fluorescent microscope (Leitz Orthoplan Microscope, Leica Microsystems AG, Wetzlar, Germany) at an excitation wavelength of 480nm, using Openlab software. Coverslips seeded with MSCs were stained using the same technique without the dewaxing step.

2.17 Phosphorylated ERK analysis

2.17.1 Immunofluorescent staining for phosphorylated ERK expression in MSCs and ERK and JNK expression in neurons

Day 1

Treated cells were fixed with 4% paraformaldehyde for 30 min at RT. Fixed cells were permeabilised by incubating in 0.2% Triton X-100 (Sigma-Aldrich, England) and 0.2% proteinase K (Sigma-Aldrich, England). Cells were refixed in paraformaldehyde for a further 30 mineralisation and then washed in TBS. Non-reactive sites were blocked with blocking buffer (5% goat serum in TBS) for 2hr at RT. Cultures were then incubated

overnight at 4°C with a mouse polyclonal anti-phospho-specific ERK antibody (Santa CruZ Biotechnology Inc, Santa Cruz, CA, USA) that recognises ERK1 and ERK2 isoforms (1:500 dilution in TBS containing 5% goat serum), or a mouse polyclonal anti-phospho-specific JNK antibody (1:500 dilution in TBS containing 5% goat serum, Santa CruZ Biotechnology Inc, Santa Cruz, CA, USA).

Day 2

Cells were washed 3 times in TBS and incubated in the dark with goat anti mouse IgG conjugated to flourescein isothiocyanate (FITC) (diluted 1:100 in 5% goat serum in TBS; Sigma, Dorset, United Kingdom) for 1hr at 37°C. Coverslips were mounted on slides cell side down in vectashield (Vector, California, USA) and viewed by fluorescence microscopy at an excitation of 491nm.

2.17.2 Immunocytochemistry for phosphorylated ERK expression in MSCs and ERK and JNK expression in neurons

Day 1

Treated cells were fixed with 4% paraformaldehyde for 30 min at RT. Fixed cells were permeabilised by incubating in 0.1% Triton X-100 in TBS (Sigma-Aldrich, England) with 0.2% proteinase K (Sigma-Aldrich, England). Cells were refixed in paraformaldehyde for a further 30 mineralisation and then washed in TBS. Endogenous peroxidases were blocked for five minutes with 0.3% hydrogen peroxide in distilled water (Sigma-Aldrich, England). Coverslips were washed 3 times in TBS. Non-reactive sites were blocked with blocking buffer (5% goat serum in TBS) for 2hr at RT. Cultures were then incubated overnight at 4°C with a mouse polyclonal anti-phospho-specific ERK antibody (Santa CruZ Biotechnology Inc, Santa Cruz, CA, USA)that recognises ERK1 and ERK2 isoforms (1:500 dilution in TBS containing 5% goat serum) or a mouse polyclonal anti-phospho-specific JNK antibody (1:500 dilution in TBS containing 5% goat serum, Santa CruZ Biotechnology Inc, Santa Cruz, CA, USA).

Day 2

Cells were washed 3 times in TBS and incubated for 1hr at room temperature with biotinylated anti-mouse IgG (1:50 in blocking buffer, Vecor, California, USA). Cells were again washed 3 times in TBS and incubated in streptavidin horseradish peroxidase for 1hr at room temperature. Coverslips were washed 3 times in TBS. DAB chromagen containing 1% hydrogen peroxide and 4% nickel ammonium sulphate was placed onto the coverslips until the colour developed. Coverslips were then washed six times in distilled water and mounted cell side down in DPX (BDH Laboratory Supplied, Poole, England). Slides were viewed under a light microscope (Olympus Model CH-2; Mason, Dublin, Ireland). ERK activity was assessed by calculation of percentage pERK positive neurons out of 400 counted per coverslip.

2.18 Immunocytochemistry for phosphorylated P38 expression in MSCs in 2-D

Day 1

Treated cells were fixed with 4% paraformaldehyde for 30 min at RT. Fixed cells were permeabilised by incubating in 0.1% Triton X-100 in TBS (Sigma-Aldrich, England) with 0.2% proteinase K (Sigma-Aldrich, England). Cells were refixed in paraformaldehyde for a further 30 mineralisation and then washed in TBS. Endogenous peroxidases were blocked for five minutes with 0.3% hydrogen peroxide in distilled water (Sigma-Aldrich, England). Coverslips were washed 3 times in TBS. Non-reactive sites were blocked with blocking buffer (5% goat serum in TBS) for 2hr at RT. Cultures were then incubated overnight at 4°C with a mouse polyclonal anti-phospho-specific P38 antibody (Santa CruZ Biotechnology Inc, Santa Cruz, CA, USA) (1:500 dilution in TBS containing 5% goat serum).

Day 2

Cells were washed 3 times in TBS and incubated for 1hr at room temperature with biotinylated anti-mouse IgG (1:50 in blocking buffer, Vecor, California, USA). Cells were again washed 3 times in TBS and incubated in streptavidin horseradish peroxidase for 1hr at room temperature. Coverslips were washed 3 times in TBS. DAB chromagen

containing 1% hydrogen peroxide and 4% nickel ammonium sulphate was placed onto the coverslips until the colour developed. Coverslips were then washed six times in distilled water and mounted cell side down in DPX (BDH Laboratory Supplied, Poole, England). Slides were viewed under a light microscope (Olympus Model CH-2; Mason, Dublin, Ireland). Percentage active ERK positive cells out of a total of 400 cells counted was calculated for each culture preparation.

2.19 Quantification of mineralisation by stereology in collagen GAG scaffolds

Scaffold was seeded with cells, cultured in osteogenic medium, fixed and embedded in paraffin wax as described in sections 2.9 and 2.10.The full scaffold was then sliced into 10μm sections. These sections were stained for calcium phosphate, using the previously describe Von Kossa method with the exception of the Toluidine Blue step. Stained sections were digitally imaged and transferred to pc. Each image was imported into Scion Image (Scion Corporation), thresholded and converted to a binary image. The percentage black pixels to white was then calculated for each slice. The average for the 85 slices was then calculated, giving the percentage of the scaffold that had been mineralised within three weeks.

2.20 Unconfined compression testing of scaffolds

The Young's Modulus of control and osteogenically treated scaffolds was calculated by unconfined compression testing, using the Zwick Z005 mechanical testing system (Zwick Roell Group, Ulm Germany). Scaffolds were placed between two custom made platens and underwent three ramp and hold compression series, corresponding to 20%, 10% and 10% of the original thickness of the scaffold sample. Each step took 10min in duration. The results from this test gave a stress relaxation curve for each scaffold from which Young's Modulus could be calculated.

2.21 Statistical Analysis

Data are expressed as means ± standard error of the means (SEM). Statistical Analysis was carried out by use of a one-way analysis of variance, followed by a post-hoc Student Newman-Keuls test when significance was indicated. When comparisons were being

made between two treatments, a paired Student's t-test was performed to determine whether significant differences existed between the conditions. In all cases the alpha level was set at 0.05. All statistical analysis was carried out using Graphpad Instat software.

2.22 Collagen glycosaminoglycan scaffold manufacture

The Collagen GAG scaffolds were fabricated in MIT by Brian Harley from a collagen-glycosaminoglycan suspension using a freeze-drying method. The collagen GAG suspension was produced from microfibrillar, type I collagen isolated from bovine tendon (Integra LifeSciences, Plainsboro, NJ) and chondroitin-6-sulfate isolated from shark cartilage (Sigma-Aldrich Chemical Co., St. Louis, MO). The collagen, chondroitin-6-sulfate, and 0.05M acetic acid (pH 3.2) were mixed at 15,000rpm in an overhead blender (IKA Works, Inc., Wilmington, NC). The temperature of the suspension was maintained at 4°C for the entire mixing process by a cooling system (Brinkman, Westbury, CT) in order to prevent denaturation of the collagen fibers. The final collagen GAG suspension contained 0.5 wt% collagen and 0.05 wt% chondroitin-6-sulfate. After mixing, the collagen GAG suspension was degassed under vacuum (50 mTorr) for 60 minutes to remove air bubbles created by the mixing process. Collagen GAG scaffolds were fabricated using a rapid-freeze (quenching) freeze-drying technique where the collagen GAG suspension, originally at room temperature, is frozen in a grade 304 stainless steel pan (VirTis, Gardiner, NY) by placing it into a pre-cooled freeze-dryer (VirTis Genesis) at –40°C for sixty minutes. The temperature of the freeze-dryer shelf is maintained at a constant temperature of -40°C via computer control and the pan is constructed from the same alloy as the freeze-dryer shelves to allow for more uniform heat transfer during freezing. The frozen suspension is then sublimated under a vacuum (<100mTorr) for 17 hours at a temperature of 0°C. All collagen GAG scaffolds were dehydrothermally crosslinked in order to stiffen the collagen network. Cross-linking was carried out under a vacuum (50mTorr) in a vacuum oven (Fisher IsoTemp 201, Fisher Scientific, Boston, MA) at a temperature of 105°C for 24 hours; this process introduces covalent cross-links between the polypeptide chains of the collagen fibers without denaturing the collagen into gelatin.

Protein	Antibody source	Secondary antibody	Antibody dilution
ERK	Mouse	Goat anti-mouse IgG FITC conjugated Biotinylated anti-mouse IgG	1° 1:500 in TBS 2° 1:100 in blocking buffer for FITC conjugated 2° 1:50 in blocking buffer for biotinylated anti mouse
JNK	Mouse	Goat anti-mouse IgG FITC conjugated	1° 1:500 in TBS 2° 1:100 in blocking buffer
p38	Mouse	Biotinylated anti-mouse IgG	1° 1:500 in TBS 2° 1:50 in blocking buffer
Collagen type I	Goat	Biotinylated anti-goat IgG	1° 1:500 in blocking buffer 2° 1:50 in blocking buffer
Collagen type II	Goat	Biotinylated anti-goat IgG	1° 1:500 in blocking buffer 2° 1:50 in blocking buffer
Osteocalcin	Goat	Biotinylated anti-goat IgG	1° 1:1000 in blocking buffer 2° 1:50 in blocking buffer

Table 2.1: Primary and secondary antibodies used for immunostaining procedures

Chapter 3

Evaluation of mesenchymal stem cell viability and potential

3.1 Introduction

Current bioengineering approaches for the repair of the bone and cartilage defects involve a choice of two main treatment regimes. The first, and generally accepted gold standard means of repair, is autologous transplantation of bone or cartilage from other body regions, thereby weakening the donor site (Younger and Chapman 1989) and causing painful recovery of a second surgical site. This method is also limited by the amount of donor tissue available. The second treatment involves the use of prosthetic implants to strengthen the defect (Zimmerman *et al.*, 2002), which often creates problems such as infection and eventual loosening of the implant. As such there is great need to develop new approaches for the repair of such clinical problems as non-union fractures and osteoarthritis. These, and other problems associated with treatment of such diseases, have led to the emergence of the field of tissue engineering, with increasing emphasis on the biological aspects. With this in mind, the aim of this research is to develop viable three dimensional bone and cartilage constructs that when implanted will interface with existing tissues *in vivo*. This first results chapter is concerned with the initial experiments that were undertaken to optimise the adult rat mesenchymal stem cell (MSC) culture protocols and assess the purity and viability of the MSCs *in vitro*. It is important to demonstrate some of the defining characteristics of adult stem cells and gain further knowledge of the processes involved in MSC differentiation in a 2-D environment before moving to the 3-D setting that is relevant to tissue engineering. Marrow derived cells isolated from the adult rat femur and tibia contain not only MSCs but also other cells of the stromal system; osteocytes, chondrocytes, tenocytes, adipocytes, smooth muscle cells (Deans *et al.,* 2000), haemopoietic stem cells and their progeny and possibly endothelial precursors (for review see Kirschstein *et al.,* 2001). The majority of work carried out in this area has been performed on MSCs of human origin (Locklin *et al.,* 1999, Schecroun and Delloye, 2003) and the potential benefits of research in smaller animal models are clear, given the limited availability of human MSCs. In addition, progress in the field of tissue engineering has been slower than initially expected with much scepticism of some of the initial claims of success and multilineage potential of various stem cell types (Verfaillie *et al.,* 2002). There is now an increasing realisation that there is a need for better understanding of the processes involved in all forms of stem cell differentiation. The small animal model is perfectly suited for this type of

research. Until recently, it was believed that the induction of osteogenesis in rat models was not possible. However, several recent papers have demonstrated the differentiation of rat MSCs along the osteogenic lineage (Tsuchida *et al.,* 2003, Herbertson and Aubin, 1997, Cassiede *et al.,* 1996).

In setting up the procedures for the isolation, purification and culture of MSCs it was necessary to demonstrate their presence in culture, not only by visual/morphological identification, but also using an accepted MSC marker. Endoglin or CD105 is a cell surface marker that has been recently termed a putative stem cell marker (Lodie *et al.,* 2002). In 1992, Haynesworth *et al.* developed a monoclonal antibody directed against MSCs and termed it SH2. Several years later they showed that this monoclonal antibody recognised an epitope on the cell surface marker Endoglin (CD105) (Barry *et al.,* 1999) and it has since been used in several studies as a MSC marker (Charbord *et al.,* 2002; Conrad *et al.,* 2002). Endoglin is a member of the transforming growth factor beta (TGF-β) receptor family and is also found on the endothelium of postcapillary venules (Zvaifler *et al.,* 2000).

Cell proliferation is an important issue in stem cell research because the low numbers of cells obtained after extraction are insufficient to carry out meaningful experiments. It is estimated that only 1 in 100,000 cells of the bone marrow is a MSC in newborns and can decrease by 10 to 50 fold by the age of 80 (Caplan 1994). As a result, MSC numbers must be expanded in culture for a period of time in order to increase the cell numbers and overall protein content for viable experiments and subsequent analysis. MSCs have been shown to proliferate for up to one hundred cell doublings for extended periods in culture (for review see Verfaillie 2002).

This chapter is also concerned with the temporal appearances of bone related proteins and matrix mineralization in 2-D monolayer cultures. There are a large number of proteins expressed during the differentiation process along the osteoblastic lineage. Initially, collagen type I is expressed, which leads to further differentiation and is believed to be necessary for the process of osteogenesis to take place (Xiao *et al.,* 1998). Following this, several bone specific proteins are expressed, including osteopontin, which is expressed several times during osteogenesis, osteonectin and bone sialoprotein (for review see Aubin *et al.,* 1995). Alkaline phosphatase levels are

also increased during the early phases of osteogenesis. All of these markers of osteogenesis are expressed before matrix mineralization occurs, an occurrence believed by many to be required before osteogenesis can be definitively said to have taken place. Osteocalcin however is not expressed until much later in the differentiation process. Osteocalcin is a vitamin K and vitamin D dependent protein produced by osteoblasts and is the most abundant of the non-collagenous proteins in bone (Lee *et al.,* 2000). Osteocalcin also has clinical relevance, being up or down-regulated in a variety of bone related diseases (for review see Lee *et al.,* 2000). This protein is involved in matrix mineralization (Hauschka *et al.,* 1981) and is upregulated only in post proliferative osteoblasts and is therefore a late stage marker of osteogenesis. The proteins collagen I and osteocalcin were chosen as early and late stage markers of osteogenesis (Aubin *et al.,* 1995) to give as much scope to this study as possible.

It was postulated that the mitogen activated protein kinase (MAPK) extra cellular-signal regulated kinase (ERK) might be involved in initiating the differentiation process along the osteogenic lineage considering its usual role in cell survival and differentiation. Jaiswal *et al.* (2000) demonstrated elevated ERK expression levels following 7-11 days treatment of MSCs with osteogenic supplements and Simmons *et al.* (2003) showed that cyclic strain-induced matrix mineralization occurred via the ERK 1/2 pathway. Both of these studies were carried out on adult human MSCs and the role of ERK in osteogenesis in the rodent stem cell model requires further investigation. p38 is also a member of the MAPK family of signalling molecules but like the third MAPK c-Jun-N-terminal Kinase (JNK), its activity has been related more to cellular apoptosis rather than differentiation and survival (Xia *et al.,* 1995, Kummer *et al.,* 1997). Jaiswal *et al.* also showed that p38 was upregulated during osteogenesis, however were unable to determine its precise function in the osteogenic process. The role of p38 in MSC differentiation along the osteogenic lineage was also a target for investigation in this study. Thus, the aim of this chapter was to validate the purity, proliferative capacity and osteogenic potential of adult rodent MSCs in 3-D. Furthermore, the role of the MAPKs, ERK and p38, in the osteogenic differentiation of these cells was assessed.

3.2 Results

3.2.1 Positive endoglin staining verifies the presence of MSCs in culture

The aim of the initial experiments was to verify the presence of MSCs in monolayer cultures, obtained using the protocol described in section 2.5.1. Figure 3.1 shows representative images demonstrating Endoglin positive cells. The cells had been maintained in culture for 3 days and all cells were found to express Endoglin immunoreactivity. The images are representative of 4 independent cultures.

3.2.2 Cells proliferate for up to ten days in culture

After plating onto culture flasks cells were observed to adhere to the surface within 1 hour. Figure 3.2 (i and ii) demonstrates an absence of BrdU staining, indicating that proliferation had not begun in cells 24 hours post plating. In contrast, following 10 days in culture cells were observed to be in a state of mitosis (Figure 3.2 iii & iv) as evidenced by BrdU incorporation. The images are representative of 5 independent cultures

3.2.3 Cell proliferation begins after approximately 3 days and continues for up to 15 days

Figure 3.3 demonstrates that proliferation of cells began after approximately 3 days in culture and increased for up to 15 days in culture (n=4). This result agreed with the BrdU analysis, showing that cell division did not occur spontaneously following plating. It is most likely that there is a lag phase following plating during which the cells adjust to their surroundings and extend their processes.

3.2.4 Cells cultured in the presence of osteoinductive factors for a period of 2 weeks stop proliferating

When cells were cultured in the presence of 0.68nM dexamethasone and 60ng/ml bone morphogenetic protein (BMP), for 14 days, MSCs did not divide (Figure 3.4 ii & iv), suggesting that they had begun to differentiate. These cells were first cultured for 3 weeks in normal supplemented DMEM, which was then supplemented with

osteoinductive factors for 14 days. This result also showed that those cells cultured in control medium (Figure 3.4 i & ii) for a period of 5 weeks, continued to proliferate. The images are representative of 4 independent cultures.

3.2.5 Collagen type I is expressed in 2-D from day three onwards

To understand the activity of MSCs *in vitro*, the cells were characterised at several timepoints in 2-D monolayer cultures. Cells were cultured with standard medium or with medium supplemented with osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). Figures 3.5 and 3.6 illustrate the temporal expression of collagen type I at 0, 3, 7 (Figure 3.5), 10, 14 and 21 days (Figure 3.6). Collagen type I was expressed weakly from day 3 onwards in osteoinductive factor-treated cultures as assessed by immunocytochemistry. By day 7, immunofluorescence could be observed in nodule-like aggregations. At no point was the expression of collagen I observed in fibroblastic shaped cells. Figure 3.6 illustrates that collagen I positive cells were cuboidal in morphology, without the characteristic cell projections indicative of fibroblasts. As can be seen in Figures 3.5 (i, iii, v) and 3.6 (i, iii, v), no collagen I expression was observed in any control cultures.

3.2.6 Osteocalcin is expressed weakly in 2-D from 3 days onwards

Figures 3.7 and 3.8 illustrate the temporal expression of the late stage osteogenic marker, osteocalcin. MSCs were cultured on coverslips for 0, 3, 7 (Figure 3.7), 10, 14, and 21 days (Figure 3.8) in the presence or absence of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). Figure 3.7 (iv) demonstrates that 3 day exposure to osteoinductive factors resulted a small number of cells displaying weak osteocalcin immunoreactivity. By day 7 more cells were osteocalcin immunopositive, but again staining was quite weak. At this timepoint, some control cells also exhibited osteocalcin immunoreactivity (Figure 3.7 v). This early staining was possibly due to the presence of some differentiating cells in the culture from the original preparation. However, by day 10, osteocalcin expression was much more evident in all osteoinductive factor treated samples (Figure 3.8 ii, iv, vi). This staining timecourse agreed with the findings of others

(Herbertson and Aubin 1997, Cassiede *et al.,* 1996). At these later time points, all cultures maintained in control medium were negative for osteocalcin immunoreactivity (Figure 3.8 i, iii, v).

3.2.7 Cells form a mineralised matrix at day 10 of culture with osteoinductive factors

Figures 3.9 and 3.10 illustrate Alizarin Red S histological staining for calcium, as a marker of matrix mineralisation. The formation of mineralised osteoid is seen as the end point of the osteogenic differentiation process. As osteocalcin is involved in the mineralisation process, its expression would be expected to precede or coincide with mineralisation. Again, MSCs were cultured on coverslips for 0, 3, 7 (Figure 3.9), 10, 14, and 21 days (Figure 3.10) in the presence (ii, iv, vi) or absence (i, iii, v) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). At days 0 and 3, cells were widely spread with extended processes in both control (Figure 3.9, i and iii) and osteoinductive factor-treated samples (Figure 3.9, ii and iv). By day 7, cells were aggregating and some positive staining for calcium was observed in individual cells following exposure to osteoinductive factors. There was a marked effect in cells exposed to osteoinductive factors for 10 days, with large mineralised nodules being observed in treated samples (Figure 3.10, ii). Mineralisation continued up to day 21 (Figure 3.10 vi) with nodules increasing in size.

3.2.8 Phospho-p42 and p44 expression are not altered significantly following osteoinductive treatment for 2 weeks

Extracellular signal regulated kinase (ERK) has been shown to be involved in the processes that lead to the osteogenic differentiation of adult MSCs of human origin (Jaiswal *et al.,* 2000). The following experiments describe the analysis of ERK activity in adult rat MSCs when stimulated with osteoinductive factors. Figures 3.11 and 3.12 show the activity of the p42 and p44 isoforms of ERK following a 2 week treatment with osteoinductive factors (0.68nM Dex and 60ng/ml BMP), as assessed by western immunoblot using an antibody that recognises the phosphorylated form of

p42 and p44 recognising an epitope corresponding to a short amino acid sequence containing phosphorylated Tyr-204. Following a 2 week treatment with the osteoinductive factors, neither phospho p42 (Figure 3.11) nor phospho p44 (Figure 3.12) expression levels were significantly altered compared to control cells cultured without osteoinductive factors. Phospho-p42 was expressed as a proportion of total ERK. In control cells, p42 expression was 7.09 ± 3.21 vs 5.31 ± 2.63 in treated cells ($p=0.2167$). Control p44 expression was 3.17 ± 0.06 vs 5.04 ± 1.43 ($p=0.2167$) in the treated cells. Results are expressed as mean arbitrary units ± SEM for 10 individual cell culture preparations with data analysed by a paired students *t* test and a Bonferroni correction.

3.2.9 Total ERK is not significantly different from control levels after 14 days treatment with osteoinductive factors

Figure 3.13 demonstrates that total ERK expression was also not significantly altered following a 14 day treatment with osteoinductive factors (0.68nM Dex and 60ng/ml BMP) compared to untreated controls. The expression of ERK was 0.9 ± 0.2 in control cultures compared to 1.0 ± 0.2 in cultures treated with osteoinductive factors for 14 days. Results are expressed as mean arbitrary units ± SEM for 10 individual cell culture preparations with data analysed by a paired students *t* test.

3.2.10 Induction of Osteocalcin expression in MSCs is ERK dependant

To determine the role of ERK in the induction of osteocalcin expression, cells were exposed to osteoinductive factors (BMP 60ng/ml and Dexamethasone 0.68nM) with and without the selective ERK inhibitor, U0126 (2μM) for 14 days. Figure 3.14 demonstrates that osteocalcin expression was significantly increased when cells were cultured with osteoinductive factors in the absence of U0126, where mean osteocalcin expression was 0.56 ± 0.038 (arbitrary units ± SEM) in control cells and 8.29 ± 1.06 in cells exposed to osteoinductive factors ($p<0.01$, ANOVA; $n=4$). In contrast, when cells were exposed to the osteoinductive factors in the presence of U0126, the induction of osteocalcin was abrogated. Thus, mean osteocalcin expression in cells exposed to U0126 alone was 0.18 ± 0.012 and 0.62 ± 0.063 in cultures exposed to

both U0126 and osteoinductive factors. This result demonstrates a role for ERK in the osteogenic potential of rodent stromal cells.

3.2.11 ERK activity is significantly increased at day 10 of osteogenic treatment

Having demonstrated that ERK is required for the induction of osteocalcin in adult rodent MSCs (Figure 3.14), in spite of an absence of ERK activity being detected at day 14 (Figure 3.11 and 3.12), a timecourse of ERK activity was determined. Expression of the phosphorylated form of ERK was determined by immunocytochemistry using a phospho-specific antibodyfollowing exposure of the cells to osteoinductive factors for 0, 3, 5, 7, 10, and 12 days. 400 cells were counted per coverslip and identified as positive or negative for ERK immunoreactivity and expressed as the percentage of positive cells of the total number counted. Figure 3.15 shows that, following 10 days treatment with osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM), ERK activity was significantly elevated compared to untreated controls. At day 10, 29.3 ± 6.5% of cells stained positively for phospho-ERK compared to 51 ± 6.1% of osteoinductive factor-treated cells. Results are expressed as means ± SEM for 6 individual cell culture preparations at day 10. A students *t* test with a Bonferroni correction was used to compare controls and osteoinductive factor-treated cells at each timepoint. At no other timepoint was ERK activity in osteoinductive factor-treated cells significantly different from untreated controls. The gradual increase of pERK expression in all samples was likely due to a combination of increasing cell numbers as a result of cell proliferation and some spontaneous differentiation as a result of overcrowding. Figure 3.15 B shows representative photomicrographs of control (i) and osteoinductive factor-treated cells (ii).

3.2.12 p38 activity is significantly increased at day 7 of osteogenic treatment

Jaiswal *et al.* (2000) also showed that the MAPK p38 was also upregulated during osteogenesis in adult human MSCs. Figure 3.16 shows a timecourse of activity of phospho p38 from day 0 to 12. After 7 days treatment with osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM),

expression of the phosphorylated form of p38 was significantly elevated from 30.3 ± 5.4% to 47 ± 1.5% phospho p38 positive cells in control vs osteoinductive factor-treated cells (p=0.0171). At no other timepoint was p38 activity in osteoinductive factor-treated cells significantly different from controls. Results are expressed as means ± SEM for 6 individual cell culture preparations at day 7. A students *t* test with a Bonferroni correction was used to compare controls and treated cells at each timepoint. As in the above result, the gradual increase in p38 activity in control cells was likely due to increasing cell numbers and spontaneous differentiation.

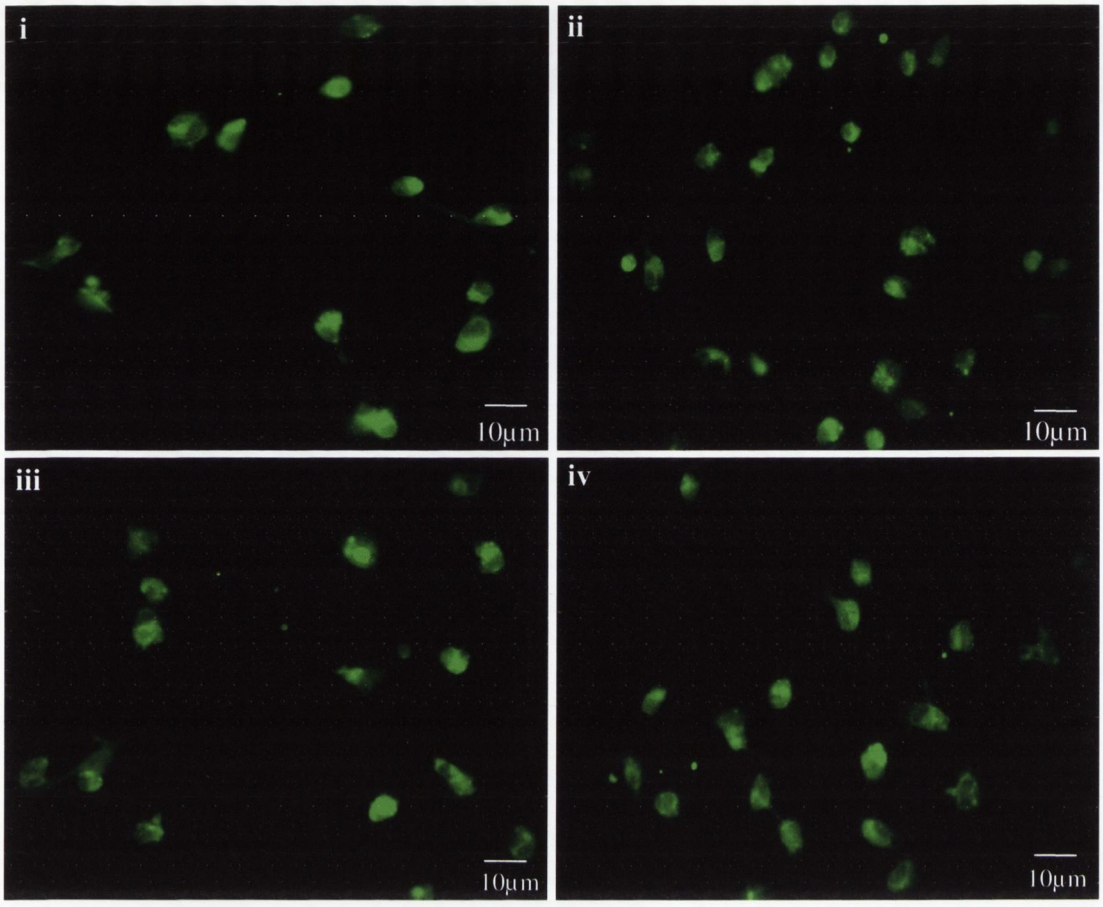


Figure 3.1: Positive endoglin staining verifies the presence of MSCs in culture

The presence of endoglin immunoreactivity is an indicator of the presence of mesenchymal stem cells in culture. A MSC culture was prepared and maintained in culture for 3 days. The images are representative of 4 independent culture preparations.

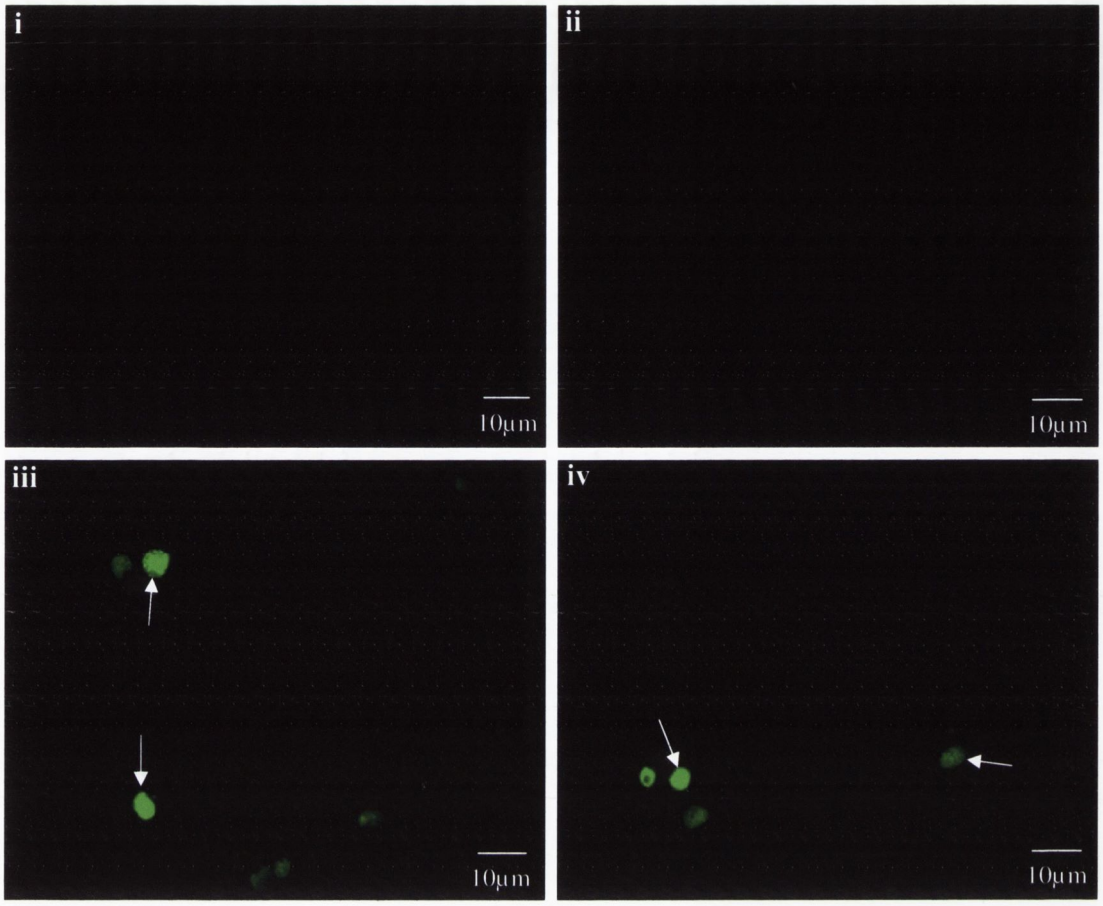


Figure 3.2: Isolated cells have proliferative capacity *in vitro*

Following 24hr in culture (i and ii), the cells were not in a proliferative phase in culture as evidenced by lack of BrdU incorporation. By 10 days in culture (iii and iv) cells were observed to be in a state of cell division, as evidenced by BrdU immunoreactivity. The images (i-iv) are representative of 5 independent culture preparations.

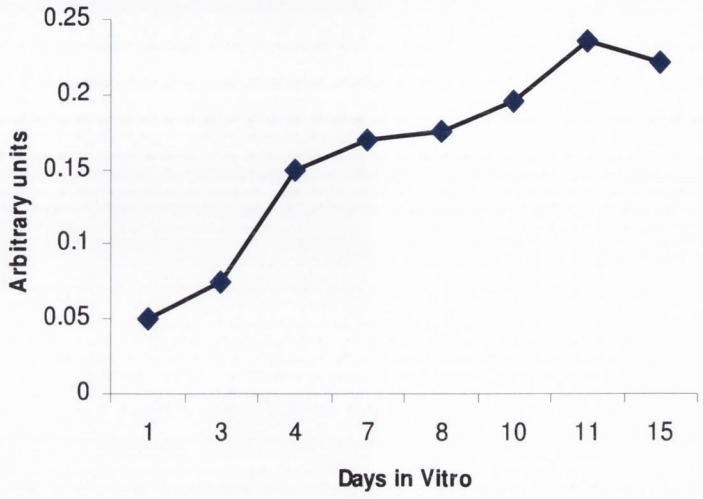


Figure 3.3: Cell proliferation begins after approximately 3 days and continues for up to 15 days

When assayed using the Aqueous One colourimetric assay for cell proliferation, MSCs were shown to begin dividing at 3 days in culture. Cell proliferation was maintained for at least 15 days in culture. Results are expressed as mean for 4 independent observations.

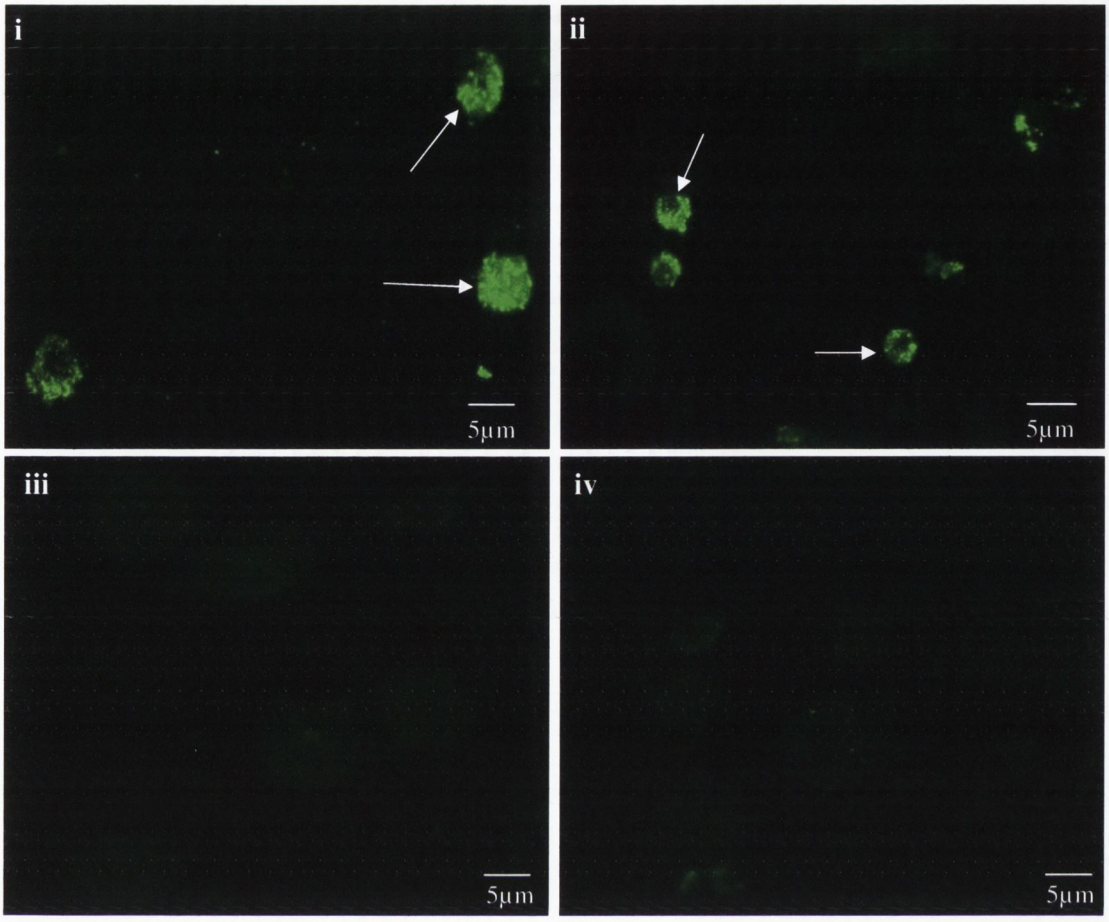


Figure 3.4: Cells cultured in the presence of osteoinductive factors for a period of 2 weeks stop proliferating

After culturing for 5 weeks in control supplemented DMEM (i and ii) cells continue to divide as assessed by BrdU incorporation. However, when cells were grown for 3 weeks in control supplemented DMEM and then exposed to osteoinductive factors (0.68nM Dex and 60ng/ml BMP; iii and iv) for a further 2 weeks, proliferation ceased as evidenced by the failure of cells to incorporate BrdU. Images are representative of 4 independent culture preparations.

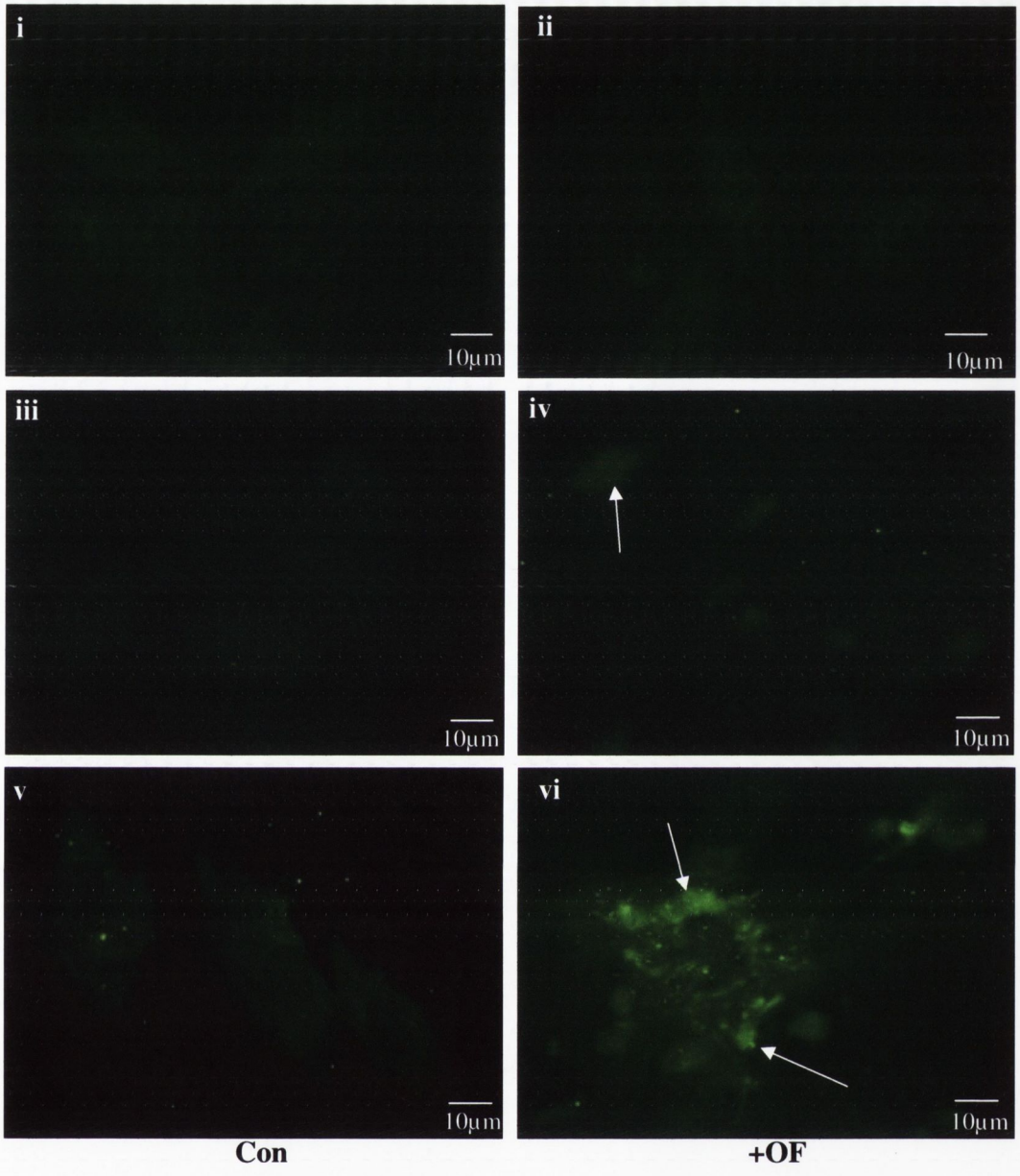


Figure 3.5: Collagen type I expression at day 0, 3 and 7 culture with osteoinductive factors

MSCs were cultured for 0 (i and ii), 3 (iii and iv) and 7 (v and vi) days in the absence (i, iii and v) or presence (ii, iv and vi) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM; n=5). At day 0 no collagen I immunoreactivity was observed (i, ii). Collagen type I expression was weakly evident by day 3 in the presence of osteoinductive factors (iv) and was expressed more strongly by day 7 (vi), as evidenced by immunofluorescent staining for collagen type I.

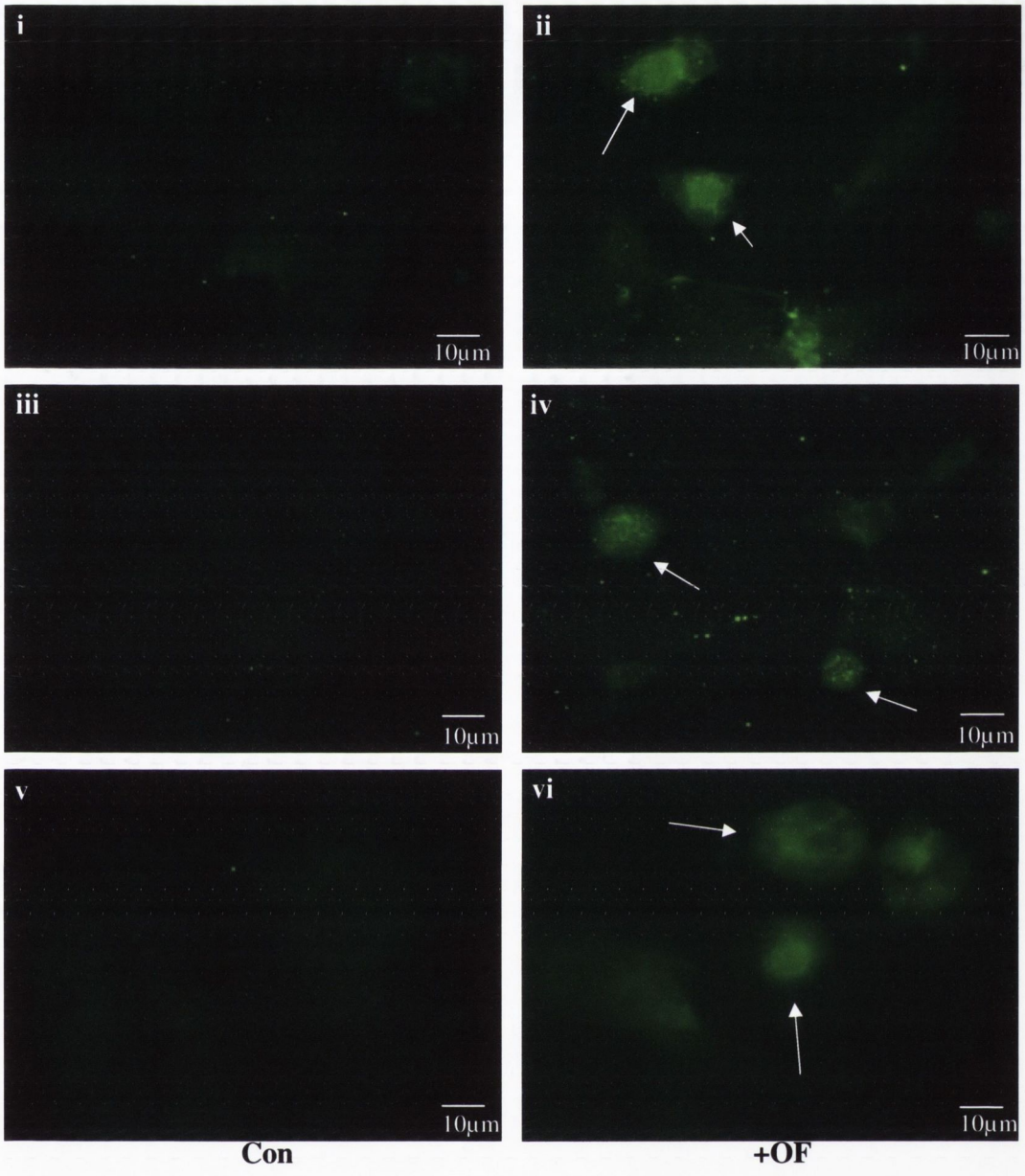


Figure 3.6: Collagen type I expression at day 10, 14 and 21 culture with osteoinductive factors

MSCs were cultured for 10 (i and ii), 14 (iii and iv) and 21 (v and vi) days in the absence (i, iii and v) or presence (ii, iv and vi) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM, n=5). At each timepoint collagen type I expression was evident in osteoinductive factor-treated samples, as evidenced by immunofluorescent analysis.

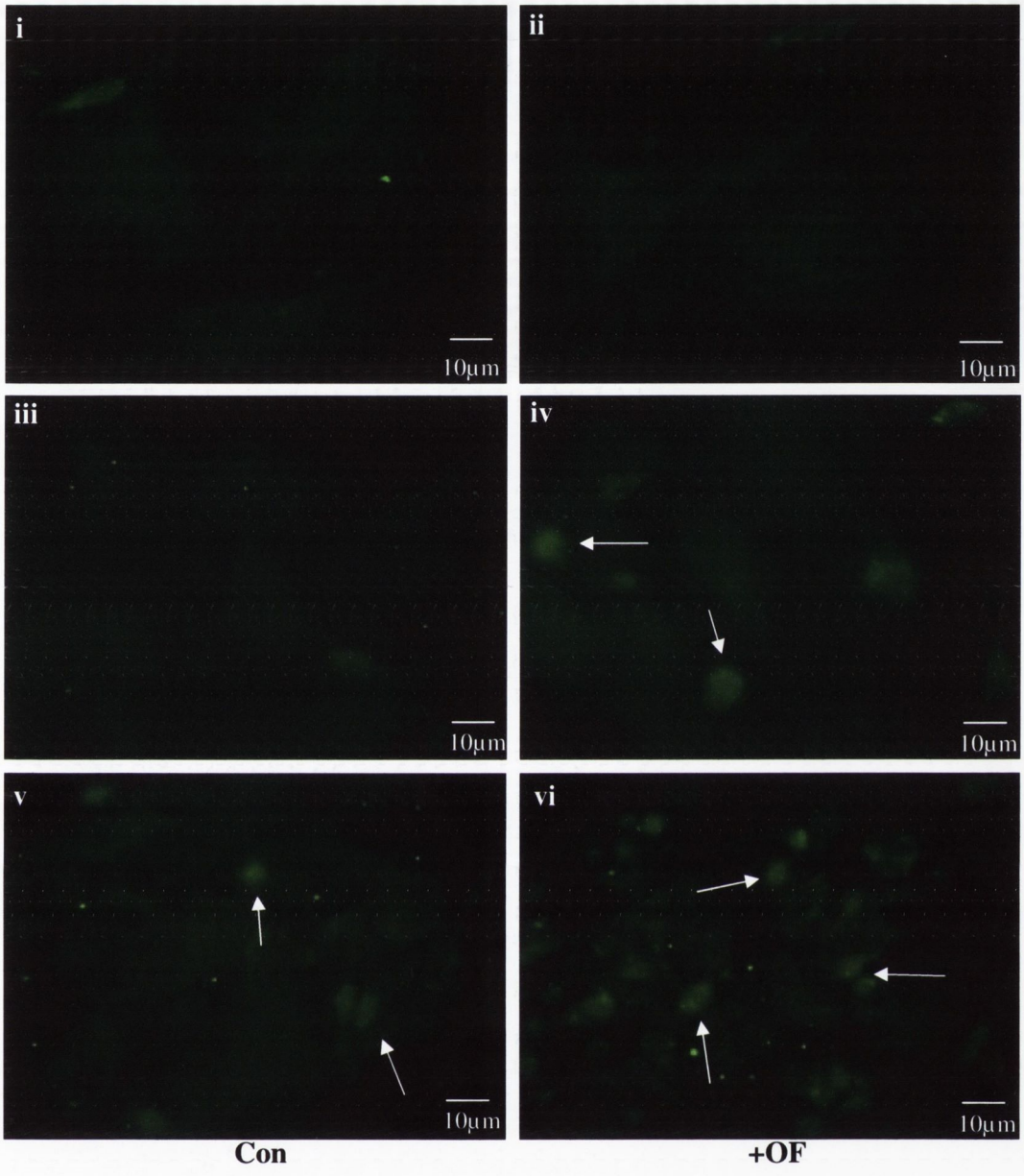


Figure 3.7: Osteocalcin expression at day 0, 3 and 7 culture with osteoinductive factors

MSCs were cultured for 0 (i and ii), 3 (iii and iv) and 7 (v and vi) days in the absence (i, iii and v) or presence (ii, iv and vi) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM, n=5). At day 0 no osteocalcin expression was observed in MSC samples cultured on coverslips (i, ii). Osteocalcin expression was weakly evident by day 3 (iv) and again at day 7 (vi) in the presence of osteoinductive factors, as evidenced by immunofluorescent analysis. Some control cells also stained positively for osteocalcin at day 7 (v).

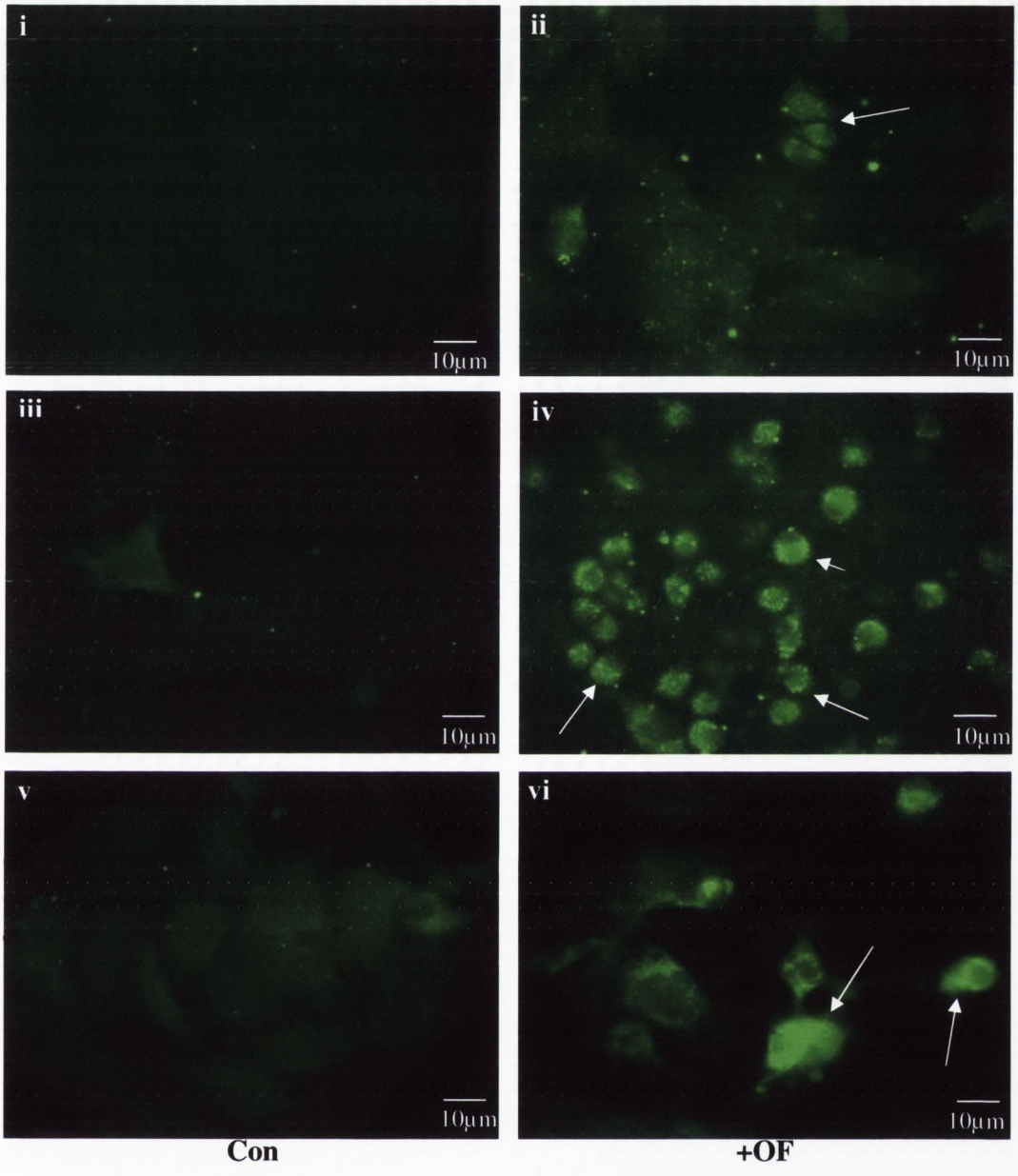


Figure 3.8: Osteocalcin expression at day 10, 14 and 21 culture with osteoinductive factors

MSCs were cultured for 10 (i and ii), 14 (iii and iv) and 21 (v and vi) days in the absence (i, iii and v) or presence (ii, iv and vi) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM, n=5). At each timepoint osteocalcin expression was evident in osteoinductive factor treated samples, as evidenced by immunofluorescent analysis.

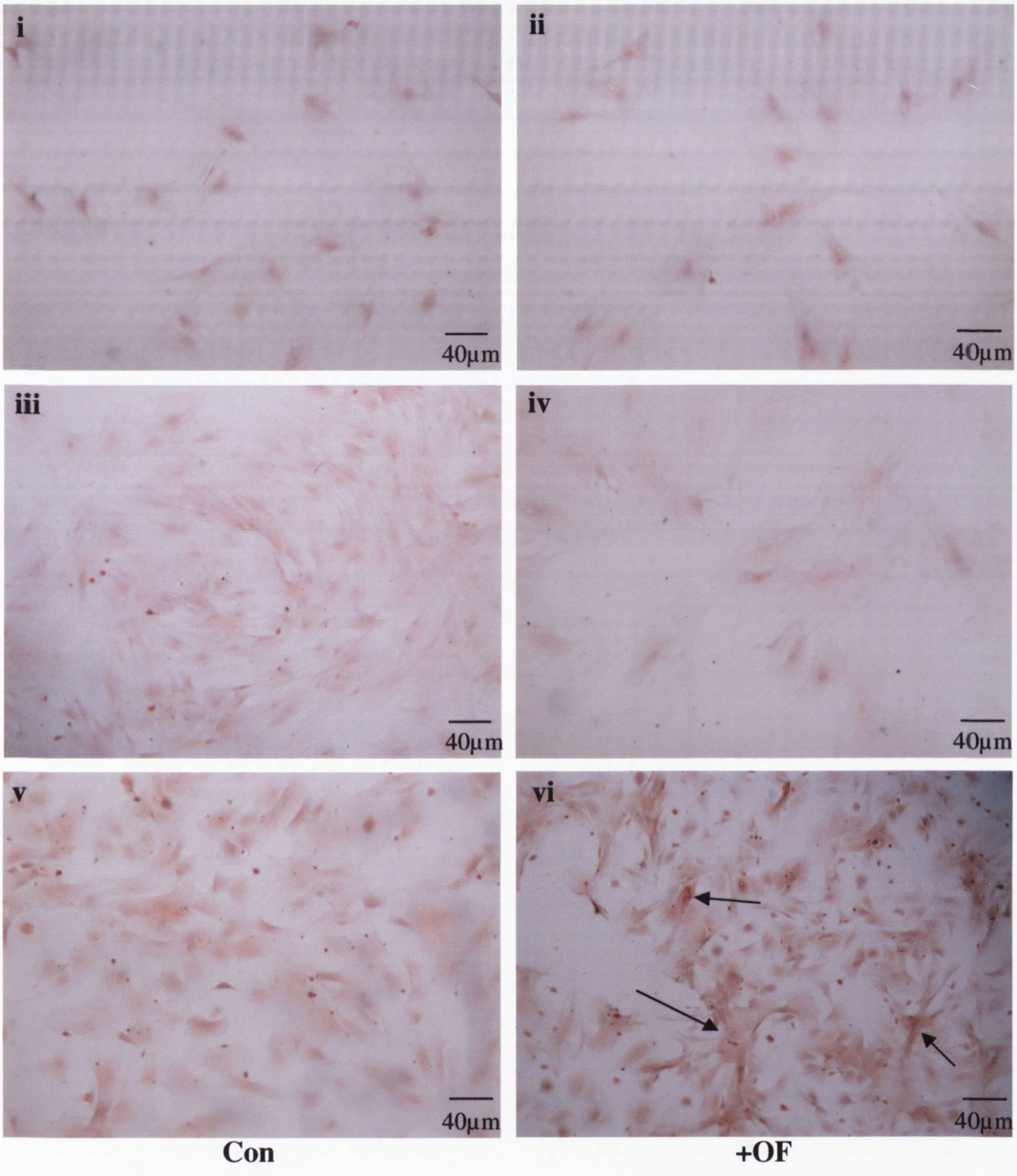


Figure 3.9: Mineralisation at day 0, 3 and 7 culture with osteoinductive factors

MSCs were cultured for 0 (i and ii), 3 (iii and iv) and 7 (v and vi) days in the absence (i, iii and v) or presence (ii, iv and vi) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM, n=5). At no timepoint was mineralisation observed in MSC samples cultured on coverslips. By day 7, cell aggregation was occurring in osteoinductive factor treated samples (vi), an indication of imminent nodule formation and mineralisation.

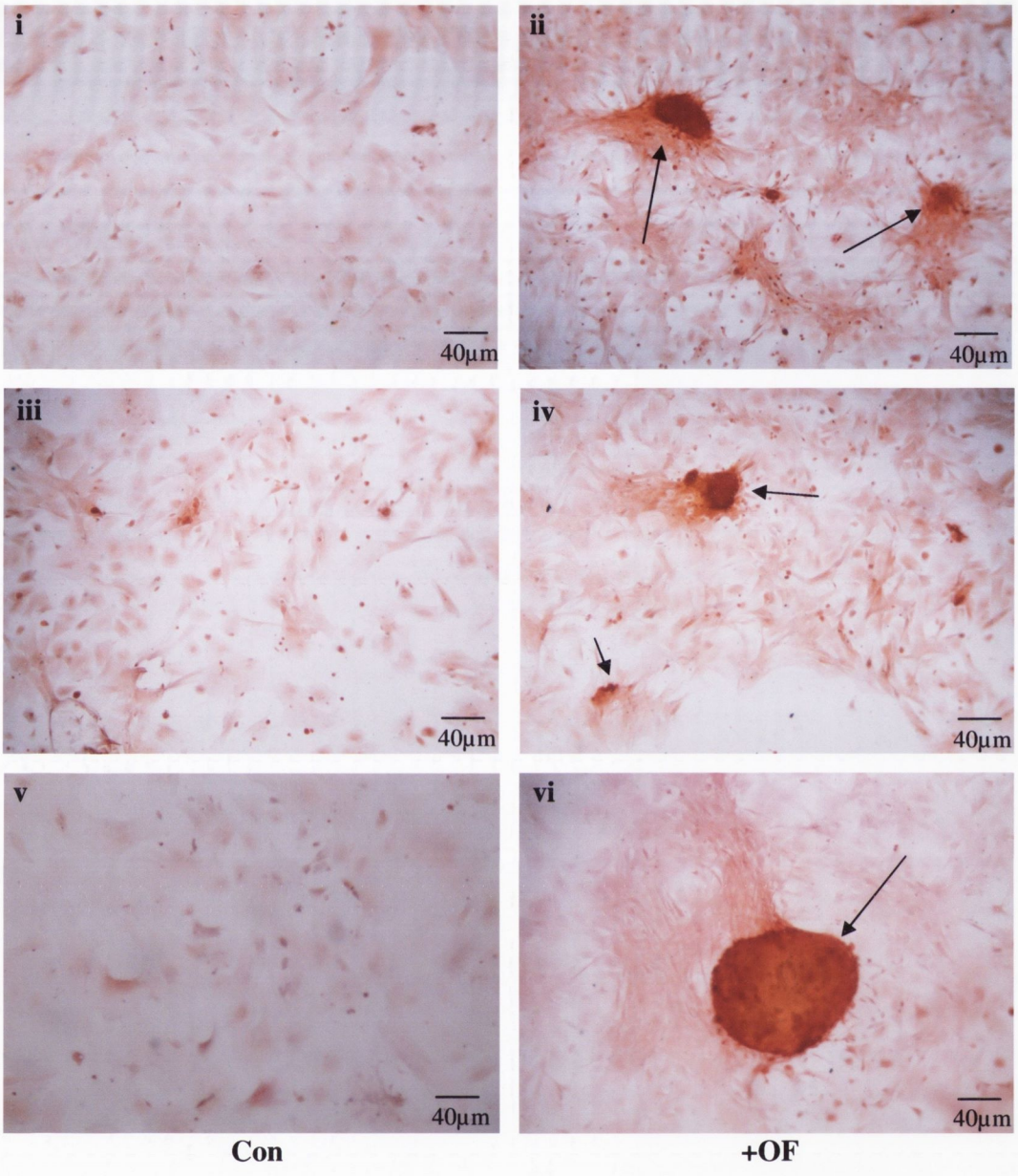


Figure 3.10: Mineralisation at day 10, 14 and 21 culture with osteoinductive factors

MSCs were cultured for 10 (i and ii), 14 (iii and iv) and 21 (v and vi) days in the absence (i, iii and v) or presence (ii, iv and vi) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) (n=5). Mineralisation was observed in osteoinductive factor-treated samples from day 10 onwards (ii, iv, vi). Nodule size was observed to increase with time in culture.

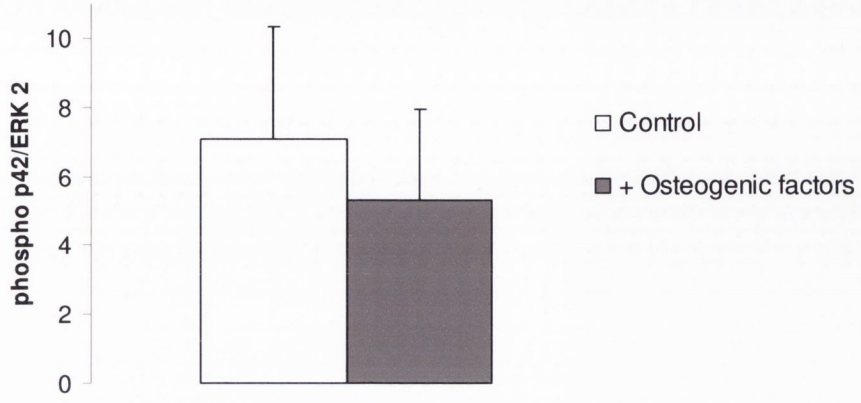


Figure 3.11: Phospho p42 expression is not altered significantly following osteoinductive treatment for 2 weeks

Following 2 weeks treatment of MSCs with osteoinductive factors (BMP 60ng/ml and Dexamethasone 0.68nM) phospho-p42 expression was not different from control values when expressed as a ratio against total p42 expression. Results are expressed as mean ± SEM for 10 independent observations.

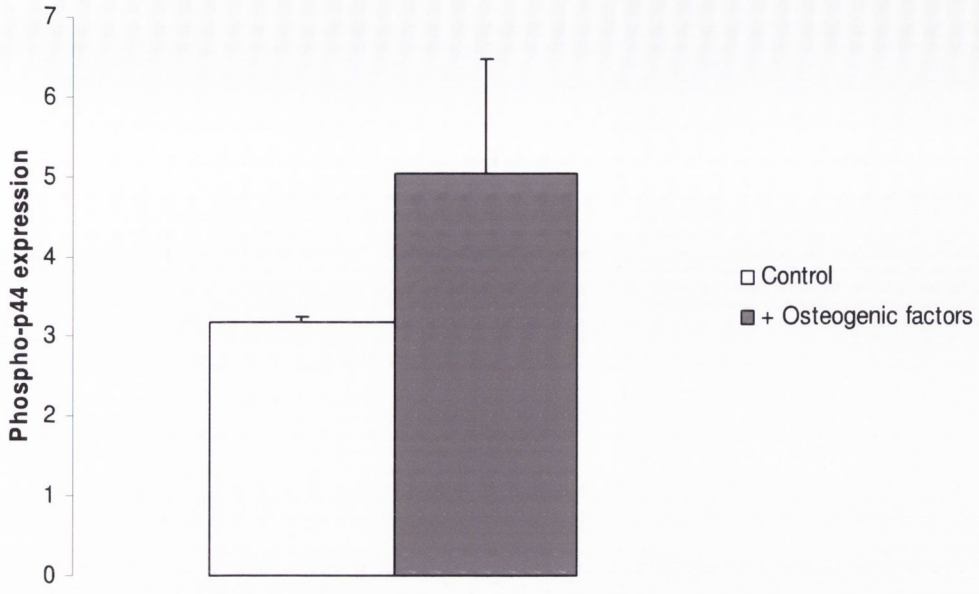


Figure 3.12: Phospho p44 expression is not altered significantly following osteoinductive treatment for 2 weeks

Following two weeks treatment of MSCs with osteoinductive factors (BMP 60ng/ml and Dexamethasone 0.68nM) phospho p44 expression was not significantly different from control values. Results are expressed as mean ± SEM for 10 independent observations.

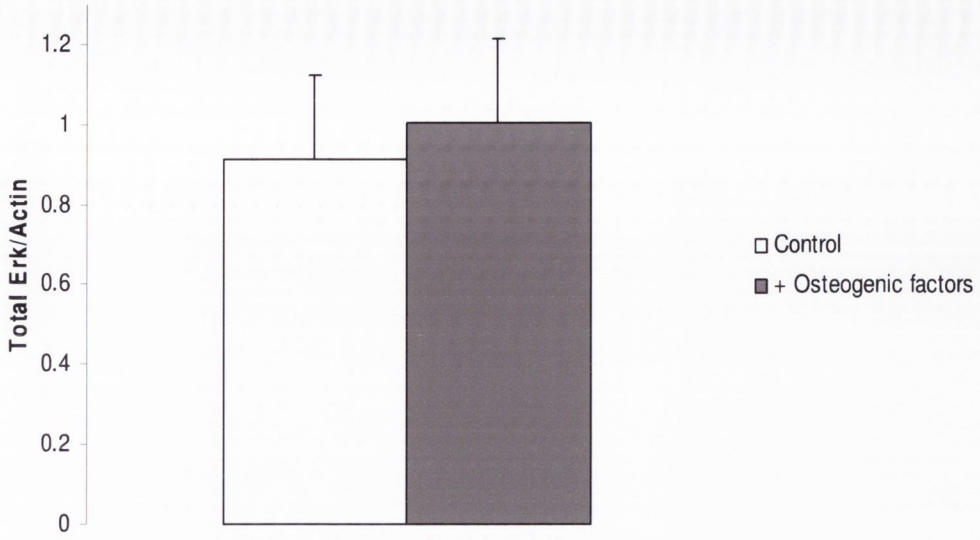


Figure 3.13: Total ERK expression is not altered significantly following osteoinductive treatment for 2 weeks

Following two weeks treatment of MSCs with osteoinductive factors (BMP 60ng/ml and Dexamethasone 0.68nM) Total p42 expression was not significantly different from control values when expressed as a ratio against total Actin. Results are expressed as mean ± s.e.m. for 10 independent observations.

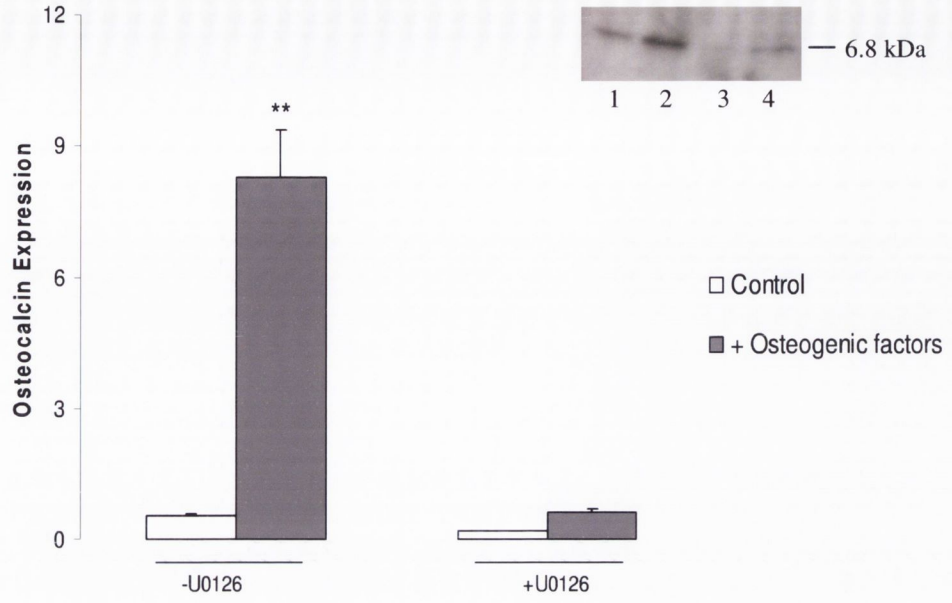
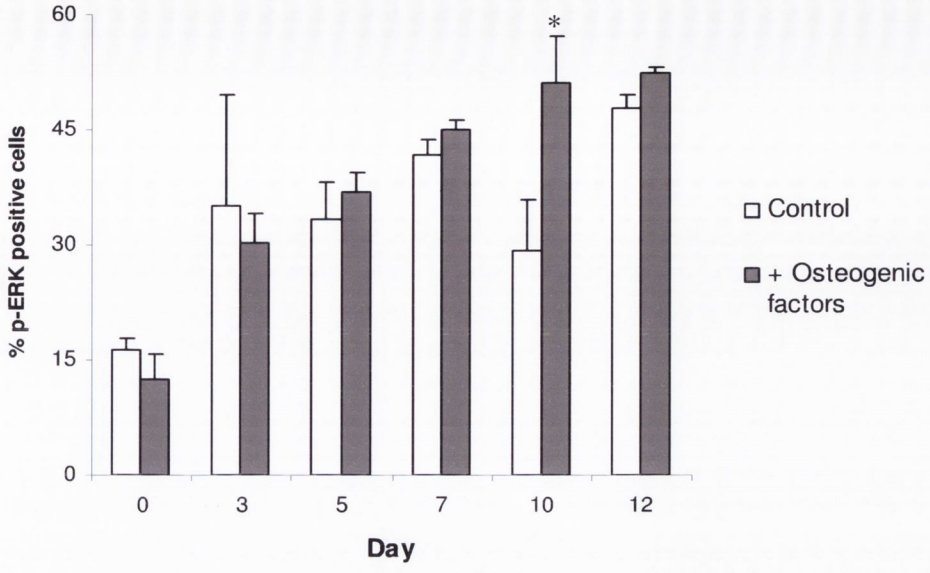


Figure 3.14: Induction of osteocalcin expression in MSCs is ERK dependant

Cellular expression of osteocalcin was significantly increased following treatment with osteoinductive factors (BMP 60ng/ml and Dexamethasone 0.68nM), for 2 weeks. This effect was abrogated when cells were treated with the ERK inhibitor, U0126 (2μM). Results are expressed as mean ± SEM for 4 independent observations. ** $P<0.01$. Inset: Representative blot shows osteocalcin expression in control cells (lane 1) cells exposed to osteoinductive factors (lane 2), cells exposed to U0126 alone (lane 3) and cells exposed to osteoinductive factors in the presence of U0126 (lane 4).

A



B

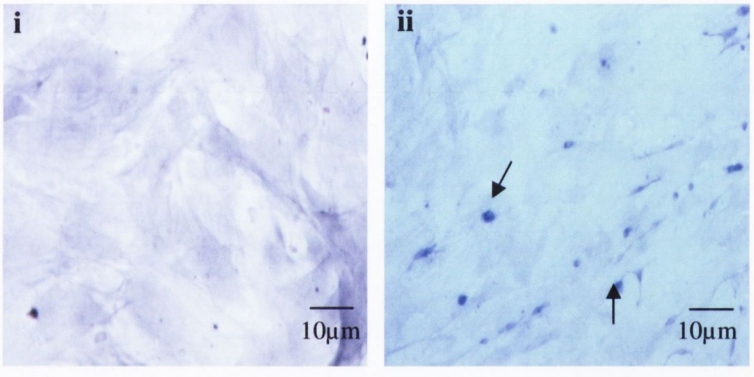


Figure 3.15: ERK activity is significantly increased following osteogenic treatment for 10 days

Cells were exposed to osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 0-12 days. Cells were fixed and assessed for ERK activity using a phospho-specific ERK antibody and immunocytochemistry. Following 10 days treatment, phosphorylated ERK expression was significantly higher than control levels. At no other timepoint was ERK activity significantly different from control. Representative micrographs illustrate cells positive for ERK activity in osteoinductive treated samples (ii). Results are expressed as mean ± SEM for 4-6 independent observations.* $P<0.05$.

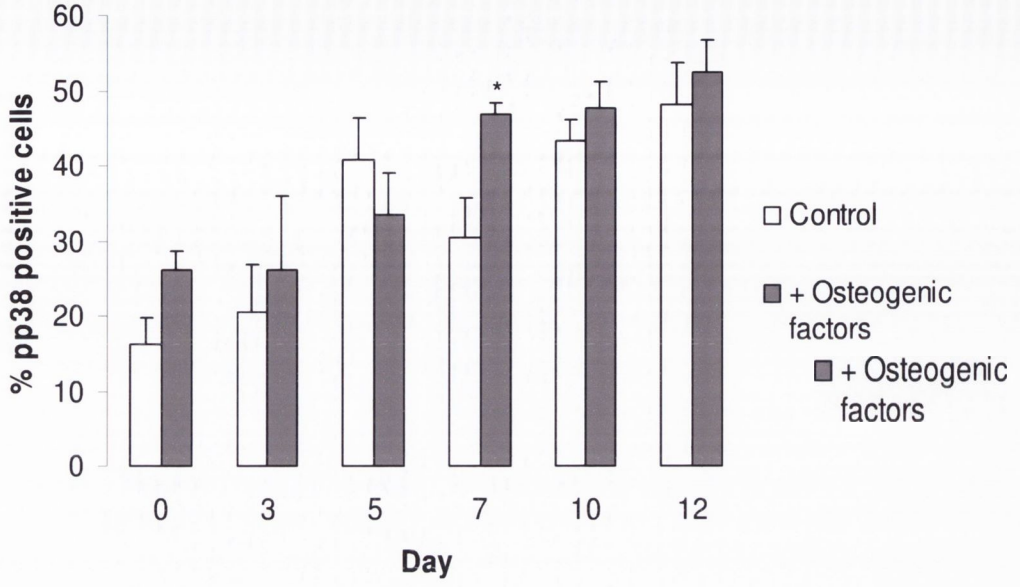


Figure 3.16: p38 activity is significantly increased following osteogenic treatment for 7 days

Cells were exposed to osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 0-12 days. Cells were fixed and assessed for p38 activity using a phospho-specific p38 antibody and immunocytochemistry. Following 7 days treatment, phosphorylated p38 expression was significantly higher than control levels. At no other timepoint was p38 activity significantly different from control. Representative micrographs illustrate cells positive for p38 activity in osteoinductive treated samples (ii). Results are expressed as mean ± SEM for 5-7 independent observations.* $P<0.05$.

3.3 Discussion

The purpose of this study was to validate the purity and viability of MSC cultures, to assess their ability to differentiate *in vitro* and to elucidate some of the signalling processes involved in the osteogenic differentiation process. The long term aims of inducing bone and cartilage formation in 3-D biocompatible scaffolds can not be carried out without these preliminary studies within a 2-dimensional environment. Endoglin immunofluorescent staining of cultures verified the presence of MSC. Endoglin is found on mesenchymal stromal cells in several tissues. It binds TGF-β1 and TGF-β3 through its association with TGF-β receptor II and modulates several responses to TGF- β1. It also binds activin, BMP-7, and BMP-2 by interacting with their respective ligand-binding receptors, suggesting that it is in the receptor complex for several growth factors of the TGF-β superfamily (Bourdeau et al., 1999). As stated in the introduction to this results chapter, the monoclonal antibody SH-2, developed as a marker against MSCs (Haynesworth et al., 1992), was discovered to recognise an epitope on Endoglin (Barry et al., 1999). As such, using Endoglin as a MSC marker in this instance, rules out the possibility of other cell types, such as haemopoeitic stem cells (HSCs), also being labelled, since Haynesworth et al demonstrated the specificity of SH-2 for MCSs.

As research in this field progresses, new approaches for the isolation and verification of the presence of a pure population of MSCs are being developed. There is now a comprehensive compliment of markers and antibodies used to define the MSC phenotype, including the Stro-1 antibody (Simmons and Torok-Storb 1991). Newer techniques are also being employed to utilise these markers. For example the use of fluorescence-activated cell sorting (Herzog *et al.,* 2003), isolation of single clonal colonies (Muraglia *et al.,* 2000) and the use of density centrifugation have further purified MSC preparations. However, to date, there is no accepted compliment of markers or single technique employable that will generate a 100% pure MSC population. This is a very important goal for the near future if we are to conclusively verify the potential clinical uses of adult MSCs and allay the fears and scepticism regarding the plasticity of this cell type (Verfaillie 2002). This is also the case in the present study, where there is some inhomogeneity in cell cultures as a result of the isolation and culture procedures. The culture procedures involved steps for the

purification of the cellular preparation to remove unwanted cells. Adherent cells such as macrophages were removed after 30 min in culture by retrieval of the cell suspension and the non-adherent cells were removed after 24 hours by two media changes. HSCs, macrophages and various blood cells are removed at these different stages, however, there are still a limited number of other stromal cells present in the culture. This is another reason for the importance of increasing MSC numbers, as doing so decreases the relative numbers of unwanted cells compared to MSCs. This limitation in all adult MSC research must be considered when results are being examined and compared.

Proliferation analyses showed that adult rat MSCs begin to proliferate after 3 days and will divide for up to 5 weeks in culture under normal conditions. Bruder et al (1997b) previously demonstrated the expansion capacity of MSC and Reyes et al (2001) demonstrated that MSCs could undergo at least 50 population doublings without obvious signs of differentiation or senescence. The ability to culture MSCs for such an extended period of time without infection and with continued proliferation is extremely useful and important for generating sufficient cell quantities for future studies that will require large numbers of cells to be seeded onto novel scaffolds and bioreactors. This high proliferation capacity that MSCs possess is one of the major advantages they have over several other cell types (Ringe *et al.,* 2002). It has been observed however, that these cells do have a limited proliferative capacity, which decreases with increasing passage number (Bruder *et al.,* 1997). Banfi *et al.* (2000) also observed a decrease in proliferation velocity after successive passages. Despite this, it should be noted that MSCs were shown to have expanded over one billion fold without the loss of their osteogenic potential (Bruder *et al.,* 1997). In this study, BrdU incorporation after five weeks in culture also indicated that cells had not progressed along any differentiation pathways, as commitment to a certain lineage leads to cessation of mitosis. When exposed to the osteoinductive factors Dex and BMP for 14 days however, proliferation ceased further demonstrating this point. This finding was in agreement with previous studies, such as that by Banfi et al (2000) and Reyes et al (2001). This indicated that the MSCs treated with osteoinductive factors were in a post-proliferative state, an indicator of the start of the differentiation phase (Banfi *et al.,* 2000). Interestingly, studies have also shown that the addition of osteoinductive supplements, particularly dexamethasone, actually increase the proliferation rate of

MSCs (Jaiswal *et al.,* 1997). It is possible that there is an initial surge in cell proliferation before lineage commitment occurs and proliferation ceases. The use of osteoinductive factors is essential for the complete differentiation of these cells. Dexamethasone, a synthetic glucocorticoid, has been found to be crucial for the development of bone-like nodules (Maniatopoulos *et al.,* 1988). Ascorbic acid has been shown to facilitate osteogenesis *in vitro* (Bellows *et al.,* 1986). This is due to its involvement in the conversion of pro-collagen to collagen, a necessary early step in the osteogenic process. β-glycerophosphate acts as a source of phosphate ions for the mineralisation of osteoid (Anderson *et al.,* 1984). BMP is also a potent inducer of osteogenesis being a member of the TGFβ superfamily.

As part of the characterisation of adult MSCs, the temporal expression of collagen type I and osteocalcin in monolayer cultures was examined. By day 3 collagen I expression was weakly evident. By day 7, collagen I was visible both within cells and also as an extracellular matrix in some instances. In the control scaffolds, some collagen I expression was also evident suggesting spontaneous differentiation was taking place. However this was the only instance where this was observed and no osteocalcin or mineralisation was observed at any subsequent timepoints in control scaffolds. The sustained expression of collagen I from day 3 onwards was expected to occur, as it is expressed from the osteoprogenitor stage of the differentiation process onwards (Aubin *et al.,* 1995). While collagen I is also expressed by fibroblasts, it is unlikely that the positive cells labelled in 2-D were fibroblasts. This is because the morphology of the cells was too rounded. Also, the controls were negative for collagen type I. If fibroblastic contamination had occurred, positive immunofluorescence would also have been observed in control samples. This demonstrates the relative purity of the culture preparations despite the limitations previously discussed.

As with similar treatments elsewhere, osteocalcin expression was shown to rise significantly following 2 weeks treatment with osteoinductive factors (Aubin *et al.,* 1995). Several early markers of osteogenesis exist, including osteopontin, osteonectin, bone sialoprotein and alkaline phosphatase, however, as osteocalcin is expressed at a post-proliferative osteoblastic stage, expression of this marker more conclusively proved that osteogenesis had occurred. This was demonstrated both in western

immunoblots and using immunofluorescent analysis. The potent expression of osteocalcin from day 10 onwards mirrors temporal expression of osteocalcin found in other laboratories (Tsuchida *et al.,* 2003, Herbertson and Aubin, 1997, Cassiede *et al.,* 1996).

Alizarin Red S staining for calcium enabled not only the analysis of mineralization, but also morphological study of the osteogenic process. In accordance with Bianco *et al* (2001), 2-D cultures treated with osteoinductive factors were observed to form colonies with the subsequent formation of nodules as evidenced from day 7 onwards. Satomura *et al* (1991) also observed similar morphological characteristics, with MSCs appearing as large flattened cells with many processes and osteoblast like cells being smaller and more cuboidal in shape. Maniatopoulos *et al* (1988) additionally observed clusters of cells by day 8. At later timepoints, there were some observed changes in cell morphology in control samples, probably due to overcrowding as a result of the lack of cell passaging. This might explain the weak staining for collagen I and osteocalcin that occurred in some control samples. However, this was the exception and in no circumstances did nodule formation or subsequent mineralisation take place. Again these findings are in accordance with previous observations by Ohgushi *et al* (1996) and Ter Brugge and Jansen (2002) who reported low alkaline phosphatase expression and no calcification in control samples. In 2-D, mineralisation was observed by day 10 in some samples and by day 14 in all.

Neither phosphorylated, nor total ERK levels were altered in those cells cultured in osteoinductive medium for a period of 14 days (Figures 3.6-3.8). A recent study by Jaiswal *et al.* (2000) showed that ERK levels are elevated by 7 days *in vitro* when cells are cultured in the presence of osteoinductive factors. It is possible that by day 14, phosphorylated ERK expression had returned to basal levels and the timepoint for activation had been missed. The studies by Jaiswal *et al.* (2000) and Simmons *et al.* (2003) were both carried out on MSCs of human origin and it could be hypothesised that osteogenesis and activation of Cbfα-1 occur via an alternate pathway in the rodent model. However, ERK has also been implicated in several other differentiation pathways both negatively in chondrogenesis (Chang *et al.,* 1998) and positively in myogenesis (Gredinger *et al.,* 1998). As such it was reasonable to assume that ERK might also play some sort of role in osteogenic differentiation.

To further analyse the effects of ERK activation on osteogenesis, ERK activity was suppressed using U0126 an inhibitor of the upstream activators of ERK, MEK 1 and MEK 2. When cells were cultured under osteoinductive conditions, osteocalcin levels were observed to be significantly higher than control and this effect was completely reversed when cells were co-cultured with U0126. This inhibitor was shown to have specific activity on only these two MAPKKs and had no effect on the upstream activators of JNK or p38. This shows that ERK is indeed responsible for MSC differentiation along the osteogenic route in the rodent model. However, given that ERK activation was not induced by osteoinductive factors at day 14, I sought to clarify a timecourse for ERK activation in this system.

To this end, a 6-point timecourse analysis of ERK activity was carried out, whereby cells were incubated with osteoinductive factors for 0, 3, 5, 7, 10 and 12 days. In this analysis ERK activity was shown to be elevated in cells exposed to osteoinductive factors for 10 days compared to controls, but not at earlier or later timepoints. This is in accordance with the findings of Jaiswal et al (2000) who showed ERK activation at the earlier time of day 7 in adult human MSCs, but showed sustained activation until day 11. It is important to understand the signalling mechanisms of osteogenesis in order to improve the quality and quantity of tissue engineered bone being produced, and elucidating this pathway is an important step. The role of ERK in osteogenesis is not simply confined to its induction however. The ERK phosphorylation of peroxisome proliferator-activated receptor γ (PPAR γ), has been shown to reduce its transcriptional activity (Adams *et al.,* 1997, Hu *et al.,* 1996). PPARγ is known to be critical in adipogenic differentiation. By preventing this process, possibly by the use of ERK agonists, a possible treatment mechanism for osteoporosis, a disease one of whose characteristics is the progressive replacement of marrow stroma by fat (Meunier *et al.,* 1971), might be developed. Also, normal ERK activity is most likely very important in maintaining healthy bones.

The activity of the more recently discovered MAPK p38 is usually related to cellular apoptosis (Kummer *et al.,* 1997; Xia *et al.,* 1995). In this study however, p38 activity was shown to be significantly increased after 7 days treatment with osteoinductive factors. p38 has also been shown to be upregulated in situations not related to cellular

stress. For example Hannigan *et al* (1998) demonstrated the activation of p38 in human neutrophils by TGF-β1 indicating a possible role for p38 in chondrogenesis. Jaiswal *et al* (2000) also observed p38 activation in human MSCs following treatment with osteoinductive factors. They observed a similar timecourse of activation as was detected for ERK, with p38 being activated from day 9 until day 13. Interestingly, in that study no effect on osteogenesis was observed when p38 activity was inhibited by the selective inhibitor SB203580. Similarly, their study found that alkaline phosphatase levels and calcium accumulation were not reduced as a result of p38 inhibition. This suggests some as yet undefined role of p38 in the osteogenic process.

These results serve to demonstrate the viability of MSC cultures and the important ability to culture these cells for extended periods of time, without infection, while maintaining their proliferative capacity. Also the osteogenic capacity of these cells is obvious, with complete differentiation and matrix mineralisation taking place within 10 days. ERK was later shown to be involved in the differentiation process of MSCs along the osteogenic route in the rodent model and a timecourse of its activity was demonstrated. The stress activated protein kinase p38 was also shown to be activated during the differentiation process. The variability in research techniques in the field of adult MSC research makes the interpretation and correlation of different results precarious. For example, the preparation procedure is carried out in several different ways, as discussed in the introduction. In addition to the techniques described, researchers will also purify their cultures using immunolabelled or magnetic beads or other cell sorters or carry out several density centrifugations, while others never pellet the cells. This can lead to the study and comparison of slightly different cell phenotypes all termed MSCs. The choice of markers of osteogenesis are broad and allow for even greater variation within the research field. Osteogenesis is often said to have occurred because of the expression of early markers (osteopontin, osteonectin), when dedifferentiation of the cells could still take place. In this study, the use of Endoglin as a MSC marker and osteocalcin as a marker of osteogenesis was deemed to be most suitable to definitively demonstrate the presence of MSCs and their ability to differentiate along the osteogenic lineage. This demonstrates the ability of adult rodent MSCs to differentiate along the osteogenic lineage in an ERK dependent manner with a concurrent rise in the activity of p38, a phenomenon previously only shown in adult MSCs of human origin. The next stage of this research is to develop a

3-D system for the osteogenic differentiation of MSCs in the development of viable bone constructs.

Chapter 4

Evaluation of the osteogenic differentiation potential of adult mesenchymal stem cells in 3-D

4.1 Introduction

A vital consideration in the development of engineered tissues is the 3-D environment in which the cells grow. This environment must not only give shape to the final tissue, but also promote cell adhesion and development, be biocompatible and if necessary give structural support. According to Chapekar *et al.* (2000), it is the combination of progenitor cells, growth factors and a suitable microenvironment that form the basis of the tissue engineering of bone and cartilage. The choice of a suitable microenvironment is as important as the choice of cells and growth factors. It is only recently that the importance of generating viable microenvironments in which to engineer tissues has been recognised (Fedewa *et al.,* 1998, Temenoff *et al.,* 2000). In order for a scaffold to promote cell adhesion and growth, it must satisfy certain parameters; it must be biocompatible and degrade in the body at a rate that allows the scaffold to remain insoluble for the duration of the critical cellular processes and the products of degradation must not be toxic (O'Brien *et al.,* 2004). Other factors are also very important. For example the chemical composition of the material in question must allow and/or promote cell adhesion. Pore size is also of critical importance, with pores large enough to allow cell migration and diffusion but small enough to have enough surface area to allow for cell adhesion (O'Brien *et al.,* 2004). The uniformity/homogeneity of the structure of the scaffold is important for similar reasons (metabolite diffusion, biomechanical properties, cellular communication). While osteogenesis and chondrogenesis have been shown to occur in several studies in 2-D (Jaiswal *et al.,* 1997, Herbertson and Aubin 1997, Pri Chen *et al.,* 1998, Johnstone *et al.,* 1998, Pittenger *et al.,*1999, Stevens and Williams 1999, Gerber and Gwynn, 2002), the importance of transferring this ability into 3-D is evident. In order to successfully treat injuries or diseases of the musculoskeletal system, it will be necessary to reproducibly generate tissues in 3-D. It has been suggested that the presence of a 3-D scaffold can enhance cellular growth and differentiation by increasing cellular interactions as a result of the close proximity to one another that the scaffold allows (Jaiswal *et al.,* 1997, Weber *et al.,* 2002). While there are several scaffold designs under investigation, the enhancement of both growth and differentiation has yet to be demonstrated. For example Weber *et al.* (2002) showed that while 3-D alginate scaffolds did improve cellular differentiation, as evidenced by the production of cartilage specific matrix proteins, proliferation was attenuated. As a

result, this scaffold would not be useful as a medium in which to increase MSC numbers prior to implantation, though it might prove very successful in promoting chondrogenesis once sufficient cell numbers had been acquired by other means. Much work has yet to be carried out to determine the optimal chemical composition of a scaffold as well as its structural design. Other scaffolds are also at varying stages of development and their suitability is yet to be determined. The sintered dicalcium pyrophosphate scaffold developed for the induction of osteogenesis has only very recently been shown to be truly biocompatible (Sun *et al.,* 2004). Other scaffolds currently in use for research in this area include the lactate derived PLGA and PLLA (Yang *et al.,* 2001,2002), alginate gels that are solidified after cellular incorporation (Wang *et al.,* 2003), PLCs (Honda *et al.,* 2003) and various collagen derivatives (Honda *et al.,* 2003).

In 1981 Burke and Yannas reported the development of a collagen GAG scaffold as an artificial skin to be used in the treatment of skin burns (Burke *et al.,* 1981). This scaffold was shown to be very effective in aiding in the healing of such injuries and the reduction of scar tissue. Until recently the importance of the glycosaminoglycan component of these scaffolds went unrecognised. However, research by Pieper *et al.* (2000) and Daamen *et al.* (2003) has demonstrated the important role that glycosaminoglycans can play in maintaining matrix integrity and how alterations in the ratios of GAGs can alter the structural properties of a scaffold. To date the collagen GAG scaffold developed by Burke and Yannas has been used not only in skin regeneration but also in regeneration of the conjunctiva and peripheral nerves Chamberlain *et al.,* 1998). Despite this, as yet no-one has attempted cartilage or bone regeneration using MSCs and a collagen GAG matrix as a 3-D environment. Since it has been shown that type I collagen is necessary for osteogenesis and actually stimulates bone formation (Xiao *et al.,* 1998), and given that collagen and glycosaminoglycans are the two main components of cartilage, the collagen GAG scaffold may serve as an ideal substrate in which to induce osteogenesis and chondrogenesis from MSCs. The following experiments describe the seeding of collagen GAG scaffolds with adult rat MSCs and the treatment of these cells *in situ* with inductive factors to promote osteogenesis in 3-D.

This chapter is also concerned with the temporal appearances of two bone related proteins (collagen type I and osteocalcin) and matrix mineralisation in 3-D, following exposure of cells to an osteoinductive cocktail of ascorbic acid, β-glycerophosphate and dexamethasone, a now standard combination for the induction of osteogenesis (Coelho and Fernandes, 2000). The proteins collagen I and osteocalcin were chosen as early and late stage markers of osteogenesis (Aubin *et al.,* 1995) to give as much scope for the study as possible. Matrix mineralisation was used as a marker of the end-point of osteogenesis.

As described previously, p38 is the third member of the mitogen activated protein kinase family, which also includes ERK and JNK. Like JNK, p38 is a stress activated protein kinase and is activated by cytokines, environmental stress and radiation (Cano *et al.,* 1995). Kummer *et al.* (1997) have shown that apoptosis, induced by the withdrawal of trophic factors is mediated by p38 and Xia *et al.* (1995) have demonstrated an inhibition of ERK activity and activation of JNK and p38, after withdrawl of nerve growth factor from rat PC-12 cells. Despite these findings, p38 has also been shown to be upregulated during osteoblastic differentiation of human MSCs in 2-D (Jaiswal *et al.,* 2000), its expression occurring between days 9 and 13, slightly later in the process than ERK. Guicheux *et al.* (2003) have also shown that BMP-2 causes activation of p38 in the MC3T3 mouse osteoblast cell line. In Chapter 3, p38 activity was shown to be upregulated after 7 days culture with osteoinductive factors in 2-D, and I demonstrated ERK activity to be tightly linked to osteogenesis. It is important however, to determine which signalling mechanisms are at work in a chemically and structurally different environment. It is hoped that through a better understanding of the mechanisms behind the differentiation of MSCs, improved methods for the generation of viable tissue constructs can be developed with less emphasis on the use of high levels of growth factors.

In summary, the purpose of this chapter is threefold. Firstly, the development of mechanically and biologically viable, three dimensional bone constructs using collagen glycosaminoglycan (Collagen GAG) scaffolds. Secondly, this chapter aims to determine a timecourse of activity of these adult rat derived MSCs during the osteogenic process in 3-D and to compare these results with those in 2-D from

Chapter 3. Finally, the role of ERK and p38 in osteogenesis will be further elucidated by examining their role in the 3-D osteogenic process.

4.2.1: In MSC-seeded scaffolds no cellular migration was observed after 7 days culture in collagen GAG scaffolds

Before attempting to induce osteogenesis in collagen GAG scaffolds, it was necessary to assess cell viability within these scaffolds and to determine whether cells would colonise the entire scaffold within a reasonable timeframe. Figure 4.1 shows MSC-seeded scaffolds after 3 (i and ii) and 7 days (iii and iv) in culture. Scaffolds were cultured with normal culture medium (Control; i and iii) or with culture medium supplemented with osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM; ii and iv). Under no conditions did cells migrate from the outer layers of the scaffolds into the centre as evidenced by toluidine blue staining. Results are representative of 2 individual cell culture preparations at each timepoint.

4.2.2: In MSC-seeded scaffolds no cellular migration was observed after 14 days with scaffolds experiencing complete penetration after 21 days

Having observed cell survival at 7 days but no migration, cells were seeded into scaffolds and cultured for 14 and 21 days. Figure 4.2 (i and ii) shows that, following 14 days culture in the scaffold, MSCs were still entirely located to the periphery of the scaffold, as evidenced by toluidine blue staining. Scaffolds were cultured in the presence (ii and iv) or absence (i and iii) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 14 (i and ii) and 21 (iii and iv) days respectively. By day 21 in both control and osteoinductive factor treated samples, cells had migrated to the centre of the scaffold and were distributed homogenously throughout. Results are representative of 5 individual cell culture preparations at day 14 and 6 preparations at day 21. This apparently long delay in scaffold penetration by MSCs is well within reported timeframes for the migration of cells through biodegradable scaffolds (Freed *et al.*, 1998).

4.2.3: Collagen I expression is evident after 3 days culture in collagen GAG scaffolds

To fully characterise the responses of adult MSCs to the collagen GAG scaffold, a timecourse similar to that carried out in Chapter 3 was undertaken. Collagen type I expression was assessed at days 3, 7, 14 and 21 post scaffold seeding. Figure 4.3 shows that following 3 days culture in the absence (i) and presence (ii) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM), collagen type I expression was observed in osteoinductive treated samples. By day 7, extracellular expression was evident in osteoinductive factor-treated samples (iv). Some collagen I expression was also observed in untreated controls (iii). It is possible that the presence of collagen I in the scaffold itself caused a positive feedback for the production of collagen I without the need for osteoinductive factors. The presence of collagen I has previously been shown to aid in the induction of differentiation along the osteogenic lineage (Xiao *et al.,* 1998). Results are representative of 2 individual MSC preparations for each timepoint.

4.2.4: Collagen type I expression was sustained in collagen GAG scaffolds

Figure 4.4 demonstrates that, at days 14 (i and ii) and 21 (iii and iv), expression of collagen I was observed in osteoinductive factor (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) treated scaffolds (ii and iv). Results are representative of 5 individual MSC preparations at day 14, and 6 preparations at day 21.

4.2.5: Osteocalcin expression is absent after 3 and 7 days in collagen GAG scaffolds

Figure 4.5 illustrates MSC seeded collagen GAG scaffolds after 3 (i and ii) and 7 days culture in the presence (ii and iv) or absence (i and iii) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). Under no conditions was osteocalcin expression observed. Results are representative of 2 individual MSC preparations for each timepoint.

4.2.6: Osteocalcin expression is evident after 14 and 21 days in collagen GAG scaffolds

Figure 4.6 demonstrates osteocalcin expression following 14 (ii) and 21 (iv) days culture in the presence of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). At day 14 staining was weakly evident and became much more pronounced by day 21. No osteocalcin expression was observed in any control scaffolds (i and iii). Expression of this protein appeared to occur later in 3-D than in samples cultured in 2-D on coverslips. Results are representative of 5 individual MSC preparations at day 14 and 6 preparations at day 21.

4.2.7: Matrix mineralisation is absent after 3 and 7 days in collagen GAG scaffolds

As can be seen in Figure 4.7, after 3 (i and ii) and 7 days (iii and iv) culture in the presence (ii and iv) and absence (i and iii) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) no mineralisation took place. This was to be expected, as not only was osteocalcin expression absent, but no mineralisation was seen at day 7 in 2-D cultures. Results are representative of 2 individual cell culture preparations at each timepoint.

4.2.8: Extensive mineralisation occurred following 21 days osteoinductive treatment

Figures 4.8 and 4.9 show that, despite the presence of osteocalcin in osteoinductive factor-treated cells at day 14, no mineralization was observed as assessed by staining for calcium, using Alizarin Red S (Figure 4.8 i and ii), and calcium phosphate using the Von Kossa technique (Figure 4.9 i and ii). Images are representative of 5 individual MSC seeded scaffolds. However, following 21 days treatment with osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM), extensive mineralisation was observed in both alizarin red

(Figure 4.8 iv) and Von Kossa stained samples (Figure 4.9 v). Results are representative of 3 individual culture preparations at 21 days.

4.2.9: Incomplete mineralisation is time related

As can be seen in Figure 4.10, complete mineralisation of collagen GAG scaffolds cultured in the presence of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) did not always take place. Instead it was more common for partial mineralisation to occur as is the case in Figure 4.10 (ii and iv) as evidenced by Von Kossa staining and Alizarin Red S staining respectively. It was hypothesised that this lack of mineralisation was caused by insufficient time in culture rather than a lack of nutrient delivery to the centre of the scaffolds. Not only is the collagen GAG scaffold 99% porous, cells were also clearly visible in the scaffold core, suggesting sufficient nutrient transport. To address this issue, scaffolds were seeded with MSCs and cultured in the presence or absence of the same osteoinductive factors for 28, 35, 42 and 49 days to verify that complete mineralisation would ultimately take place. Figures 4.11-4.14 show control (i and iii) and osteoinductive factor-treated scaffolds (ii and iv) having been stained for calcium (i and ii) and calcium phosphate (iii and iv) at each of these timepoints respectively. At each later timepoint, levels of mineralisation were observed to increase until almost complete at both 42 (Figure 4.13) and 49 (Figure 4.14) days. Results are representative of 6 individual MSC preparations at day 21, 3 preparations at 28, 35 and 42 days and a single preparation at day 49.

4.2.10: The ERK inhibitor U0126 reduced the level of matrix mineralisation in osteoinductive factor treated scaffolds

As ERK activity was shown to be involved in 2-dimensional osteogenic differentiation, its role in the chemically and physically different environment of the collagen GAG scaffold was assessed. Figures 4.15 and 4.16 illustrate analysis of mineralisation in scaffolds cultured for 21 days in the absence (i and iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. In this study, scaffolds were also cultured

with the ERK inhibitor U0126 (iii and iv; 2μM). Alizarin Red S staining for calcium showed no evidence of calcium in scaffolds cultured with osteoinductive factors in the presence of U0126 (Figure 4.15 iv) and Von Kossa staining for calcium phosphate was less intense when scaffolds were treated with osteoinductive factors and U0126 (Figure 4.16 iv), showing the need for ERK in the 3-D differentiation of adult MSCs along the osteogenic lineage. Results are representative of 6 individual MSC preparations.

4.2.11: The ERK inhibitor U0126 prevented the expression of osteocalcin in osteoinductive factor treated scaffolds

Figure 4.17 demonstrates the inhibition of osteoinductive factor-induced osteocalcin expression by U0126. MSCs were seeded onto collagen GAG scaffolds in the absence (i and iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds (iii and iv) were co-cultured with the ERK inhibitor U0126 (2μM). Osteocalcin expression was absent when scaffolds were treated with osteoinductive factors and U0126 (Figure 4.17 iv). This result coupled with that of 4.2.27 suggests that ERK has an important role to play in mineralisation. Results are representative of 6 individual MSC preparations.

4.2.12: In MSC seeded scaffolds the p38 inhibitor SB203580 did not appear to inhibit osteogenesis in osteoinductive factor treated samples

In order to shed more light on the part p38 has to play in osteogenic differentiation of adult MSCs, its activity was inhibited by the selective inhibitor SB203580 (10μM). MSCs were seeded onto collagen GAG scaffolds in the absence (i and iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds (iii and iv) were co-cultured with SB203580 and osteogenesis was assessed. Figures 4.18 and 4.19 illustrate Alizarin Red and Von Kossa staining respectively. Osteogenesis was not inhibited when scaffolds were treated with osteoinductive factors and SB203580 (Figures 4.18 iv and 4.19 iv). Results are representative of 4 individual cell culture preparations.

4.2.13: SB203580 inhibited osteocalcin expression in osteoinductive factor treated scaffolds

Despite the mineralisation of scaffolds in the p38 inhibited scenario, Figure 4.20 (iv) clearly shows that osteocalcin expression was inhibited when cells were cultured with osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) in the presence of the p38 inhibitor SB203580 (10μM). It is not known how osteocalcin expression could be inhibited without a decrease in the quantity or quality of mineralisation. Perhaps upregulation of other matrix related proteins, for example matrix gla protein, compensated for the lack of osteocalcin expression, thereby maintaining the integrity of mineralisation. Results are representative of 4 individual cell culture preparations.

4.2.14: The Young's Modulus of osteoinductive factor treated scaffolds is significantly greater than controls

Figures 4.21 and 4.22 show the equilibrium moduli of control and osteoinductive factor-treated scaffolds that underwent unconfined compression testing to measure their stiffness. As this research is concerned with the development of viable bone constructs for the use as implants in the repair of skeletal defects, it is important that these constructs are strong enough to withstand the loads that will be imposed upon them *in vivo*. These preliminary mechanical tests give an indication of the progress that has been made. In Figure 4.21, scaffolds were seeded with MSCs and cultured for 21 days in the presence or absence of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). While there was an increase in the stiffness of the treated scaffolds from 2.1 ± 1.2kPa in controls to 3.1 ± 0.8kPa (Mean ± SEM), this did not reach significance. This result is representative of four individual cell culture preparations. In figure 4.22 however, the same experiment was carried out using cells from the same animal for all scaffolds to remove inter-animal variation. As can be seen, in these tests there was a significant increase in the modulus of osteoinductive factor-treated samples to 6.7 ± 1.1kPa compared to 3.4 ± 0.4kPa in untreated controls ($p<0.01$; paired students *t* test). This result shows the

effects that biological variation can have on experimental work and that scaffolds can be made significantly stiffer using the cells themselves.

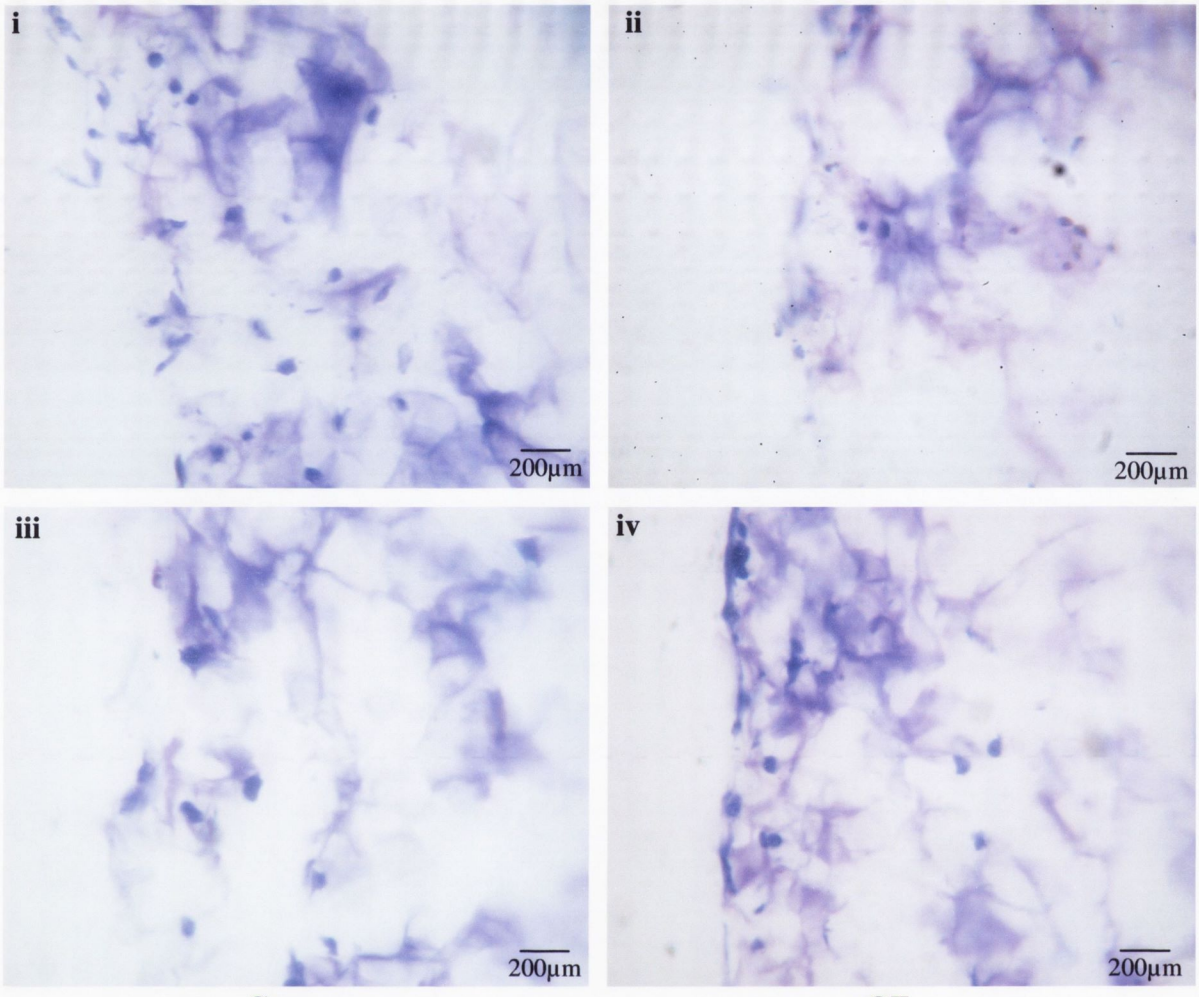


Figure 4.1: In MSC-seeded scaffolds no cellular migration was observed after 3 or 7 days

MSCs were seeded onto collagen GAG scaffolds for 3 (i and ii) and 7 days (iii and iv) in the absence (i and iii) or presence (ii and iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) By 7 days cells were still confined to the periphery of the scaffold in both control and osteoinductive factor-treated samples. Cells were stained using toluidine blue. Results are representative of 2 individual MSc preparations for each timepoint.

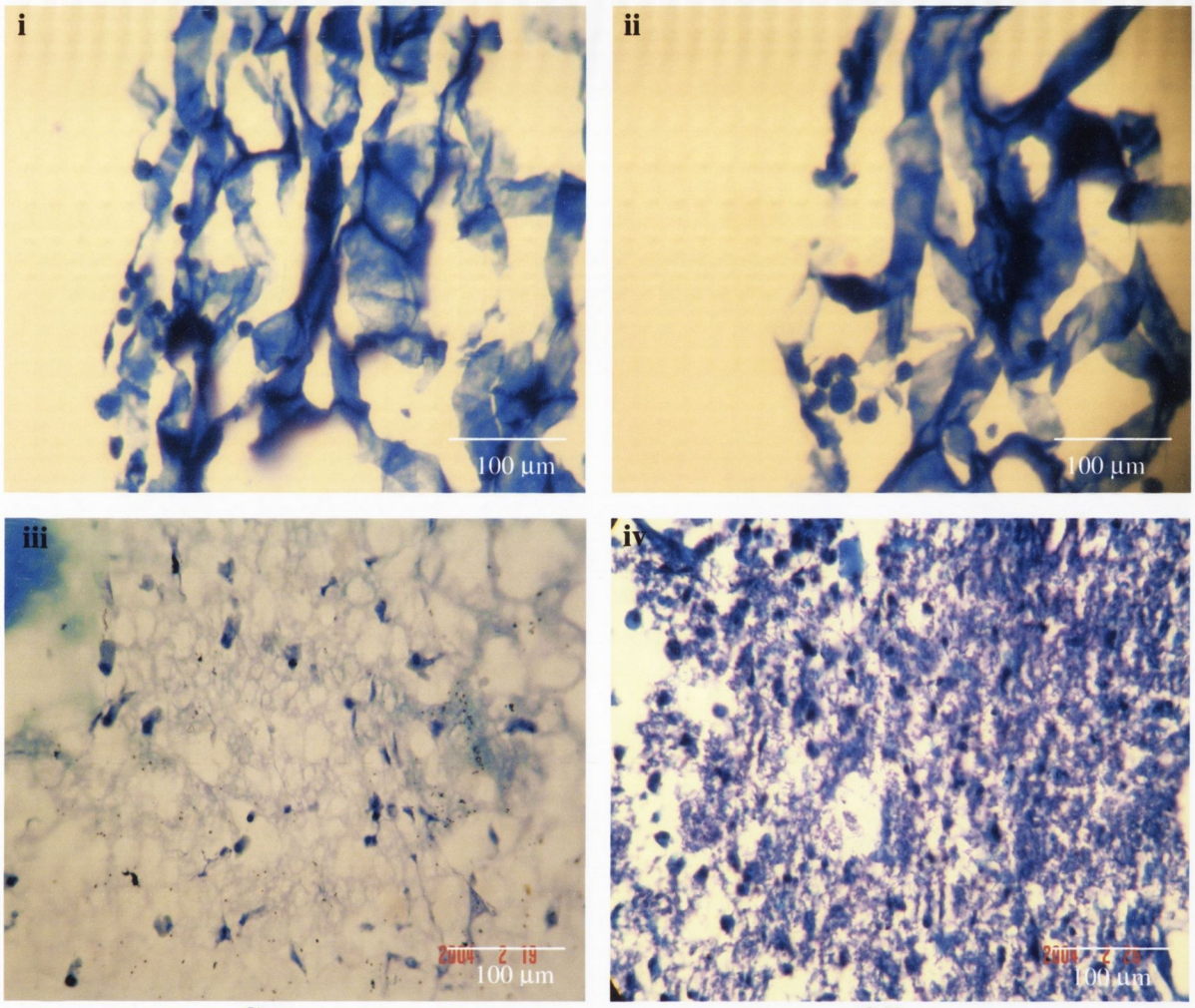


Figure 4.2: In MSC-seeded scaffolds no cellular migration was observed after 14 days with scaffolds experiencing complete penetration after 21 days

MSCs were seeded onto collagen GAG scaffolds for 14 and 21 days in the absence (i and iii) or presence (ii and iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). By 14 days, cells had not migrated past the outer parts of the scaffold. By 21 days there was a homogenous spread of cells throughout the scaffold. Cells were stained using toluidine blue. Results are representative of 6 individual MSc preparations for each timepoint.

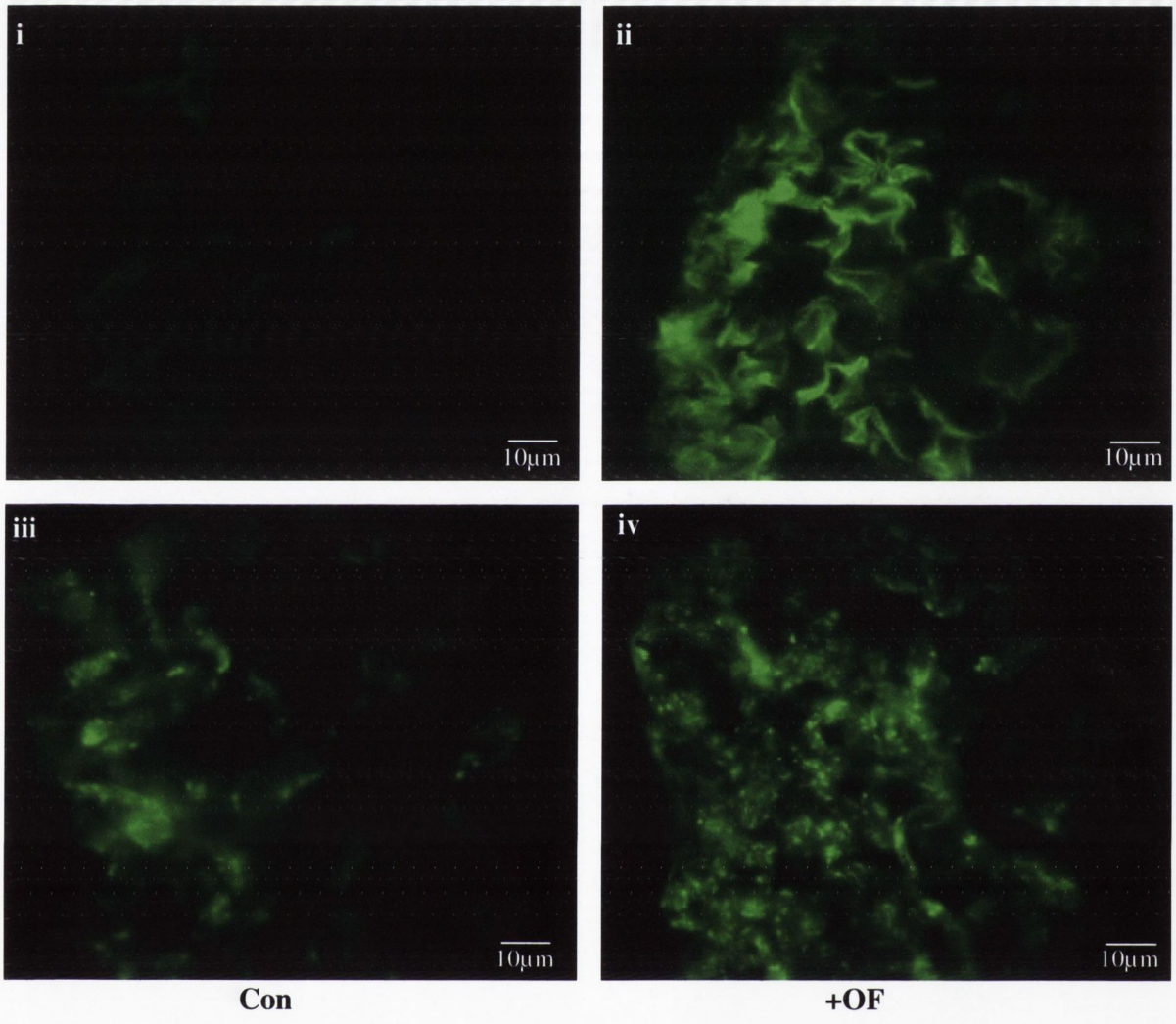


Figure 4.3: Collagen I expression is evident after 3 and 7 days in collagen GAG scaffolds

Following 3 (i and ii) days culture in collagen GAG scaffolds, MSCs expressed Collagen type I when cultured in the presence of osteoinductive factors (ii) (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) compared to control scaffolds cultured in the absence of osteoinductive factors (i). Collagen type I expression was also observed in osteoinductive treated samples at day 7 (iv). Some collagen I expression was also weakly evident in control scaffolds after 7 days (iii). Results are representative of 2 individual MSC preparations for each timepoint.

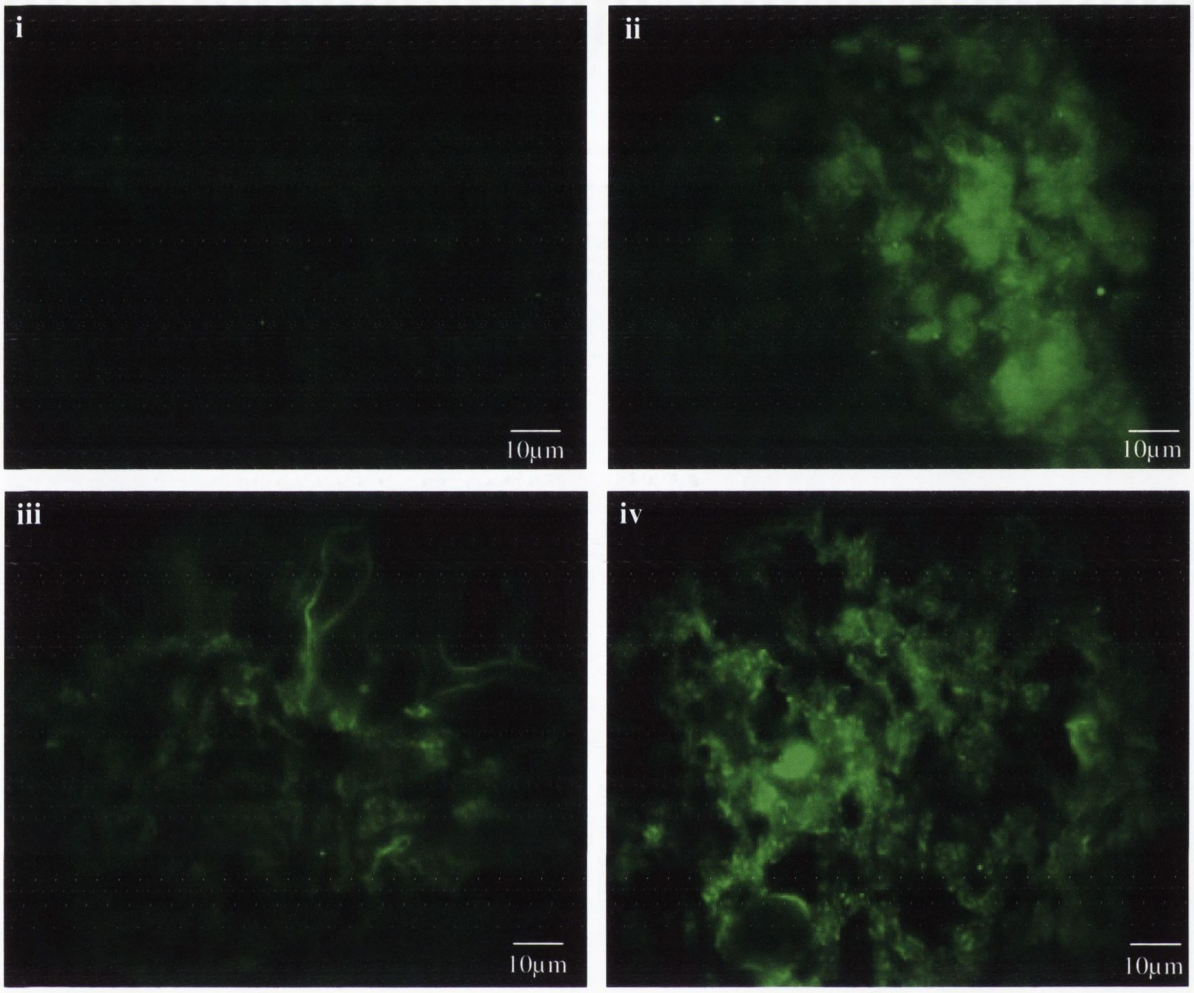


Figure 4.4: Collagen I expression is evident after 14 and 21 days in collagen GAG scaffolds

Following 14 (i and ii) and 21 (iii and iv) days culture in collagen GAG scaffolds, Collagen type I expression was sustained when cells were cultured in the presence of osteoinductive factors (ii and iv) (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) compared to control scaffolds cultured in the absence of osteoinductive factors (i and iii). Results are representative of 5 individual MSC preparations for each timepoint.

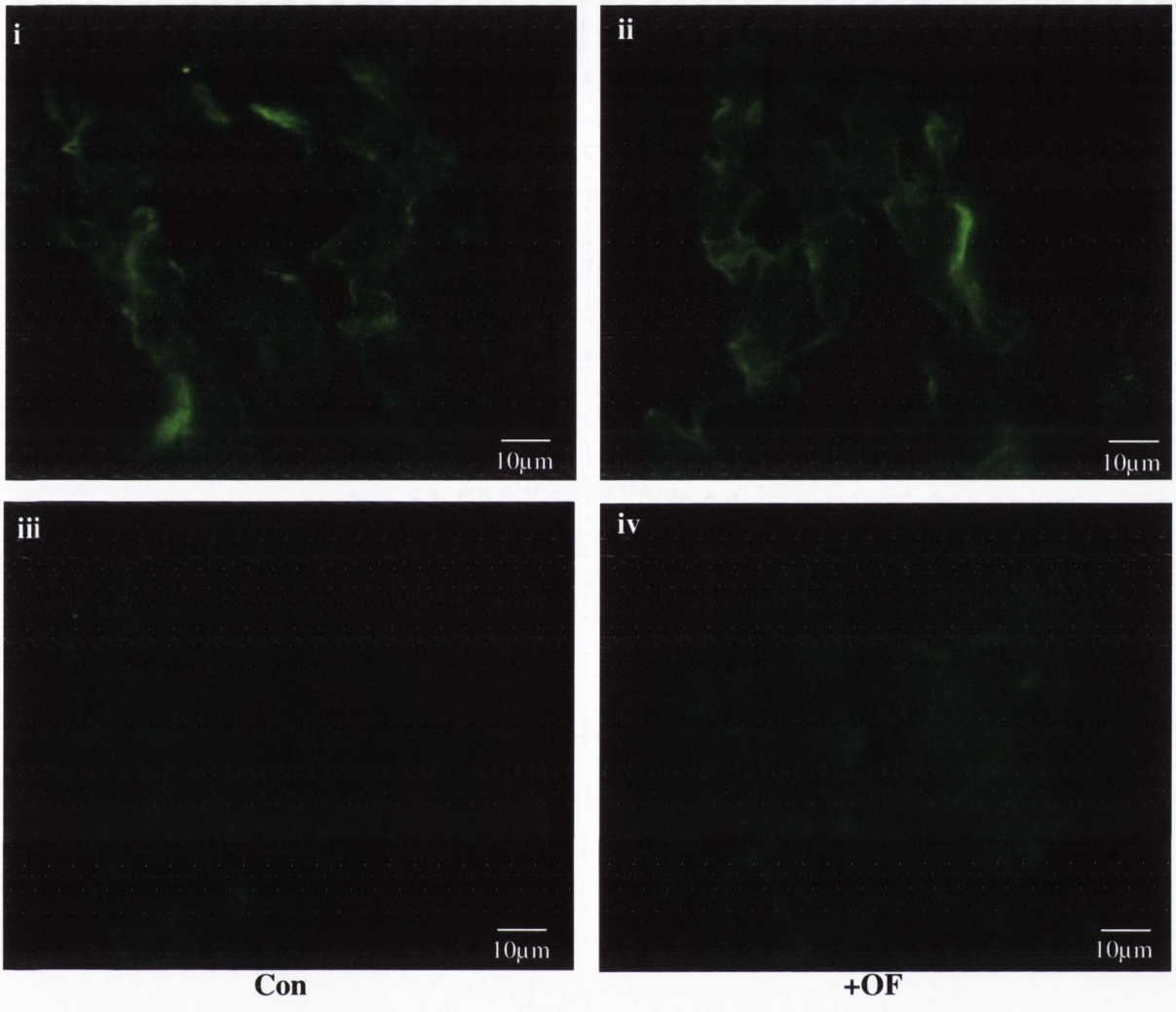


Figure 4.5: Osteocalcin expression is absent after 3 and 7 days in collagen GAG scaffolds

Following 3 (i and ii) and 7 days (iii and iv) culture in collagen GAG scaffolds, no osteocalcin was observed in scaffolds cultured with (ii and iv) or without (i and iii) osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). Results are representative of 2 individual MSC preparations for each timepoint.

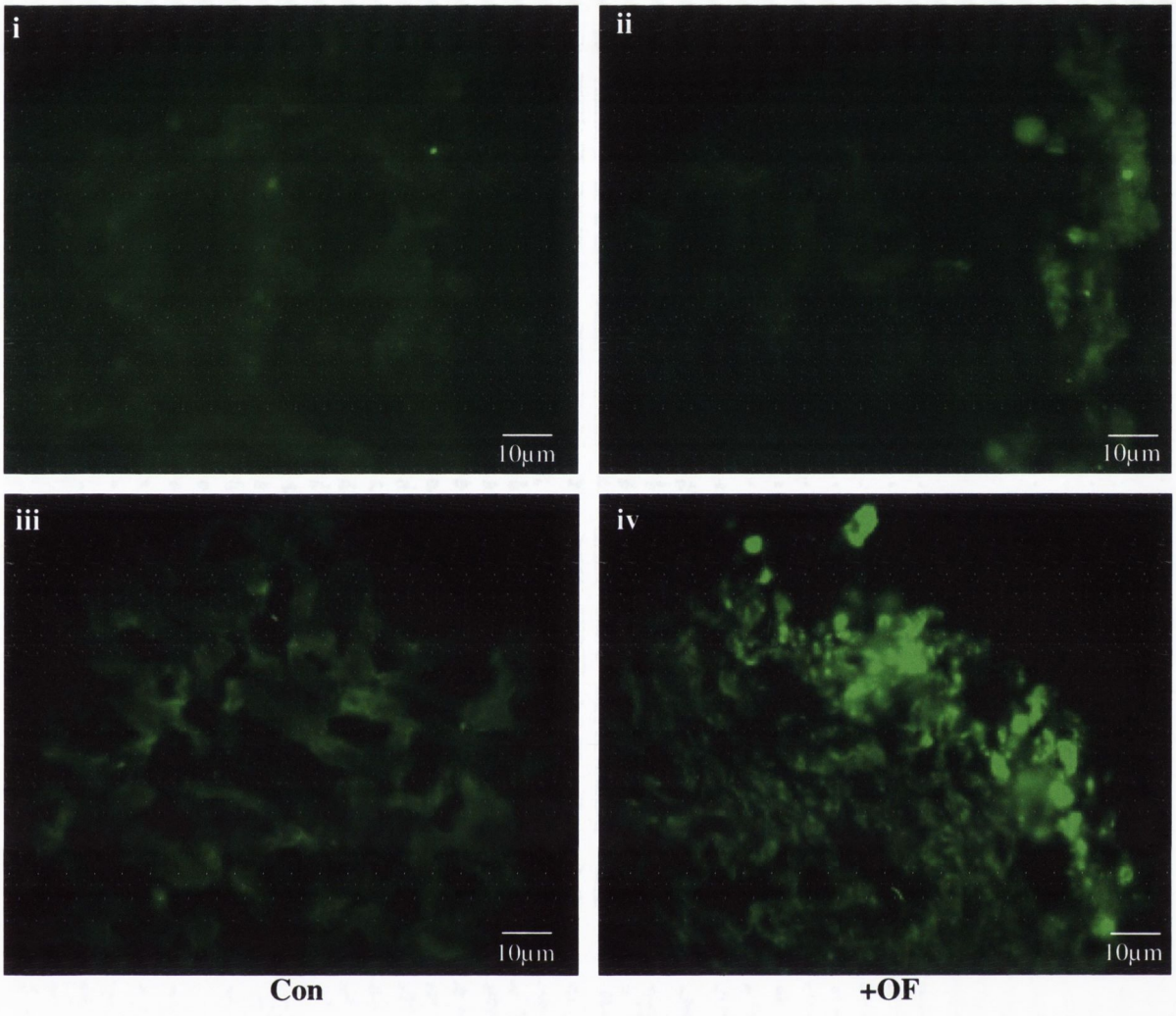


Figure 4.6: Osteocalcin expression is evident after 14 and 21 days in collagen GAG scafolds

Following 14 (i and ii) and 21 (iii and iv) days culture in collagen GAG scaffolds, osteocalcin expressed in scaffolds when cells were cultured in the presence of osteoinductive factors (ii and iv) (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) compared to control scaffolds cultured in the absence of osteoinductive factors (i and iii). The intensity of staining for osteocalcin was much greater at day 21 compared to day 14. Results are representative of 5 individual MSC preparations for each timepoint.

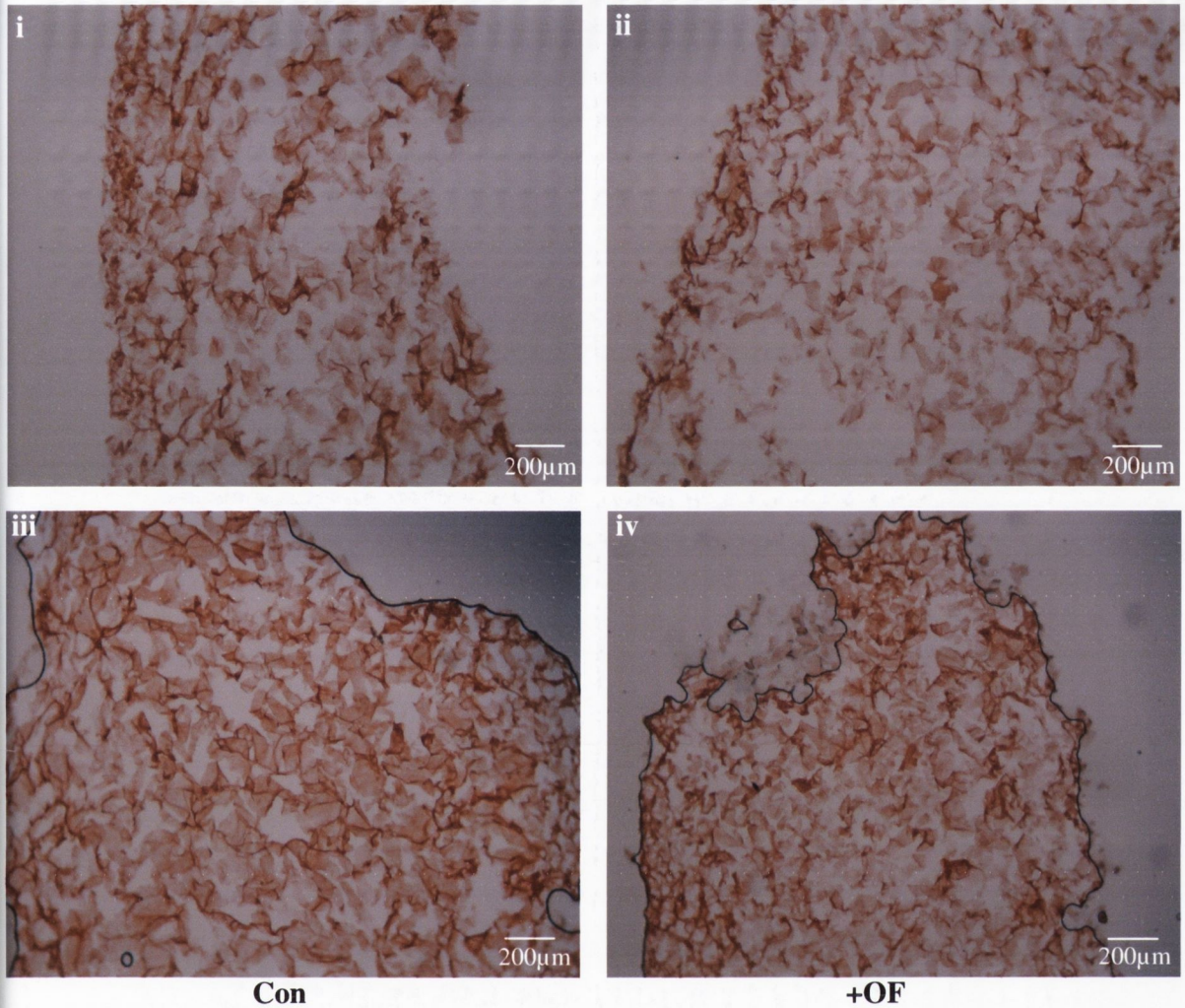


Figure 4.7: Matrix mineralisation is absent after 3 and 7 days in collagen GAG scaffolds

Following 3 (i and ii) and 7 days (iii and iv) culture in collagen GAG scaffolds, no mineralisation was observed in scaffolds cultured with (ii and iv) or without (i and iii) osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). Results are representative of 2 individual MSC preparations for each timepoint.

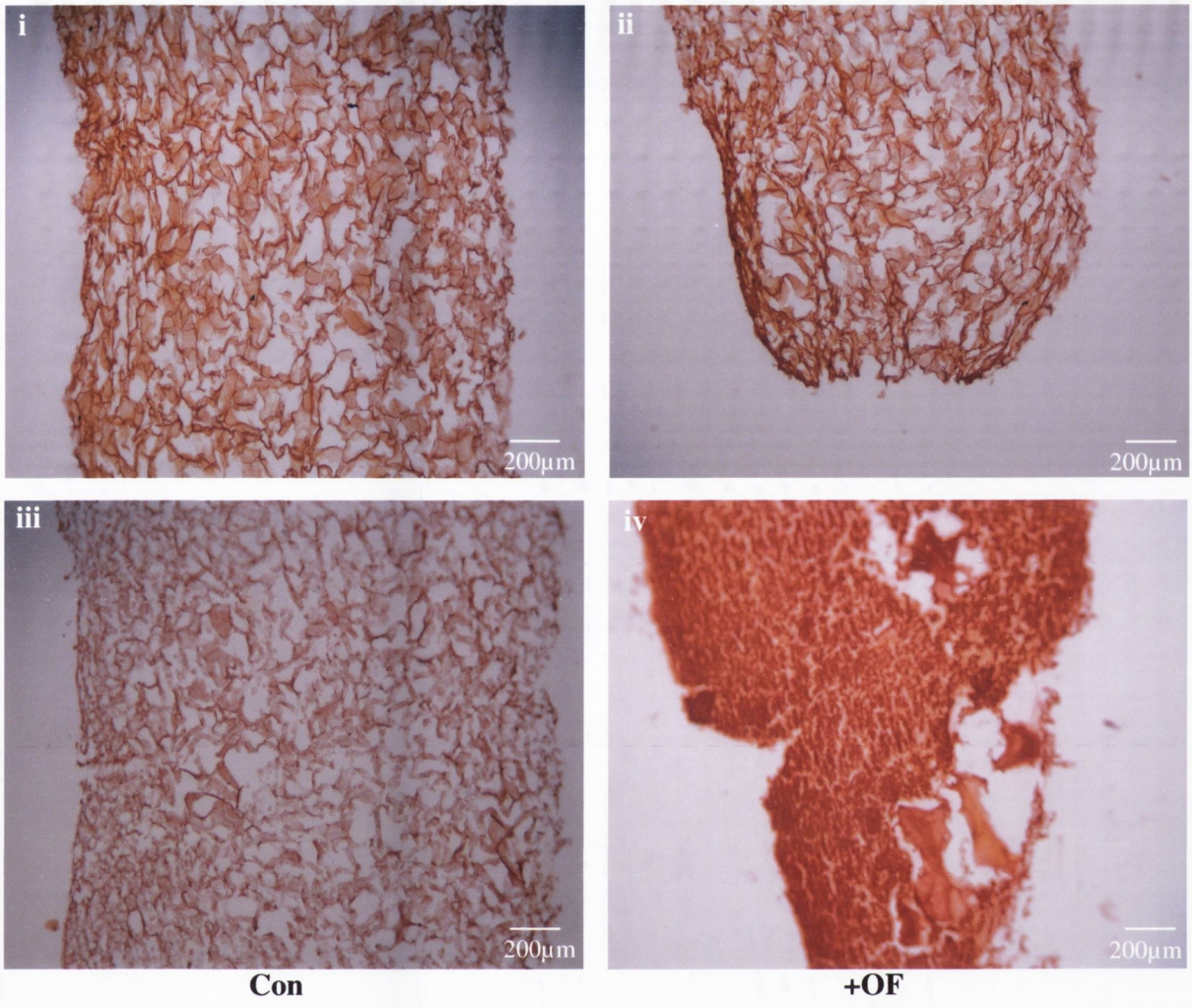


Figure 4.8: Extensive mineralisation occurred following 21 days osteoinductive treatment

Following 14 days culture in collagen GAG scaffolds, in absence (i) or the presence (ii) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM), no mineralisation was observed in either control or osteoinductive factor-treated samples as evidenced by Alizarin Red S staining for calcium. After 21 days however, extensive mineralisation occurred in osteoinductive factor treated samples (iv) but not in controls (iii). Results are representative of 5 individual MSC preparations at 14 days and 3 MSC preparations at 21 days.

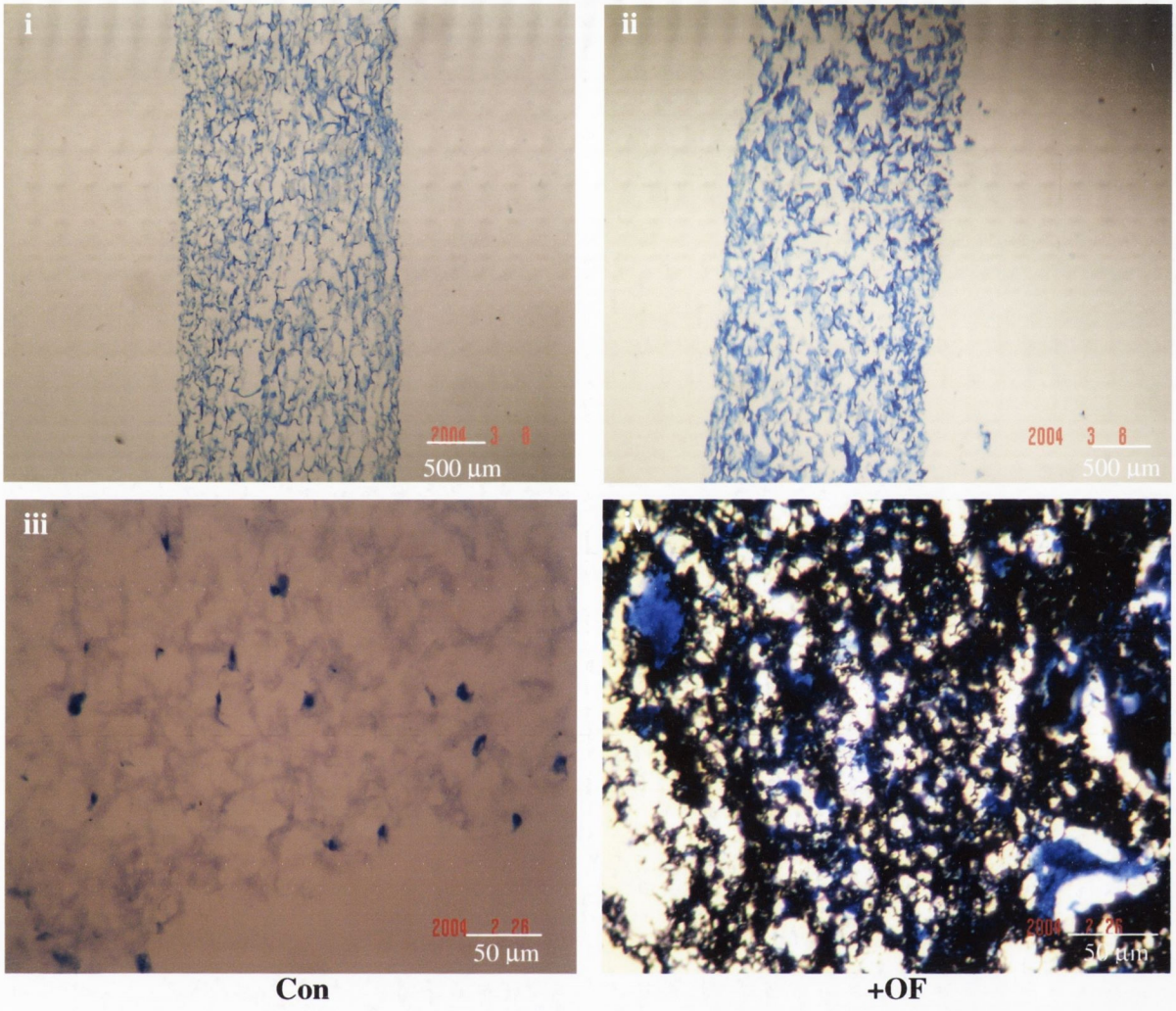


Figure 4.9: Extensive mineralisation occurred following 21 days osteoinductive treatment

Following 14 days culture in collagen GAG scaffolds, in the presence (ii) or absence (i) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM), no mineralisation was observed in either control or osteoinductive factor treated samples as evidenced by Von Kossa staining for calcium phosphate. After 21 days however, extensive mineralisation occurred in osteoinductive factor treated samples (iv) but not in controls (iii). Results are representative of 5 individual MSC preparations at 14 days and 3 MSC preparations at 21 days.

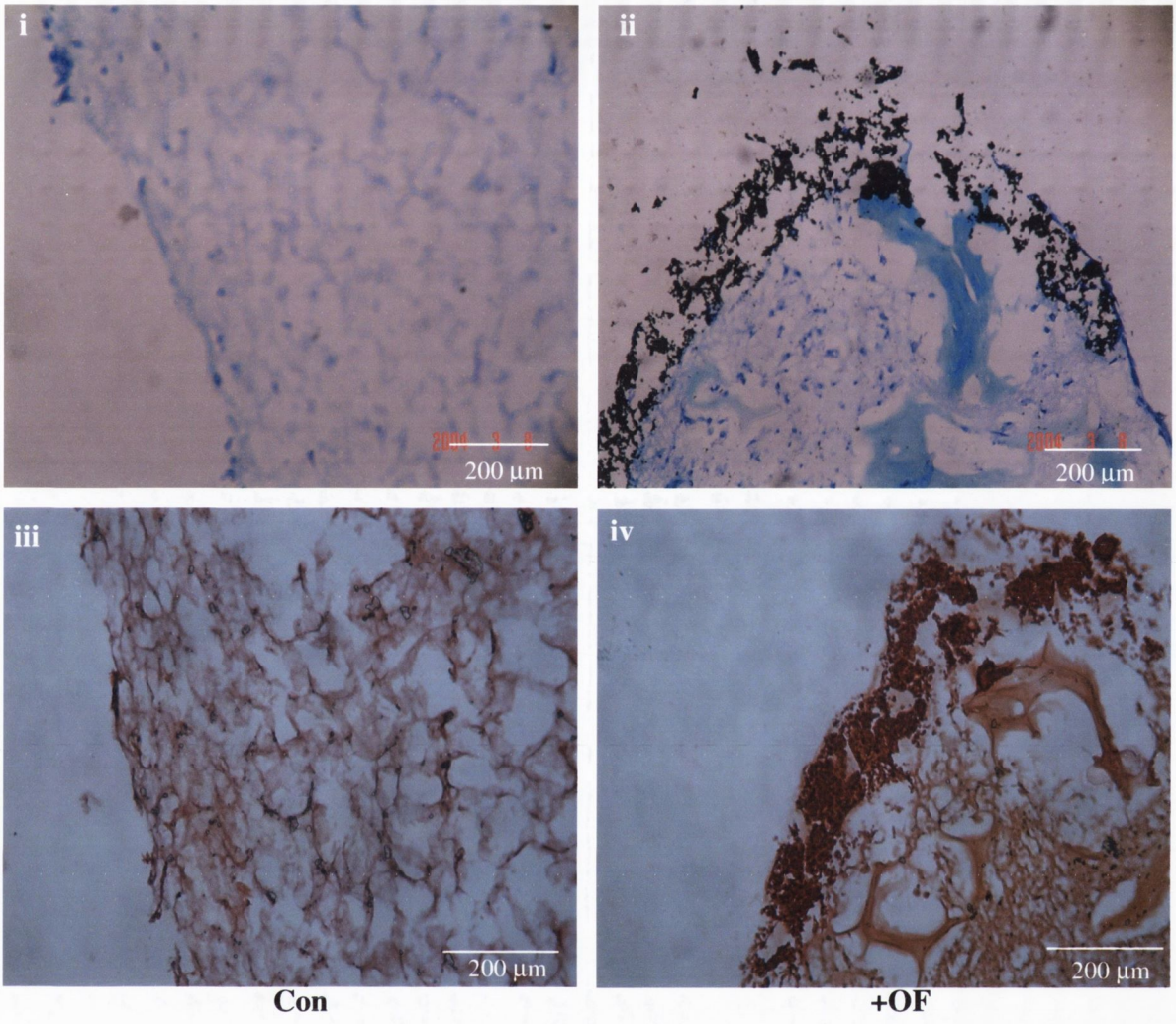


Figure 4.10: In MSC-seeded scaffolds, 21 days treatment with osteoinductive factors led to matrix mineralization

MSCs were seeded onto collagen GAG scaffolds for 21 days in the absence (i & iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM). Cells were stained using the Von Kossa method (i and ii) and Alizarin Red S (iii and iv). 21 days treatment led to matrix mineralization only at the periphery of scaffolds when cultured in the presence of osteoinductive factors. Results are representative of 6 individual cell culture preparations

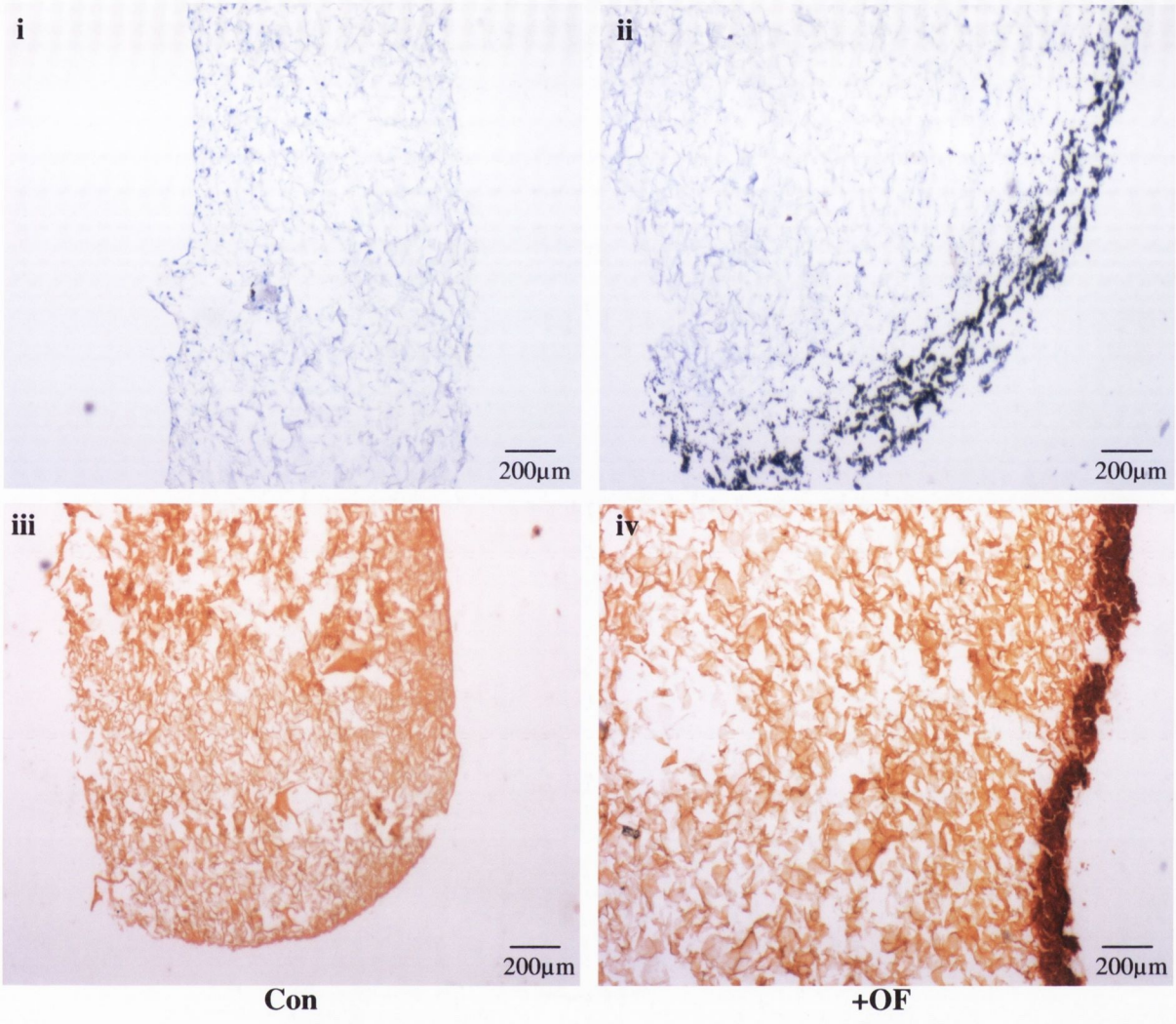


Figure 4.11: Matrix mineralisation is evident after 4 weeks culture in 3-D in the presence of osteoinductive factors

Following 28 days culture in collagen GAG scaffolds in the presence (ii and iv) and absence (i and iii) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM), matrix mineralisation was evident at the periphery of scaffolds in osteoinductive treated samples as evidenced by Von Kossa (i and ii) and Alizarin Red S (iii and iv) staining. Results are representative of 3 individual MSC preparations.

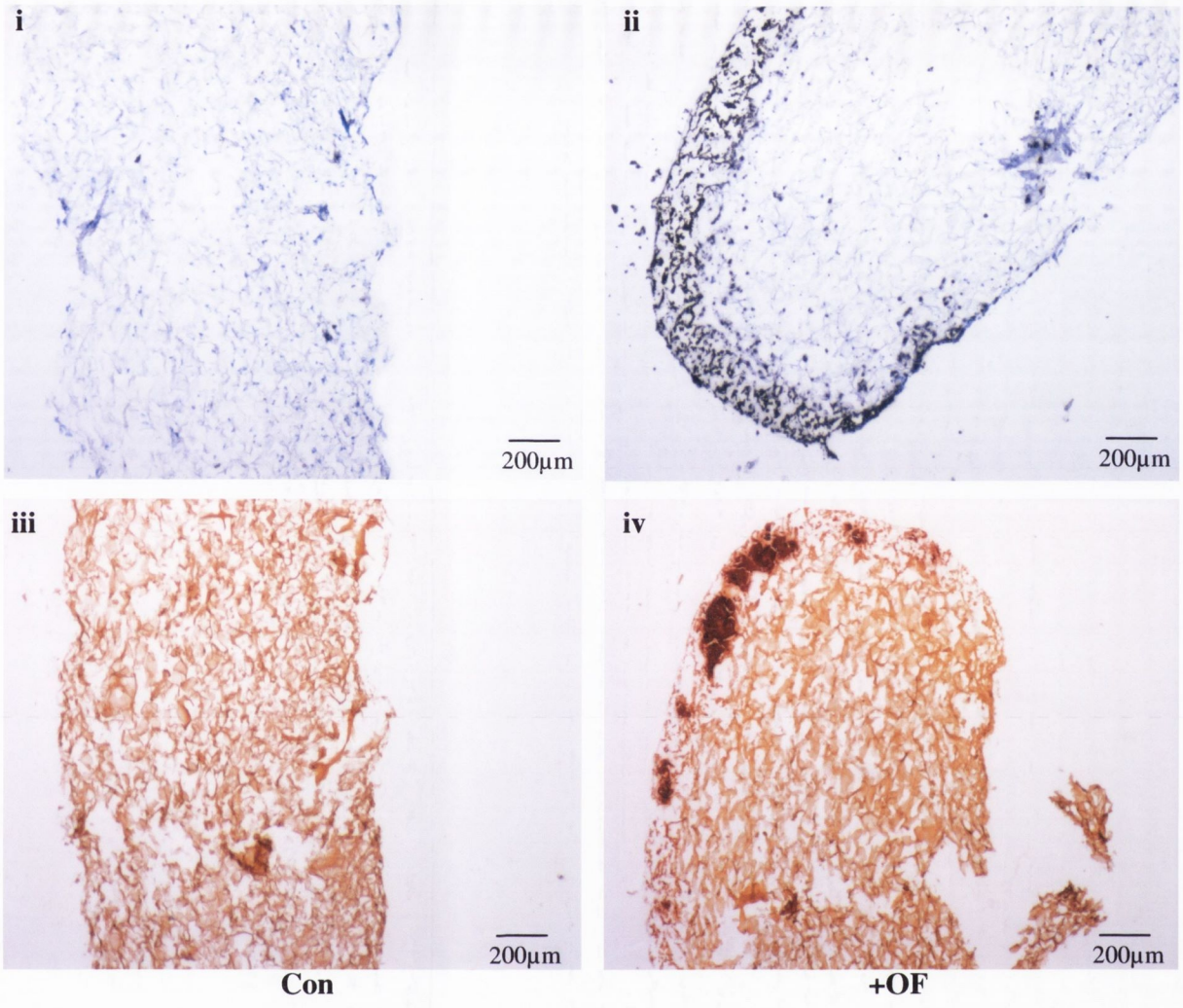


Figure 4.12: Matrix mineralisation is evident after 5 weeks culture in 3-D in the presence of osteoinductive factors

Following 35 days culture in collagen GAG scaffolds in the presence (ii and iv) and absence (i and iii) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM), matrix mineralisation was evident at the periphery of scaffolds on both sides, in osteoinductive treated samples as evidenced by Von Kossa (i and ii) and Alizarin Red S (iii and iv) staining. Results are representative of 3 individual MSC preparations.

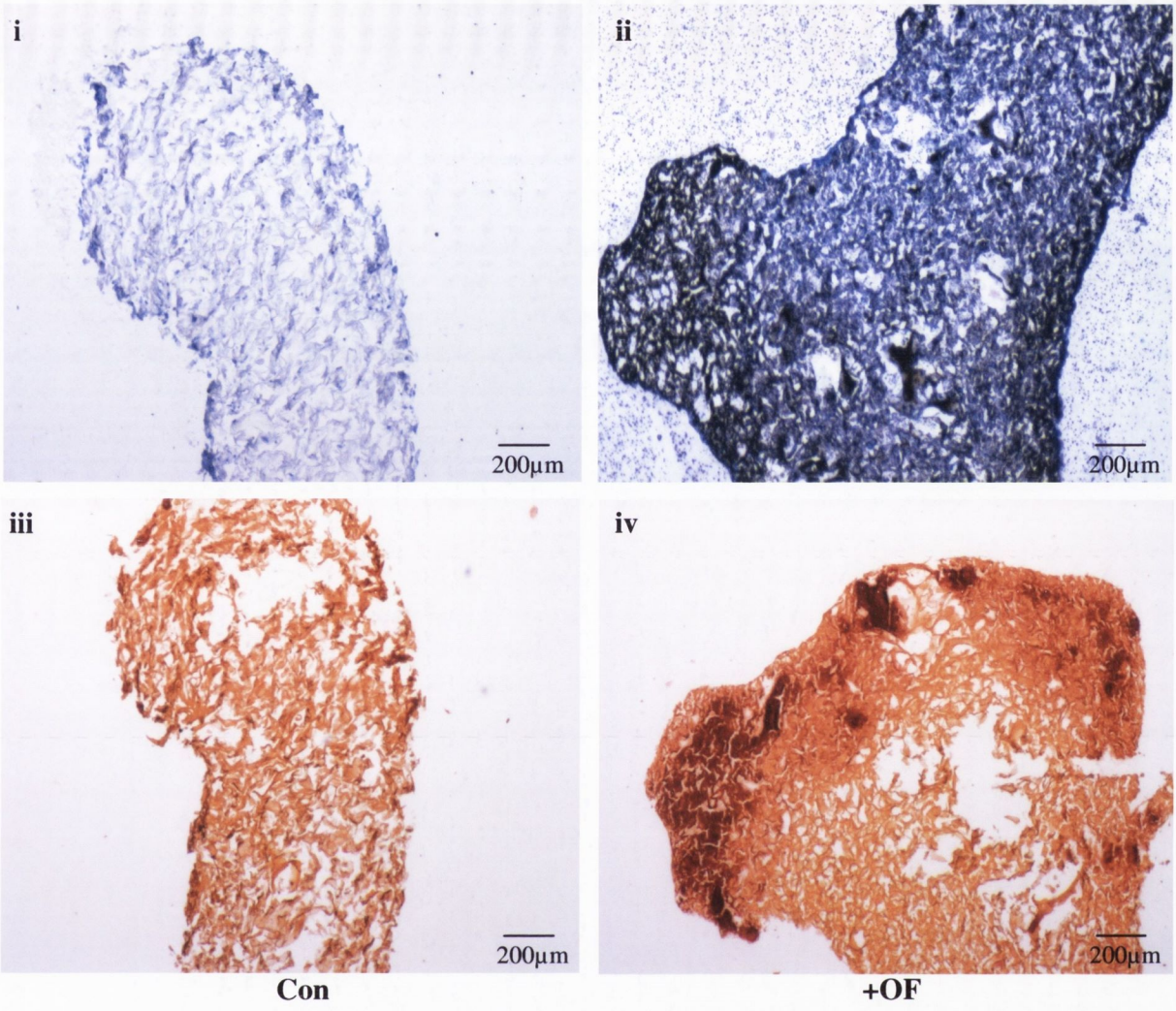


Figure 4.13: Matrix mineralisation is evident after 6 weeks culture in 3-D in the presence of osteoinductive factors

Following 42 days culture in collagen GAG scaffolds in the presence (ii and iv) and absence (i and iii) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM), matrix mineralisation was evident throughout scaffolds, in osteoinductive treated samples as evidenced by Von Kossa (i and ii) and Alizarin Red S (iii and iv) staining. Results are representative of 3 individual MSC preparations.

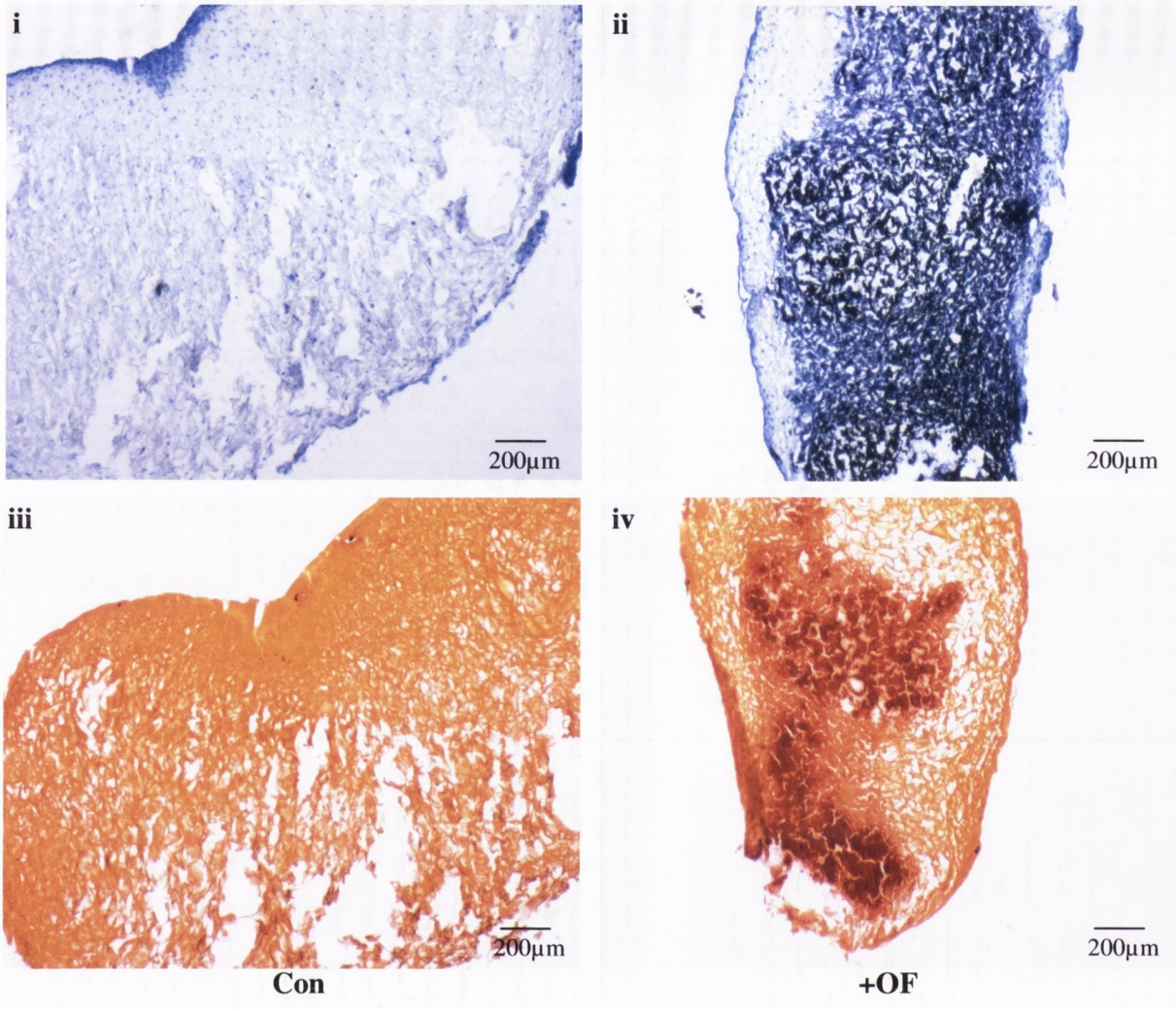


Figure 4.14: Matrix mineralisation is evident after 7 weeks culture in 3-D in the presence of osteoinductive factors

Following 49 days culture in collagen GAG scaffolds in the presence (ii and iv) and absence (i and iii) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM), matrix mineralisation was evident throughout scaffolds, in osteoinductive treated samples as evidenced by Von Kossa (i and ii) and Alizarin Red S (iii and iv) staining. Results are representative of 1 individual MSC preparation.

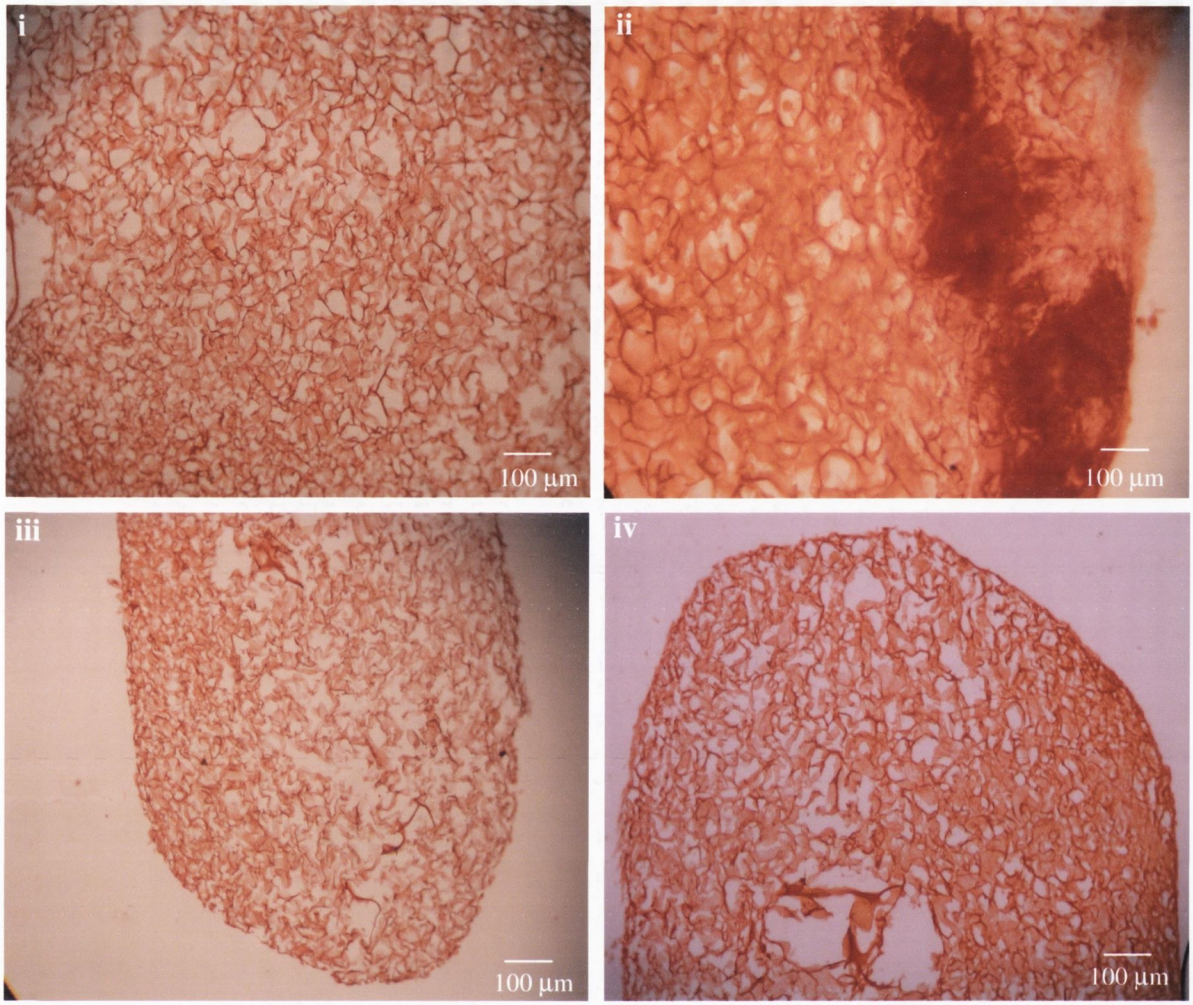


Figure 4.15: The ERK inhibitor U0126 reduced the level of matrix mineralisation in osteoinductive factor treated scaffolds

MSCs were seeded onto collagen GAG scaffolds in the absence (i and iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds (iii and iv) were co-cultured with the ERK inhibitor U0126 (2µM). Mineralisation was absent when scaffolds were treated with osteoinductive factors and U0126 (iv) as evidenced by Alizarin Red S staining for calcium. Results are representative of 6 individual MSC preparations.

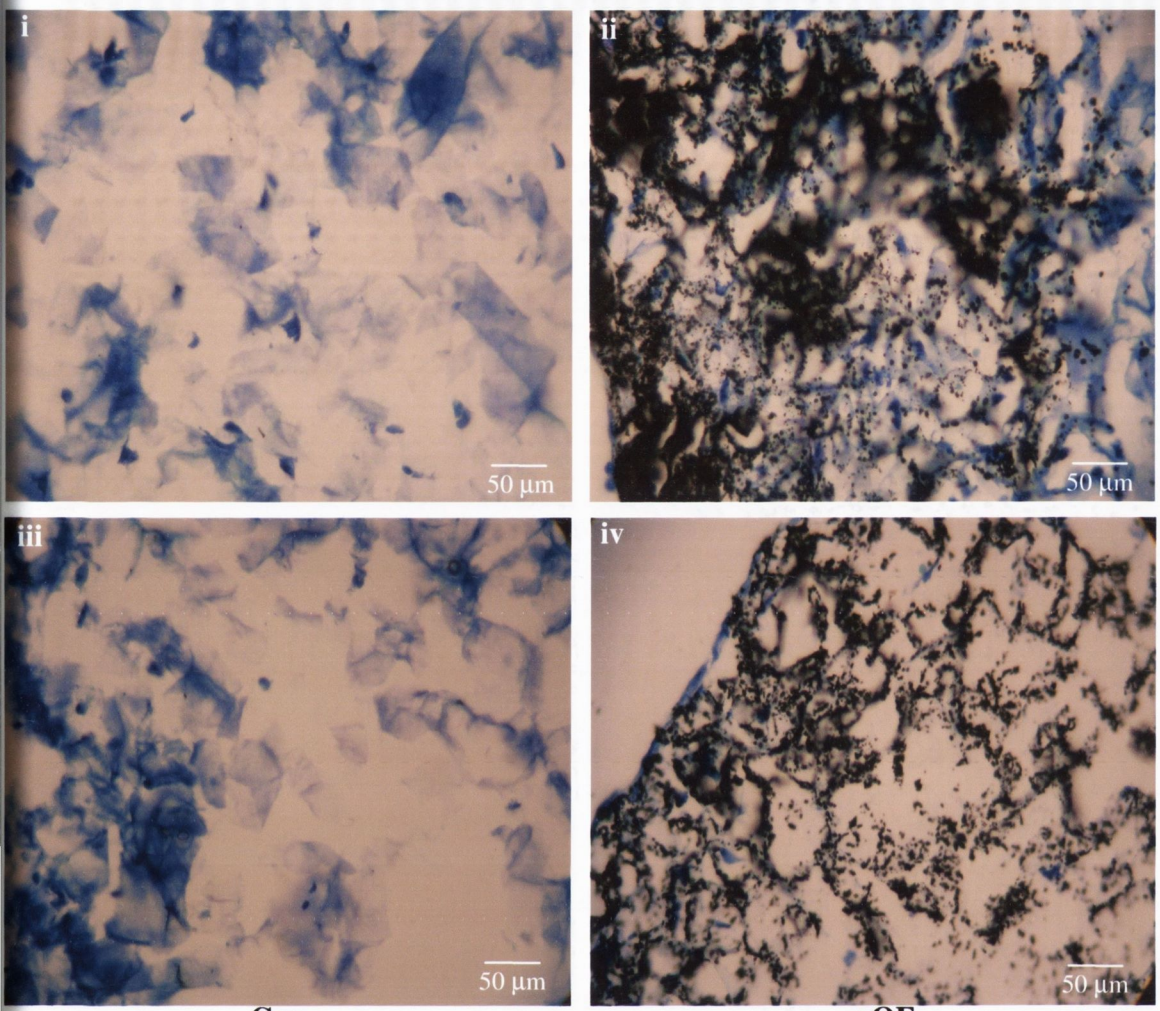


Figure 4.16: The ERK inhibitor U0126 reduced the level of matrix mineralisation in osteoinductive factor treated scaffolds

MSCs were seeded onto collagen GAG scaffolds in the absence (i and iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds (iii and iv) were co-cultured with the ERK inhibitor U0126 (2µM). Mineralisation was less intense when scaffolds were treated with osteoinductive factors and U0126 (iv) as evidenced by Von Kossa's technique for calcium phosphate. Results are representative of 6 individual MSC preparations.

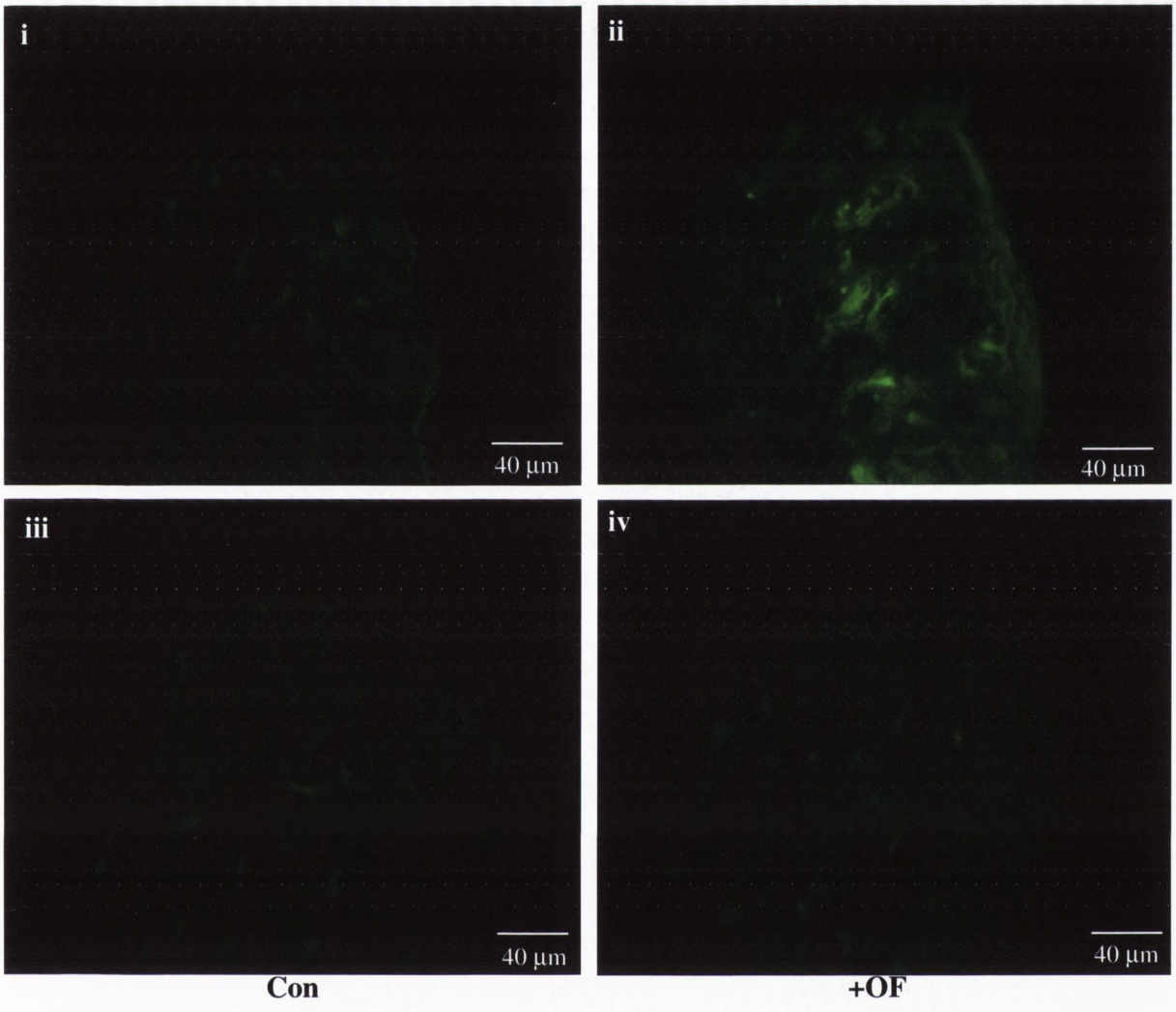


Figure 4.17: The ERK inhibitor U0126 prevented the expression of osteocalcin in osteoinductive factor treated scaffolds

MSCs were seeded onto collagen GAG scaffolds in the absence (i and iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds (iii and iv) were co-cultured with the ERK inhibitor U0126 (2μM). Osteocalcin expression was absent when scaffolds were treated with osteoinductive factors and U0126 (iv) as evidenced by immunofluorescent staining for osteocalcin. Results are representative of 6 individual MSC preparations.

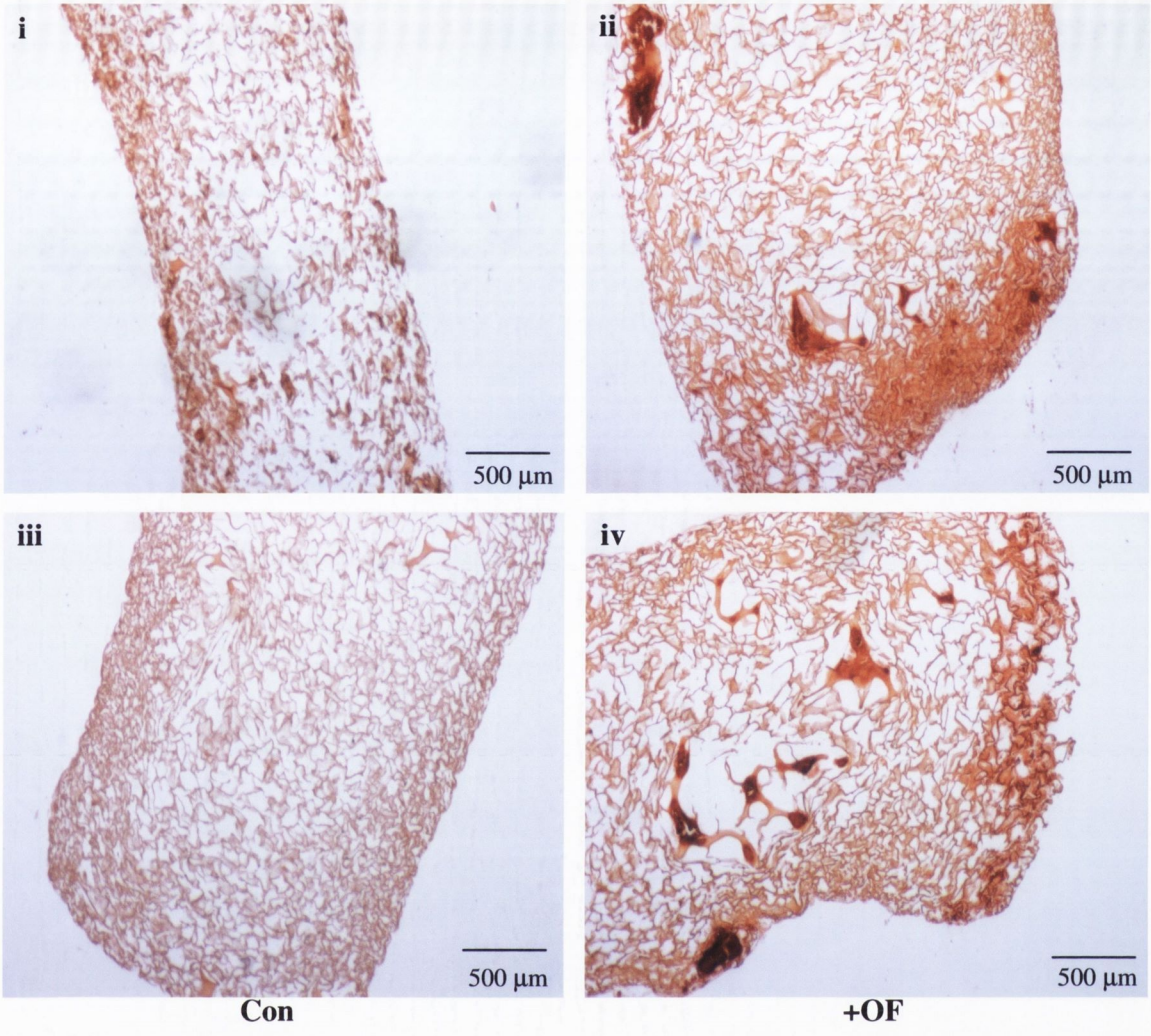


Figure 4.18: In MSC seeded scaffolds the p38 inhibitor SB203580 did not appear to inhibit osteogenesis in osteoinductive factor treated samples

MSCs were seeded onto collagen GAG scaffolds in the absence (i and iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds (iii and iv) were co-cultured with the p38 inhibitor SB203580 (10µM). Osteogenesis was not inhibited when scaffolds were treated with osteoinductive factors and SB203580 as evidenced by Von Kossa's technique for calcium phosphate (iv). Results are representative of 4 individual MSC preparations.

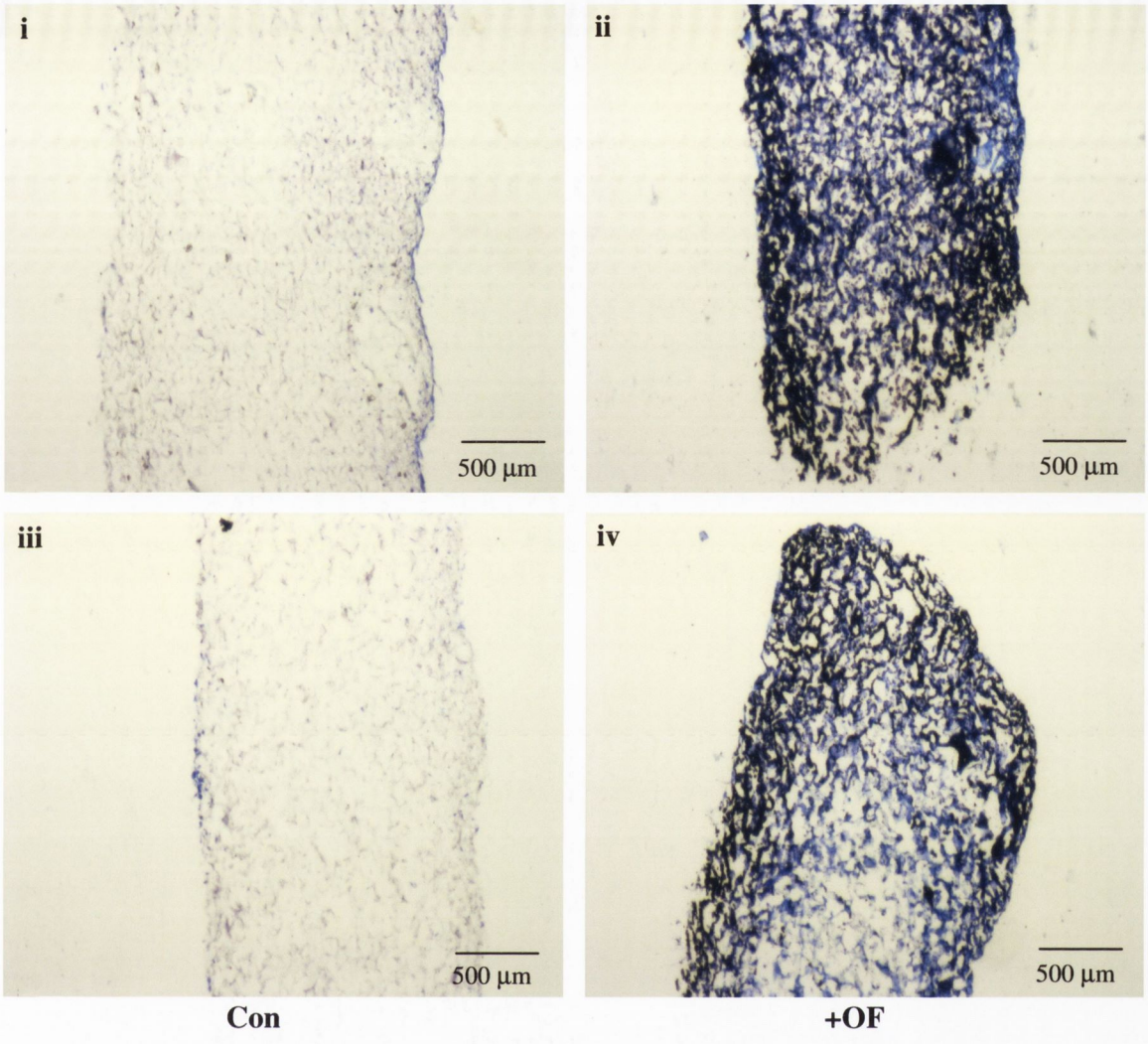


Figure 4.19: In MSC seeded scaffolds the p38 inhibitor SB203580 did not appear to inhibit osteogenesis in osteoinductive factor treated samples

MSCs were seeded onto collagen GAG scaffold in the absence (i and iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds (iii and iv) were co-cultured with the p38 inhibitor SB203580 (10µM). Osteogenesis was not inhibited when scaffolds were treated with osteoinductive factors and SB203580 as evidenced by Von Kossa's technique for calcium phosphate (iv). Results are representative of 4 individual MSC preparations.

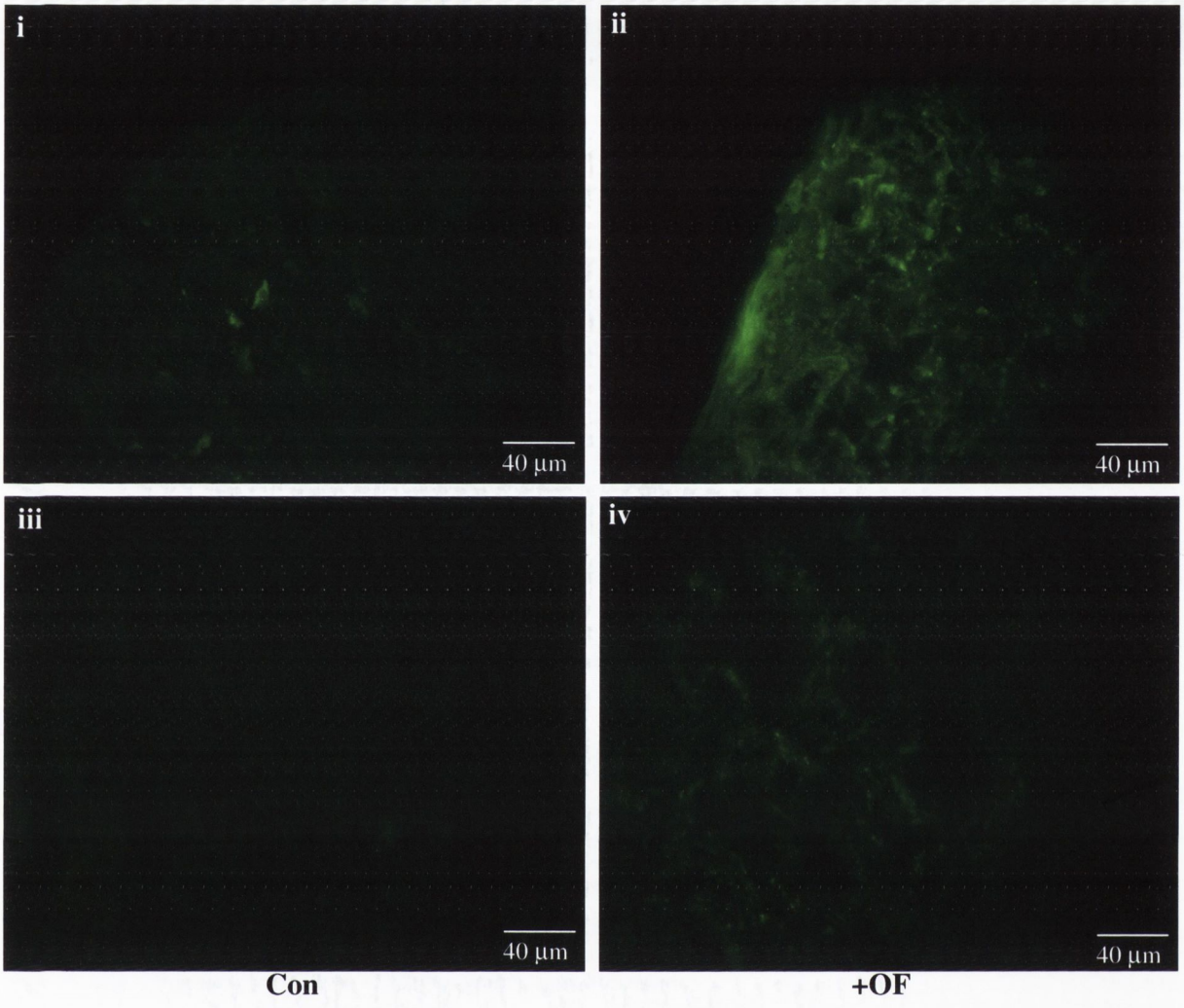


Figure 4.20: SB203580 inhibited osteocalcin expression in osteoinductive factor treated scaffolds

MSCs were seeded onto collagen GAG scaffolds in the absence (i and iii) or presence (ii & iv) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds (iii and iv) were co-cultured with the p38 inhibitor SB203580 (10μM). Osteocalcin expression was inhibited when scaffolds were treated with osteoinductive factors and SB203580 as evidenced by immunofluorescent staining for osteocalcin (iv). Results are representative of 4 individual MSC preparations.

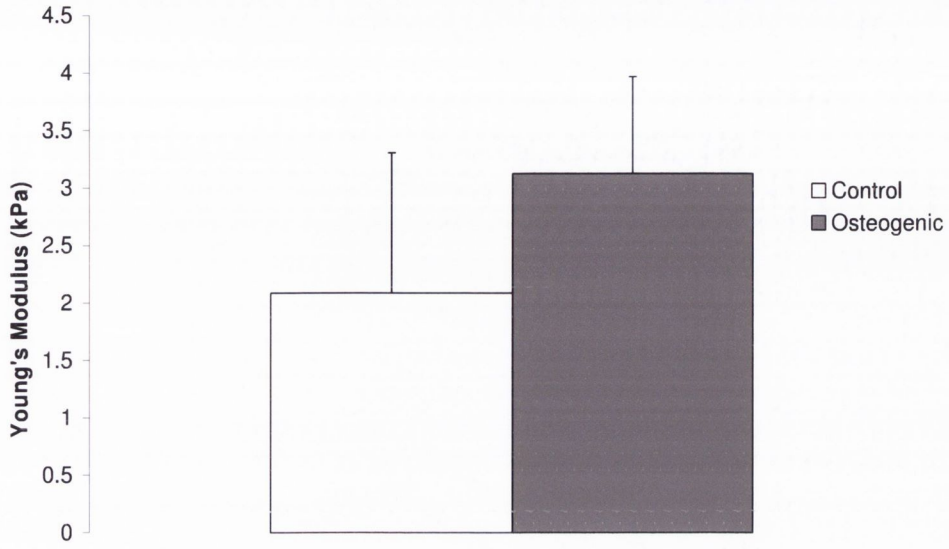


Figure 4.21: The stiffness of osteoinductive factor treated scaffolds is not significantly greater than controls

MSCs were seeded onto collagen GAG scaffolds in the absence (Control) or presence (Osteogenic) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds underwent unconfined compression testing to determine the Young's Modulus of each sample. Osteoinductive factor treated samples were not significantly stiffer that untreated controls. Results are representative of 4 individual MSC preparations.

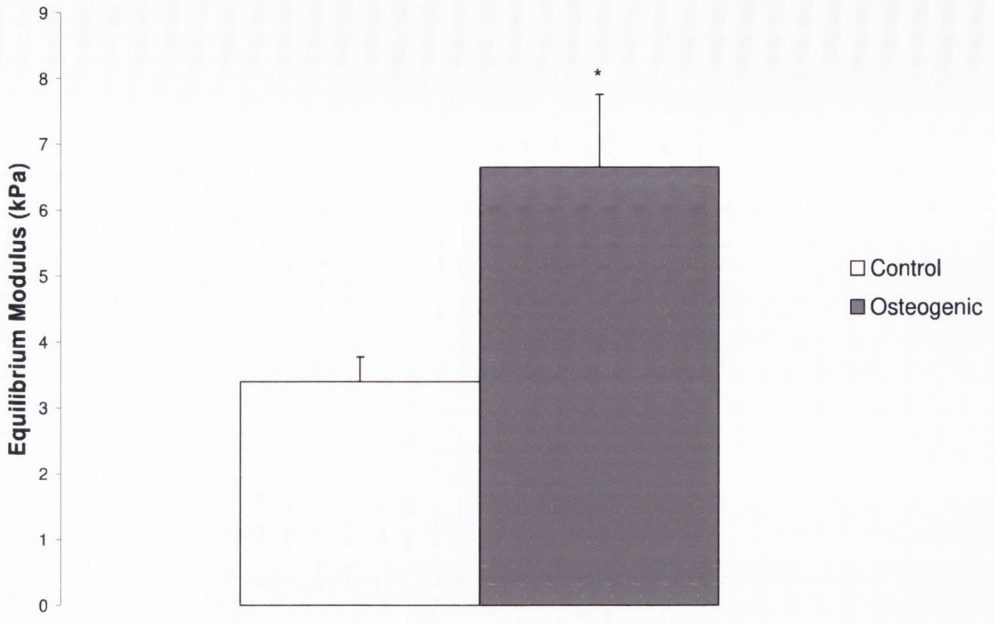


Figure 4.22: The stiffness of osteoinductive factor treated scaffolds is significantly greater than controls in repeated samples

MSCs were seeded onto collagen GAG scaffolds in the absence (Control) or presence (Osteogenic) of osteoinductive factors (dexamethasone, 0.68nM; β-glycerophosphate, 10 mM; ascorbic acid, 0.5 mM) for 21 days. Scaffolds underwent unconfined compression testing to determine the Young's Modulus of each sample and osteogenic scaffolds were found to be significantly stiffer (Students paired *t* test, $P<0.01$). In this experiment however, 4 repetitions from the same culture preparation were used to rule out inter-animal variation.

4.3 Discussion

The aim of this study was to successfully seed novel 3-D collagen GAG scaffolds with adult MSCs and to induce differentiation of these cells along the osteogenic pathway. Following 21 days culture within the collagen GAG scaffold, MSCs show the ability to differentiate fully along the osteogenic lineage. Furthermore, matrix mineralisation is evident in those scaffolds cultured in the presence of osteoinductive factors. Following 14 days in culture, cell migration through the scaffold had not occurred. Though this was unexpected, it has been shown that cells take a longer period of time to respond to stimuli in 3-D as opposed to the 2-D environment of the tissue culture flask (Laurencin *et al.* 1996), and this may be dependant in part upon the initial cell seeding density (Goldstein *et al.,* 2001). Freed *et al* (1998) also showed that it took forty days for chondrocytes to migrate through a PLGA scaffold. With this in mind it is not surprising that after 14 days, complete penetration of the scaffold had not occurred. Following 21 days in culture, complete penetration of the scaffold occurred with a homogeneous distribution of the cells throughout. This result, when compared with that of Freed *et al.* (1998) is well within the apparent timeframe of cellular migration through a biodegradable scaffold.

Having demonstrated the ability of these scaffolds to maintain MSC viability for up to 3 weeks, the expression of osteogenic markers and the appearance of mineralisation was assessed, compared to 2-D cultured samples and also to results from other labs. As the major organic component of bone (Lodish *et al.,* 2000), and one of the first proteins expressed in the osteogenic process (Aubin *et al.,* 1995), collagen type I was an important protein to examine. By day 3, some collagen I expression was evident in 3-D demonstrating a comparable initial cellular response to osteoinductive factors as was seen in 2-D. Positive collagen I immunofluorescence was observed from day 3 until the last timepoint of 21 days in 3-D also. This staining was confined to the edges of the scaffolds where, as previously shown, the cells remain until at least day 14. By day seven, collagen I was seen to be produced extracellularly forming a matrix within the scaffold structure. This is a very important step since collagen I is known to be necessary for osteogenesis, and has been shown to stimulate bone formation (Xiao *et al.,* 1998). Xiao *et al* (1999) suggested the possibility that the stimulation of the pre-osteoblast cell surface integrin $\alpha_2\beta_1$ leads to the activation of the core binding factor α1 (Cbf-α1) gene, that is necessary for osteogenesis to occur, via a MAP kinase signalling cascade. Activation of this surface

integrin is by contact with type I collagen, demonstrating the importance of collagen I production and the possible benefit of using a scaffold primarily composed of same material. Again, the appearance of extracellularly produced collagen I at day 7 coincided with the 2-D expression. By day 21 collagen I expression seemed to have decreased somewhat. I hypothesise that this was not the case, but that expression was masked by the mineralisation that had taken place in the scaffold. From these results it would appear that cells respond at a similar rate in 3-D as they do in monolayer cultures.

The post-proliferative osteoblastic marker osteocalcin was the other protein marker used to assess the differentiation progress of MSCs in 2-D and 3-D. Osteocalcin is involved in matrix mineralisation (Hauschka *et al.,* 1981, Kasugai *et al.,* 1991) and as was expected, its expression closely mirrored the mineralisation timecourse in 2-D. Expression occurred weakly from day 3 onwards, but this was probably related to the small number of partially differentiated MSCs in culture, as staining was low and not very widespread. By day 10 osteocalcin expression was much more evident, with expression taking place in large nodule-like clusters of cells by day 14. This timecourse of events is in accordance with the findings of several others in 2-D (Tsuchida *et al.,* 2003, Herbertson and Aubin, 1997, Cassiede *et al.,* 1996). In 3-D there was unfortunately no 10 day sample due to insufficient cell numbers. Due to the lack of mineralisation by day 14 in 3-D however, it is unlikely that osteocalcin would have been expressed this early. By day 14, osteocalcin expression was only weakly evident at the periphery of the scaffold. Localisation of bone specific osteocalcin was confined to the regions of the scaffolds in which cells were evident at 14 days, suggesting that although the cells had not penetrated the scaffold, differentiation was taking place. Taking into account the late stage expression of osteocalcin during osteogenesis (Bilezikian *et al.,* 2001), it appears that the cells were far along the differentiation pathway before migration into the centre of the scaffolds took place. Osteocalcin expression was greater in 21 day treated samples compared to 14 day samples. This expression profile appeared to be slightly slower than was observed in 2-D. As shown by Laurencin *et al.* (1996), cells take longer to respond to a 3-D environment and this is clear from this study.

Despite expression of osteocalcin at the periphery of scaffolds at day 14, no mineralization was observed in any scaffolds. This is most likely due to insufficient time for the cells to attach to the scaffolds and move far enough along the differentiation

pathway to mineralize osteoid in 14 days partly because the response time of these cells increases in the 3-D setting (Laurencin *et al.,* 1996). The difference between 14 and 21 days in culture was marked in all cases, with substantial mineralization occurring in osteoinductive factor-treated scaffolds after 21 days, the first time this has ever been demonstrated in collagen GAG scaffolds. The mineralization of scaffolds was 11 days later in scaffolds compared to 2-D samples, ossurring at day 21 rather than day 10. It appears that while cells initially respond at the same rate in a 3-D setting, it takes longer for each step on the osteogenic differentiation process to occur compared to 2-D cultures. Despite this apparent delay in osteogenesis, Xiao *et al* (2003) observed a similar timecourse of activity with collagen scaffolds seeded with osteoblasts. Considering these were already differentiated cells, matching the duration of time required for mineralisation with adult stem cells is impressive, illustrating the biocompatible nature of the collagen GAG scaffold. The presence of collagen I in the scaffold, with which the MSCs had intimate contact, no doubt aided in the induction of differentiation of these cells along the osteogenic pathway, as has been demonstrated by Xiao *et al.* (1998).

It was observed that matrix mineralisation did not always take place throughout the entire scaffold. This is not surprising. Since full scaffold penetration had not even occurred by day 14, one would not expect complete mineralisation to occur within a week. In addition to the findings of Freed *et al* (1998), Yang *et al* (2001) also observed that it took 6 weeks for the internal matrix of PLGA scaffolds to be penetrated by MSCs. In this study, we observed complete scaffold penetration after only 21 days. It is interesting to note that the scaffold employed by Freed *et al* was only 2mm thick whereas the one used in this study was 3-4 mm when wet. Unfortunately Yang *et al* do not provide the dimensions of their scaffold for comparison. Additionally Laurencin *et al.* (1996) demonstrated the increased time required for cells to respond in a 3-D environment. As a result of these findings, and the lack of consistency of complete mineralisation of the scaffolds in this study, it was decided to determine if this was caused by insufficient time for mineralisation to take place or lack of nutrients in the centre of the scaffolds. A timecourse study of scaffold mineralisation was carried out. Scaffolds were cultured for 4, 5, 6 and 7 weeks in control and osteoinductive medium. Scaffold mineralization was subsequently assessed. The results clearly demonstrate that, while mineralisation was a very slow process, if given enough time the entire scaffold will be mineralised. As discussed by Kelly and Prendergast (2003) and demonstrated by Pei *et al.* (2002), a current problem with

scaffolds and tissue engineered constructs is core degradation. This can be as a result of insufficient nutrient delivery and waste removal, particularly in static cultures. The results from this study are promising. They demonstrate complete mineralisation of a scaffold 3-4mm thick without dynamic stimulation or any other means of nutrient delivery by 6-7 weeks. Longer cultures in larger samples should obviously be carried out to ensure core degradation does not subsequently take place. Importantly, this study also demonstrated the ability to maintain these scaffolds in culture for extended periods without matrix degradation or resorption by the cells. The glycosaminoglycan content in the scaffold would have played a role in the maintenance of scaffold integrity as shown by Pieper *et al.* (2000). In order to quantify the level of mineralisation, stereology was employed to calculate the percentage mineralisation of a single osteoinductive factor-treated scaffold. 85 10μm thick slices were stained, using Von Kossa's technique with no counterstain, and digitally photographed. Using Scion Image software the percentage of black pixels to white in the scaffold were calculated for each slice and averaged. It was found that a scaffold displaying incomplete mineralisation was 33.1% mineralised after 21 days in culture (Result not shown). This analysis was carried out on only one scaffold sample as a proof of principle for the quantification of mineralisation of a collagen GAG scaffold.

As has been shown in the 2-D setting both in the static (Chapter 3 & Jaiswal *et al.,* 2000) and dynamic (Simmons *et al.,* 2003) environments, the mitogen activated protein kinase ERK is involved in the process of differentiation. It is possible that ERK is also involved in inducing osteogenesis in these 3-D scaffolds. Hirota *et al.* (2000) demonstrated the activation of ERK via its upstream activator MEK in chondrocytes exposed to TGF-β, suggesting that ERK is also responsible for the differentiation of MSCs along the chondrogenic lineage. ERK has also been shown to be activated in differentiating muscle cells and positively regulates the expression and activity of the MyoD protein (Gredinger *et al.,* 1998). This transcription factor controls the differentiation of muscle cells. If ERK is indeed responsible for all of these processes, then how the same precursor cells respond to the same signal with a different response is not known. Perhaps more than one signal is activated in response to stimuli, inducing differentiation, or the differential activation of several signalling cascades occurs in a specific order. This is quite possible as crosstalk between the different MAPKs and even between MAPKs and protein kinase C (PKC) has been demonstrated (Chang *et al.,* 1998). Jaiswal *et al.* (2000) showed that other mitogen activated protein kinases, c-Jun-N-terminal Kinase (JNK) and p38 were also activated

during osteogenesis. While some of the signalling mechanisms of the differentiation of MSCs along specific lineages have been elucidated in 2-D models, no work has been carried out on 3-D models of osteogenesis. Much of the focus to date has simply been on attaining the induction of differentiation in a 3-D setting. This study investigated the role of both ERK and p38 in 3-D osteogenic differentiation. Having demonstrated the requirement for ERK in 2-D using the specific MEK inhibitor U0126, the same inhibitor was employed for the 3-D study. Inhibition of ERK activity dramatically reduced mineralisation levels but did not completely inhibit it. The quality of mineralisation was definitely affected, with less dense Von Kossa staining, and no positive Alizarin Red S staining. Alizaring Red S appears to require higher levels of calcium for a positive result than Von Kossa's technique. This is related to the pH of the stain. If the pH is raised, the stain will pick up calcium more easily but also stains much more non-specifically. The cause of the decrease in the quality of mineralisation is possibly the absence of osteocalcin, whose expression was dramatically decreased as a result of ERK inhibition. This draws a parallel with the result in the previous chapter, demonstrating complete inhibition of osteocalcin in 2-D following inhibition of ERK activity.

In the study by Jaiswal *et al.* (2000), p38 activity was shown to be elevated under osteogenic conditions after 9 days until day 13. In chapter 3 it was demonstrated that p38 activity was elevated at day 7. Despite the finding of Jaiswal *et al.*, they did not observe a decrease in either alkaline phosphatase activity or calcium accumulation when p38 activity was inhibited with SB203580. Jaiswal was unable to propose a role for p38 in osteogenic differentiation. In this study, a similar result was observed. Inhibition of p38 did not cause a decrease in matrix mineralisation. However, as with the inhibition of ERK activity, osteocalcin expression was prevented by inhibition of p38 activity. This correlates well with the timing of p38 and ERK activity in 2-D and the appearance of osteocalcin expression at day 10. Unlike the inhibition of ERK however, the quality of matrix mineralisation did not appear to be affected by p38 inhibition. How p38 mediated osteocalcin expression can be so abrogated without affecting mineralisation is not known. Simmons *et al.* (2003) also observed interesting results regarding p38 activity. They too observed that inhibition of p38 activity did not have an effect on mineralisation levels after 16 days in osteogenic culture medium. However they did observe a large increase in alkaline phosphatase activity when cells were co-cultured with SB203580. Interestingly, when cells were cyclically strained for 16 days, the opposite effect was seen. Inhibition of

p38 activity led to reduced alkaline phosphatase levels in strained cells with a dramatic increase in mineralisation compared to strained cells without p38 inhibition. The results from this chapter and those of Jaiswal *et al.* and Simmons *et al.* clearly show that p38 plays an integral role in the osteogenic process, though one that requires further investigation in order to understand precisely what that role is.

Chapter 5

Evaluation of the chondrogenic differentiation potential of adult mesenchymal stem cells in 3-D

5.1 Introduction

Articular chondrocytes synthesise cartilage specific extracellular matrix components such as type II collagen and sulphated proteoglycan to maintain cartilage homeostasis (Shum and Nuckolls, 2002, DeLise *et al.,* 2000, Sandell and Adler, 1999). During disease states this balance is destroyed leading to cartilage loss and joint damage. Articular cartilage functions to provide uncompromised movement by minimising friction between joints (Tuan *et al.,* 2003). Cartilage acts as a load bearing interface between articulating joints and is capable of resisting strong compressive forces. Its regenerative capacity is very limited however. Damage as a result of joint disease (osteoarthritis) or injury can lead to major pain and long term disability. Current treatments for repair of articular cartilage include, but are not exclusive to, total joint arthroplasty, autografting, allografting, intentional drilling of full thickness defects to stimulate invasion of cells from the underlying periosteum and autologous cell transplantation. There has been some progress, for example Wakitani *et al.* (1994) have succeeded in repairing full thickness defects on the weight-bearing surface of medial femoral condyles. Despite some successes, these procedures have a far from perfect success rate, particularly in younger patients (O'Driscoll *et al.,* 1998).

Although hyaline cartilage, the type that comprises articular cartilage, appears to be a simple material composed of only a single cell type and its extracellular matrix, it possesses the ability to resist compression and distribute loads so successfully, that no replacement tissue has yet been designed to carry out the same task to the same degree (Temenoff *et al.,* 2000, Buckwalter *et al.,* 1998, Cohen *et al.,* 1998). The arrangement of the extracellular matrix in cartilage creates an electrical double layer within its structure. Positive ions from the synovial fluid are attracted to negative ions on proteoglycans in the matrix. When the tissue is compressed, the distance between layers is decreased. As the layers overlap, strong electrostatic repulsion develops resulting in a significant increase in matrix stiffness (Bonassar *et al.,* 2002). This effect has been estimated to contribute to as much as half of the stiffness of the tissue (Frank *et al.,* 1987). The fluid phase of cartilage also plays a role in maintaining stiffness. As the tissue is compressed, fluid leaves the tissue. The frictional forces generated from this movement relative to the solid phase also tend to stiffen the tissue. Clearly, articular cartilage is a much more complex tissue that it appears to be. The best option for the treatment of damaged cartilage is to regenerate it or

replace it with new cartilage tissue rather than try to develop an alternative. The use of MSCs to tissue engineer new cartilage offers a potential solution to the problem of insufficient cartilage repair.

A 3-D environment is critical for the induction of chondrogenesis, since it is not possible to fully induce the differentiation of MSCs along the chondrogenic lineage in a 2-D environment. For this reason, it was not until very recently that a reproducible system for the differentiation of MSCs into cartilage was developed (Johnstone *et al.,* 1998), much later than for osteogenic differentiation. In general, scaffolds are generated for the specific culture of a single cell type or else for stem cells to be directed specifically along one lineage. For example PLGA and PLLA are designed for the induction of bone formation (Yang *et al.,* 2001,2002), while alginates are designed for chondrogenesis (Wang *et al.,* 2003), as are collagen gels (Temenoff and Mikos, 2000). There are also several other scaffold materials specifically designed for the support of either osteogenesis or chondrogenesis as was discussed in chapter 1. This chapter aims to investigate the potential for the collagen GAG scaffold to support the chondrogenic differentiation of adult rat MSCs having already demonstrated its ability to sustain osteogenesis

Chondrogenic differentiation is a process that begins with aggregation of MSCs, cellular proliferation, differentiation, hypertrophy and eventually apoptosis (Shum *et al.,* 2002). Several cell-cell interactions and signalling cascades occur during this complex process. The role of the MAP kinase family in this differentiation process has been the subject of much research of late. The majority of this work has been on the mouse embryonal carcinoma cell line ATDC5 (Nakamura *et al.,* 1999) and on embryonic chick limb bud mesenchymal cells (Chang *et al.,* 1998) with little or no focus on adult MSCs. The findings of these studies have been somewhat varied, particularly in relation to ERK and its role in chondrogenesis. Studies have shown ERK to be necessary for chondrogenesis (Lee *et al.,* 2004, Murakami *et al.,* 2000) and also that ERK inhibits chondrogenesis (Oh *et al.,* 2000, Yoon *et al.,* 2000, Chang *et al.,* 1998). Clearly there is some confusion in the literature with regard to the precise role of ERK in the chondrogenic pathway. On the other hand, p38 has been consistently shown to be necessary for successful chondrogenesis in ATDC5 cells and in embryonic chick mesenchymal cells (Oh *et al.,* 2000, Yoon *et al.,* 2000). Hidaka *et al.* (2001) and Oh *et al.* (2003) have also

demonstrated a role for PI3K in the chondrogenesis in these cell types although there have also been some contrasting findings in this area (Fujita *et al.*, 2004) and the precise involvement of PI3K is not yet clear. The purpose of this chapter is to demonstrate the capability of a collagen GAG scaffold to support the chondrogenic differentiation of adult MSCs along the chondrogenic lineage and to assess the function of both ERK and p38 in this process. Carrying out this research in a primary cell culture will hopefully shed some light on this confusing signalling pathway.

5.2.1: In MSC seeded scaffolds, cellular migration was observed following 21 days culture in collagen GAG scaffolds

To determine the capability of the collagen GAG scaffold to support chondrogenic differentiation, it was necessary to demonstrate cell survival under control and chondroinductive factor treatment conditions. Figure 5.1 shows that, following 14 days culture in the absence (i) and presence (ii) of chondroinductive factors (6.8nM dexamethasone and 10ng/ml TGFβ), cell survival was observed but cells did not migrate past the periphery of the scaffold. By 21 days, cells had completely penetrated the scaffold in both untreated control scaffolds (iii) and chondroinductive factor-treated scaffolds (iv). Results are representative of 6 independent observations.

5.2.2 In MSC-seeded scaffolds, 14 days treatment with chondroinductive factors led to type II collagen expression

Figure 5.2 shows that, following 14 days culture in the presence of chondroinductive factors (6.8nM dexamethasone and 10ng/ml TGFβ), collagen type II expression was observed in the periphery of the scaffolds (ii) but not in the centre (iv) as evidenced by immunohistochemical staining for collagen II. No collagen II expression was observed in untreated control scaffolds (i and iii). Results are representative of 5 individual cell culture preparations. This demonstrates that the MSCs are differentiating along the chondrogenic lineage within the 14 day time period.

5.2.3: In MSC-seeded scaffolds, 21 days treatment with chondroinductive factors led to extensive type II collagen expression

Figure 5.3 demonstrates collagen type II expression both at the periphery of the (ii) and in the centre (iv) of the MSC seeded scaffold when treated with chondroinductive factors (6.8nM dexamethasone and 10ng/ml TGFβ) for a period of 21 days (ii and iv). Immunofluorescent analysis showed low levels of immunoreactivity for collagen II in untreated control samples (i and iii). Results are representative of 5 individual MSC culture preparations.

5.2.4: ERK is not involved in chondrogenic differentiation of MSCs

Figure 5.4 shows scaffolds seeded with MSCs for 21 days in the absence (i and iii) or presence (ii and iv) of chondroinductive factors (6.8nM dexamethasone and 10ng/ml TGFβ). Scaffolds were also treated with the selective ERK inhibitor U0126 (2μM, iii and iv). Under all conditions collagen type II expression was observed within cells in the scaffolds. This suggests that chondrogenic differentiation of adult rat MSCs in collagen GAG scaffolds does not involve ERK. Also, spontaneous chondrogenic differentiation occurred in the absence of chondroinductive factors (i and iii). As a positive control, a sample of rat neck was used to stain the trachea to ensure specific collagen type I staining (v). As can be seen, the surrounding tissue is negative for collagen II. Results are representative of 7 individual cell culture preparations.

5.2.5: p38 is not involved in chondrogenic differentiation of MSCs

Figure 5.5 shows scaffolds seeded with MSCs for 21 days in the absence (i and iii) or presence (ii and iv) of chondroinductive factors (6.8nM dexamethasone and 10ng/ml TGFβ). Scaffolds were also treated with the selective p38 inhibitor SB203580 (10μM, iii and iv). Under all conditions collagen type II expression was observed within cells in the scaffolds. p38 inhibition had no effect on collagen type II expression (iii and iv) suggesting no role for p38 in this chondrogenic event. Spontaneous chondrogenesis was again observed in seeded scaffolds cultured in the absence od chondroinductive factors (i and iii). As a negative control, a chondroinductive factor-treated scaffold was stained without the primary antibody. A sample of neck block was used as a positive control to stain the trachea and verify the activity of the primary antibody (vi). Results are representative of 7 individual cell culture preparations.

5.2.6: Phosphoisositide-3-kinase reduces collagen type II expression in MSC seeded scaffolds

Having observed no effect of either ERK or p38 inhibition on the chondrogenic differentiation of MSCs, I decided to investigate the role of phosphoinositide-3-kinase (PI3K) in this process. PI3K has previously been implicated in the chondrogenic differentiation of chick limb bud mesenchymal cells and the ATDC5 cell line (Hidaka

et al., 2001). In Figure 5.6, scaffolds were seeded with MSCs and cultured in the absence of chondroinductive factors for 7 days. Scaffolds were additionally cultured with the PI3K inhibitor LY294002 (50μM, Figure 5.6 ii), the p38 inhibitor SB203580 (10μM, Figure 5.6 iii), the ERK inhibitor U0126 (2μM, Figure 5.6 iv) or without any inhibitors (Figure 5.6 i). As was previously observed, spontaneous chondrogenesis took place under untreated control conditions (Figure 5.6 i). Neither ERK nor p38 inhibition had any effect on the expression of collagen II (Figures 5.6 iii and iv). In contrast, when PI3K activity was inhibited, expression of collagen II was reduced (Figure 5.6 ii) suggesting a role for PI3K in 3-D MSC chondrogenic differentiation. Figure 5.6 (v) demonstrates negative collagen II immunoreactivity in a scaffold with no cells and Figure 5.6 (vi) again illustrates positive collagen II immunoreactivity in a section of rat trachea. Figure 5.7 demonstrates the effects PI3K, p38 and ERK inhibition on MSC differentiation along the chondrogenic lineage when scaffolds were cultured in the presence of chondroinductive factors (6.8nM dexamethasone and 10ng/ml TGFβ). Figure 5.7 (i) demonstrates positive collagen II immunofluorescence in scaffolds cultured with chondroinductive factors alone. In Figures 5.7 (iii) and (iv), inhibition of p38 and ERK with SB203580 (10μM) and U0126 (2μM) respectively had no effect on collagen type II expression. However, inhibition of PI3K with LY294002 (50μM) caused a reduction on collagen type II expression as evidenced by immunofluorescent staining. In Figure 5.7 (v) a section of oesophagus adjacent to the trachea in a neck block sample shows no collagen II immunoreactivity and Figure 5.7 (vi) again shows positive staining in the trachea. Results are representative of 4 individual cell culture preparations.

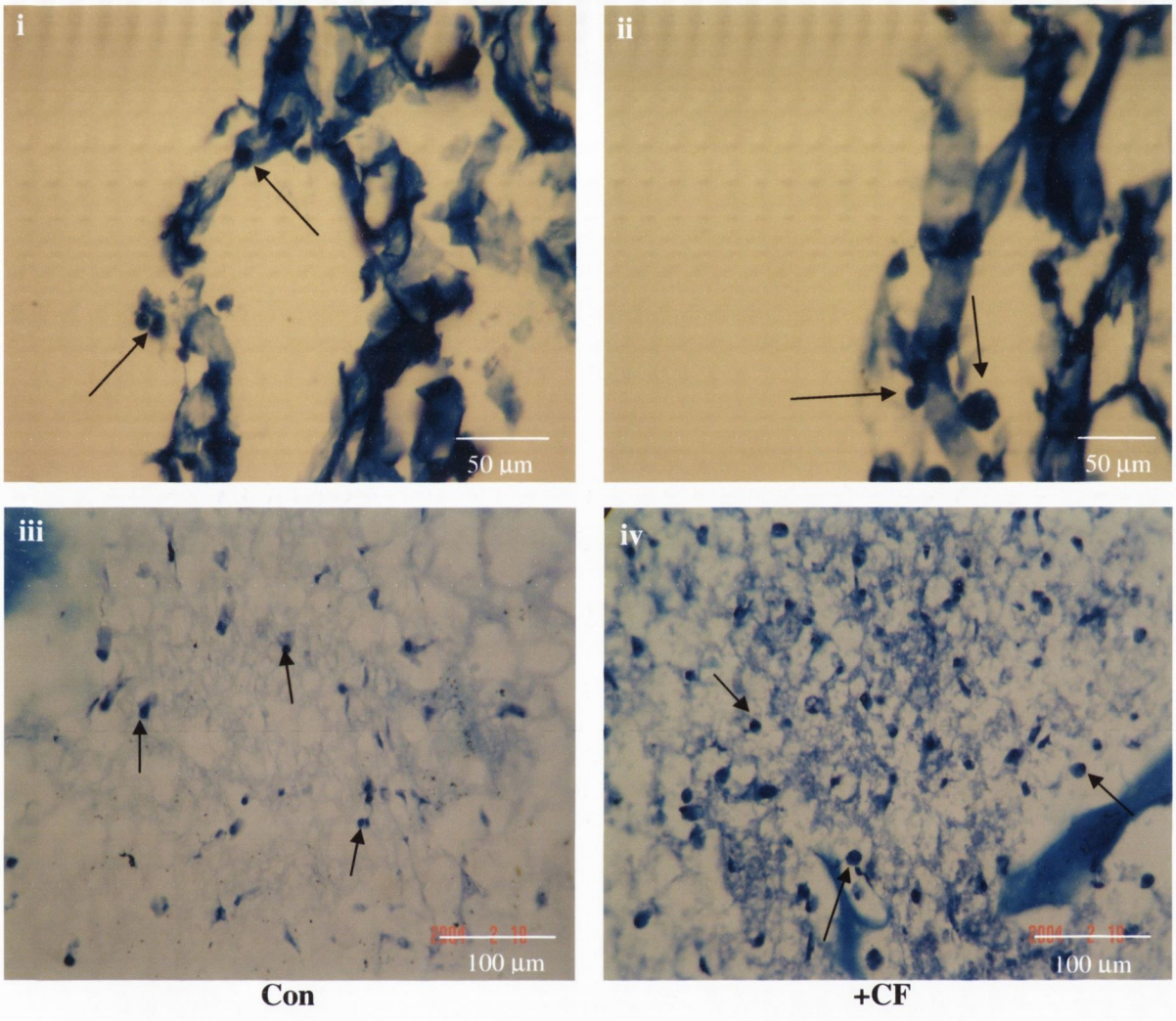


Figure 5.1: In MSC-seeded scaffolds no cellular migration was observed until 21 days

MSCs were seeded onto collagen GAG scaffolds for 14 days (i and ii) and 21 days (iii and iv) in the absence (i and iii) or presence (ii and iv) of chondroinductive factors (CF; dexamethasone, 6.8nM; TGFβ-1, 10ng/ml). Cells were stained using toluidine blue. At 14 days, MSCs did not move past the outer layer of the scaffold and penetrate the centre. By 21 days, complete scaffold penetration had occurred in both control and chondroinductive factor treated samples. Homogenous cellular distribution was observed throughout scaffolds. Results are representative of 6 individual cell culture preparations. Arrows indicate cells in the scaffold.

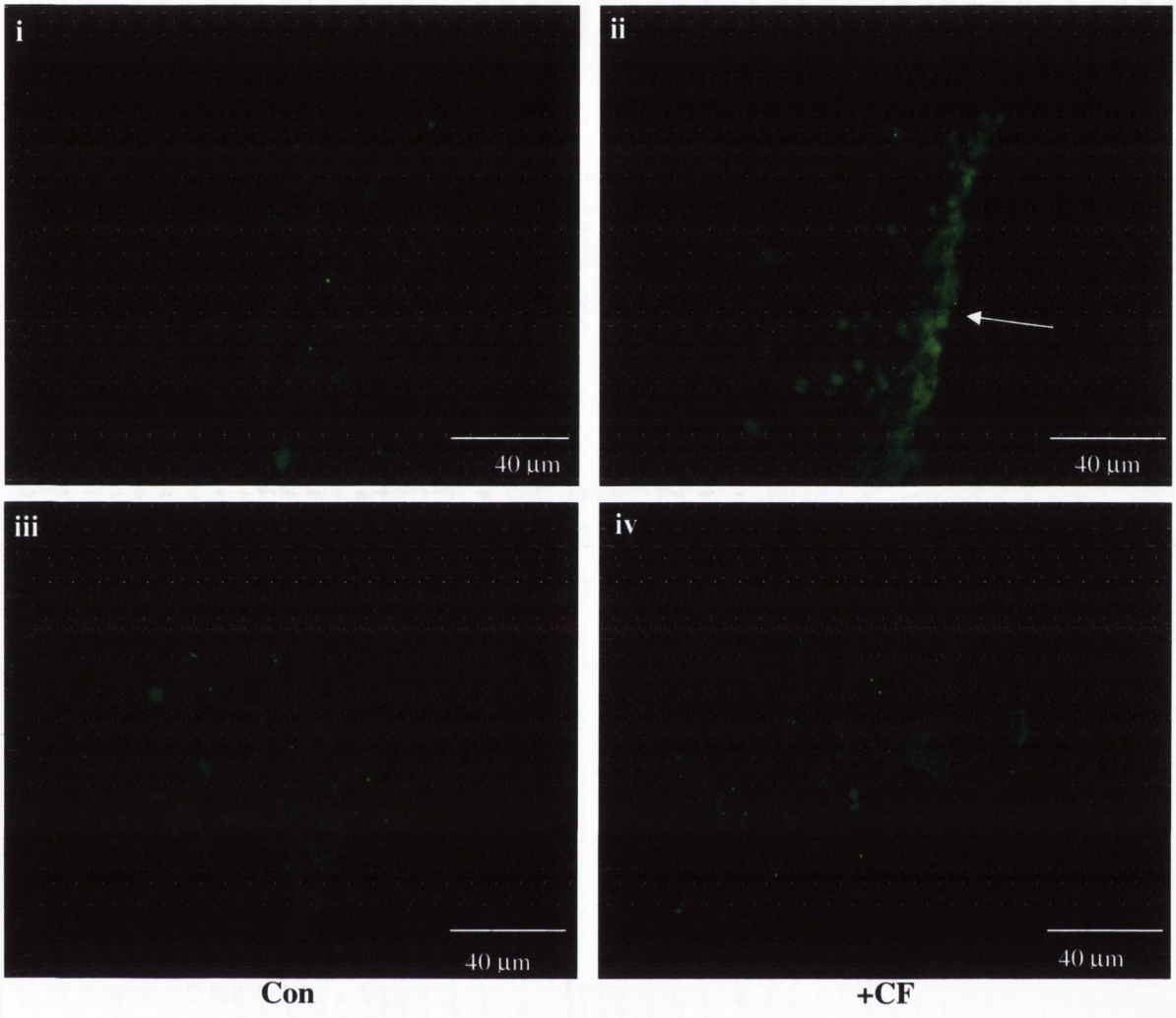


Figure 5.2: In MSC-seeded scaffolds, 14 days treatment with chondroinductive factors led to type II collagen expression

MSCs were seeded onto collagen GAG scaffolds for 14 days (n = 5) in the absence (i & iii) or presence (ii & iv) of chondroinductive factors (CF; dexamethasone, 6.8nM; TGFβ-1, 10ng/ml). Immunofluorescent showed weak immunoreactivity for collagen type II at the periphery of the treated scaffold (ii) but not at the center (iv). Arrows indicate collagen II immunoreactivity.

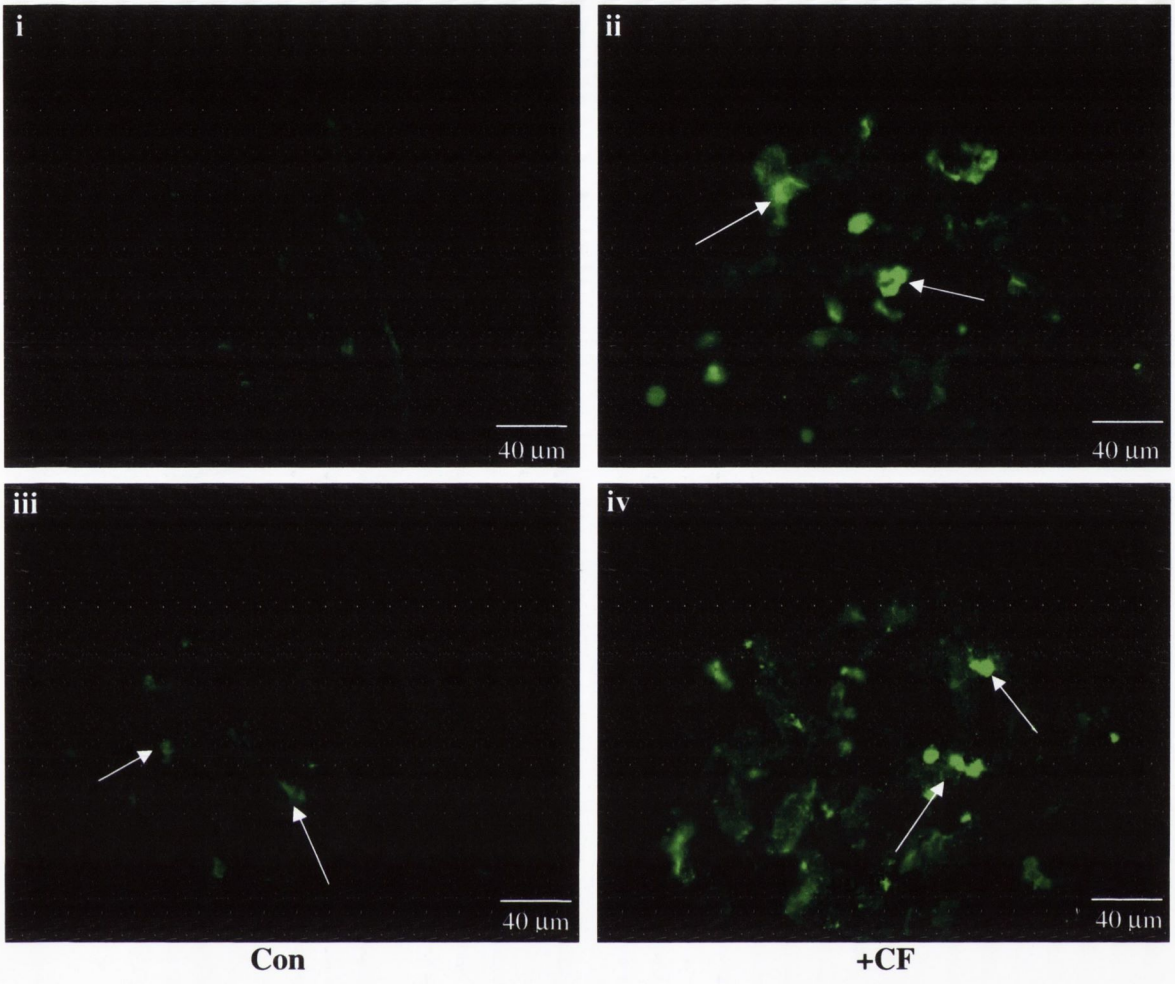


Figure 5.3: In MSC-seeded scaffolds, 21 days treatment with chondroinductive factors led to extensive type II collagen expression

MSCs were seeded onto collagen GAG scaffolds for 21 days (n = 5) in the absence (i & iii) or presence (ii & iv) of chondroinductive factors (CF; dexamethasone, 6.8nM; TGFβ-1, 10ng/ml). Immunofluorescent analysis showed immunoreactivity for collagen type II at both the periphery (ii) and center (iv) of the scaffold. There was some evidence of weakly positive staining cells in control samples. Arrows indicate collagen II immunoreactivity.

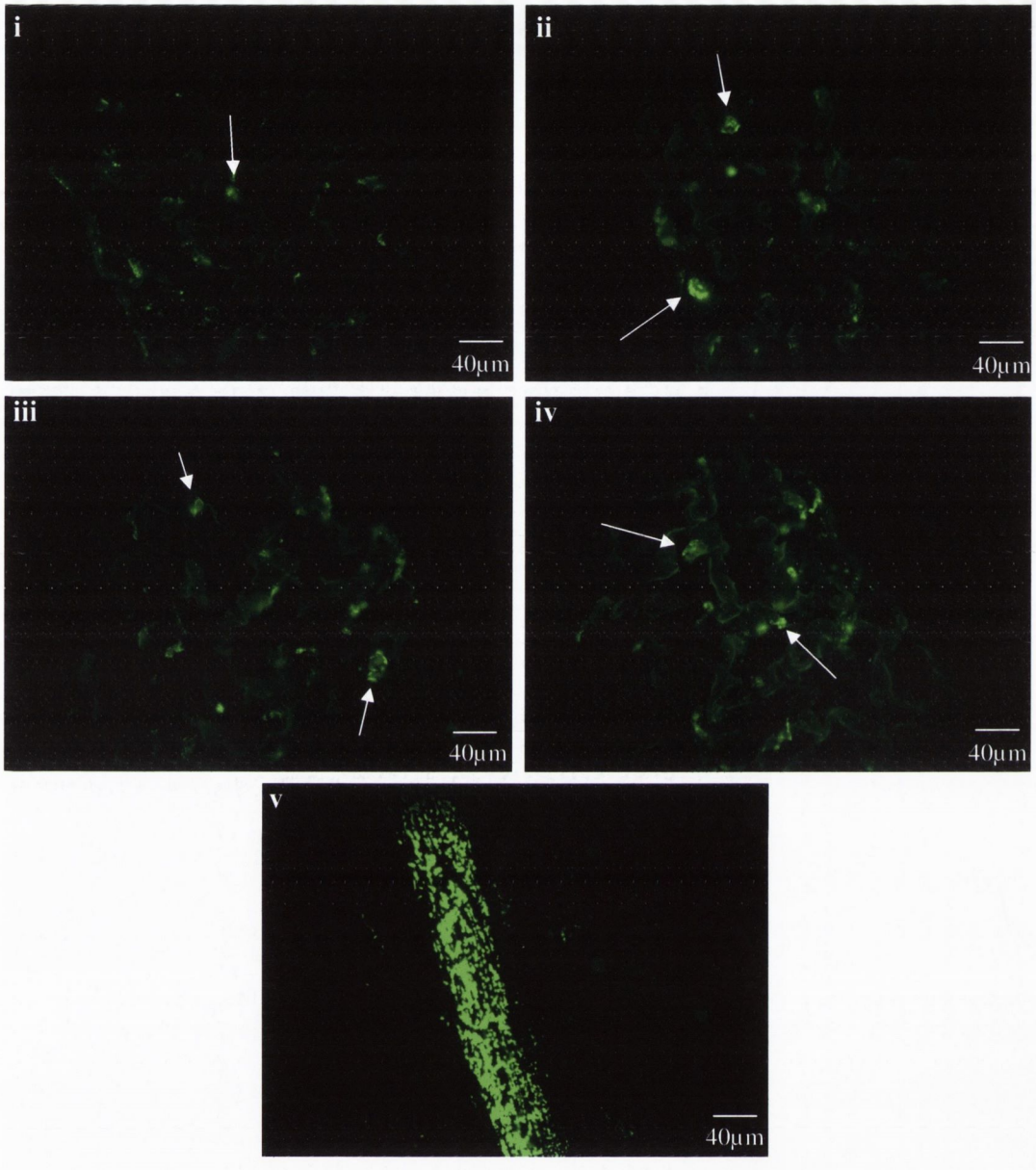


Figure 5.4: Inhibition of ERK activity did not have any effect on chondrogenesis

MSCs were seeded onto collagen GAG scaffolds for 21 days (n = 7) in the absence (i & iii) or presence (ii & iv) of chondroinductive factors (dexamethasone, 6.8nM; TGFβ-1, 10ng/ml). ERK activity was inhibited (iii and iv) using U0126 (2μM). Immunofluorescent analysis showed immunoreactivity for collagen type II in all samples, even in the absence of chondroinductive factors. A sample of rat trachea was used as a positive control to ensure collagen type II specific staining. Arrows indicate collagen II immunoreactivity.

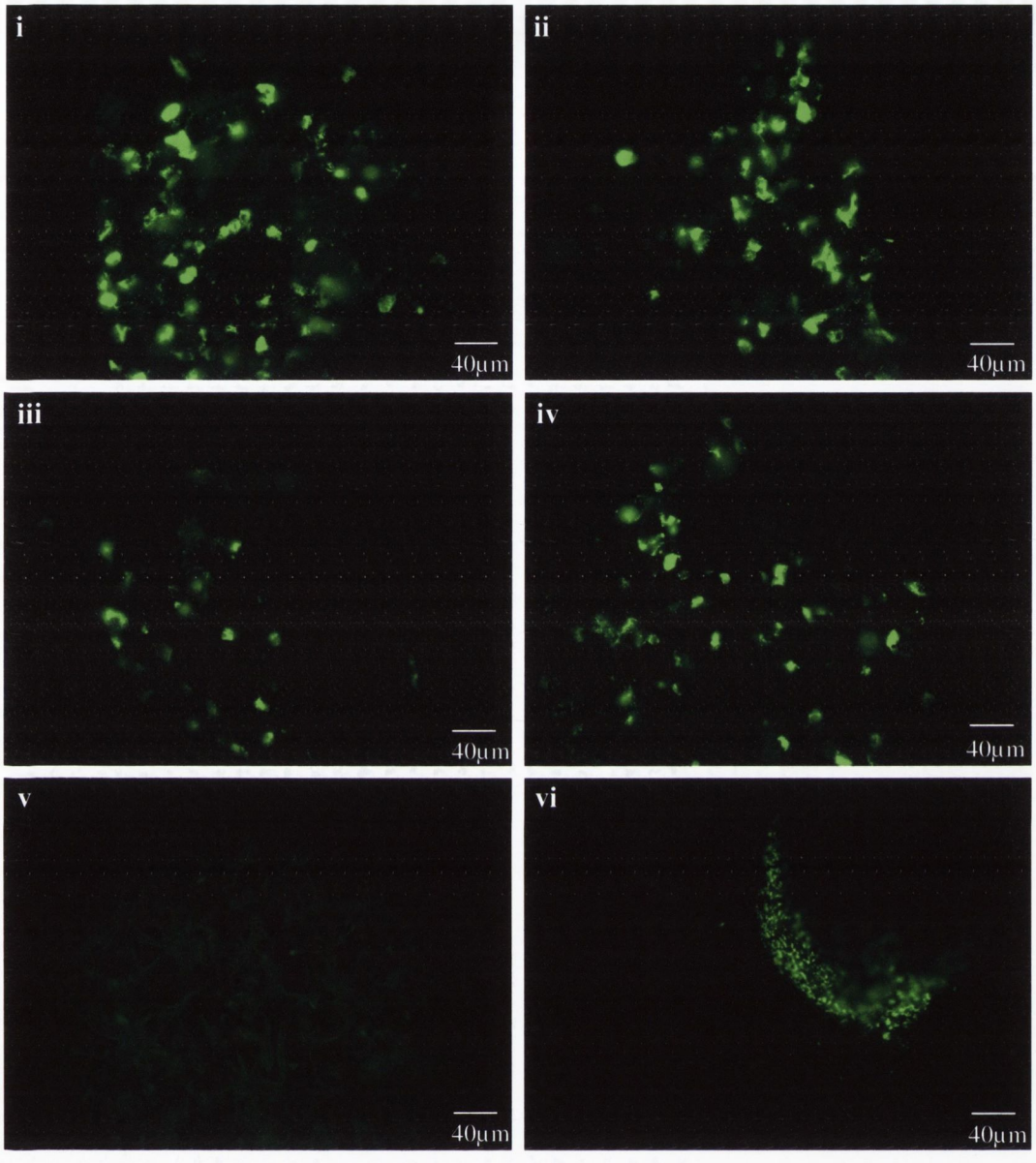


Figure 5.5: Inhibition of p38 activity did not have any effect on chondrogenesis

MSCs were seeded onto collagen GAG scaffolds for 21 days (n = 7) in the absence (i & iii) or presence (ii & iv) of chondroinductive factors (dexamethasone, 6.8nM; TGFβ-1, 10ng/ml). p38 activity was inhibited (iii and iv) using SB203580 (10μM). Immunofluorescent analysis showed immunoreactivity for collagen type II in all samples, even in the absence of chondroinductive factors. A chondroinductive factor-treated scaffold was stained without a primary antibody as a negative control (v) and a sample of rat trachea was used as a positive control to verify collagen type II specific staining (vi).

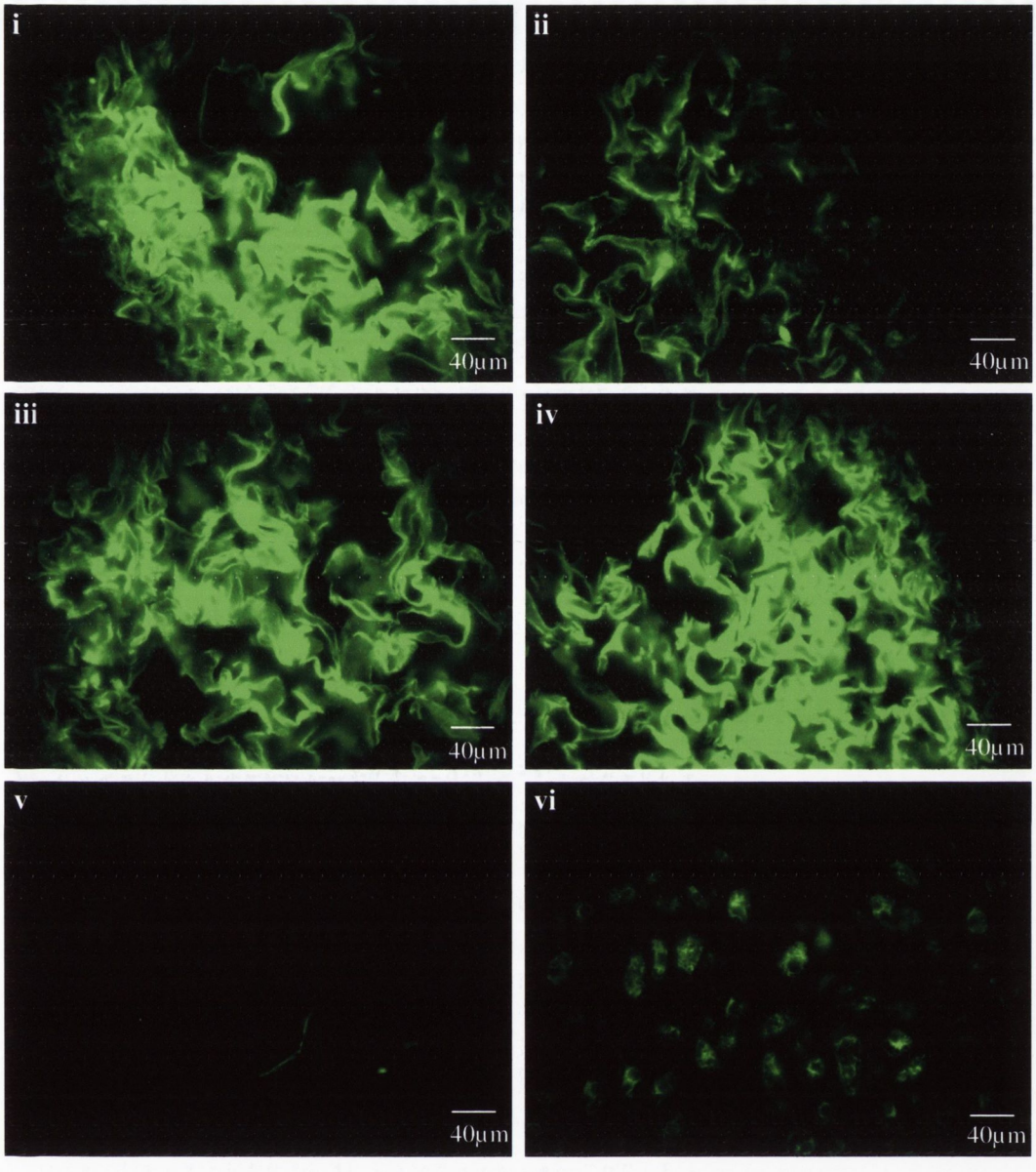


Figure 5.6: Spontaneous chondrogenesis was inhibited by the PI3 kinase inhibitor LY294002

MSCs were seeded onto collagen GAG scaffolds for 21 days ($n = 4$) in the absence (i-iv) of chondroinductive factors. Spontaneous chondrogenesis was observed in control scaffolds (i). Collagen II expression was reduced in scaffolds cultured in the presence of LY294002 but SB203580 (iii) and U0126 (iv) had no effect on collagen II expression. An empty scaffold was used as a negative control (v) and a section of trachea was used as a positive control (vi) to verify specific staining of collagen type II.

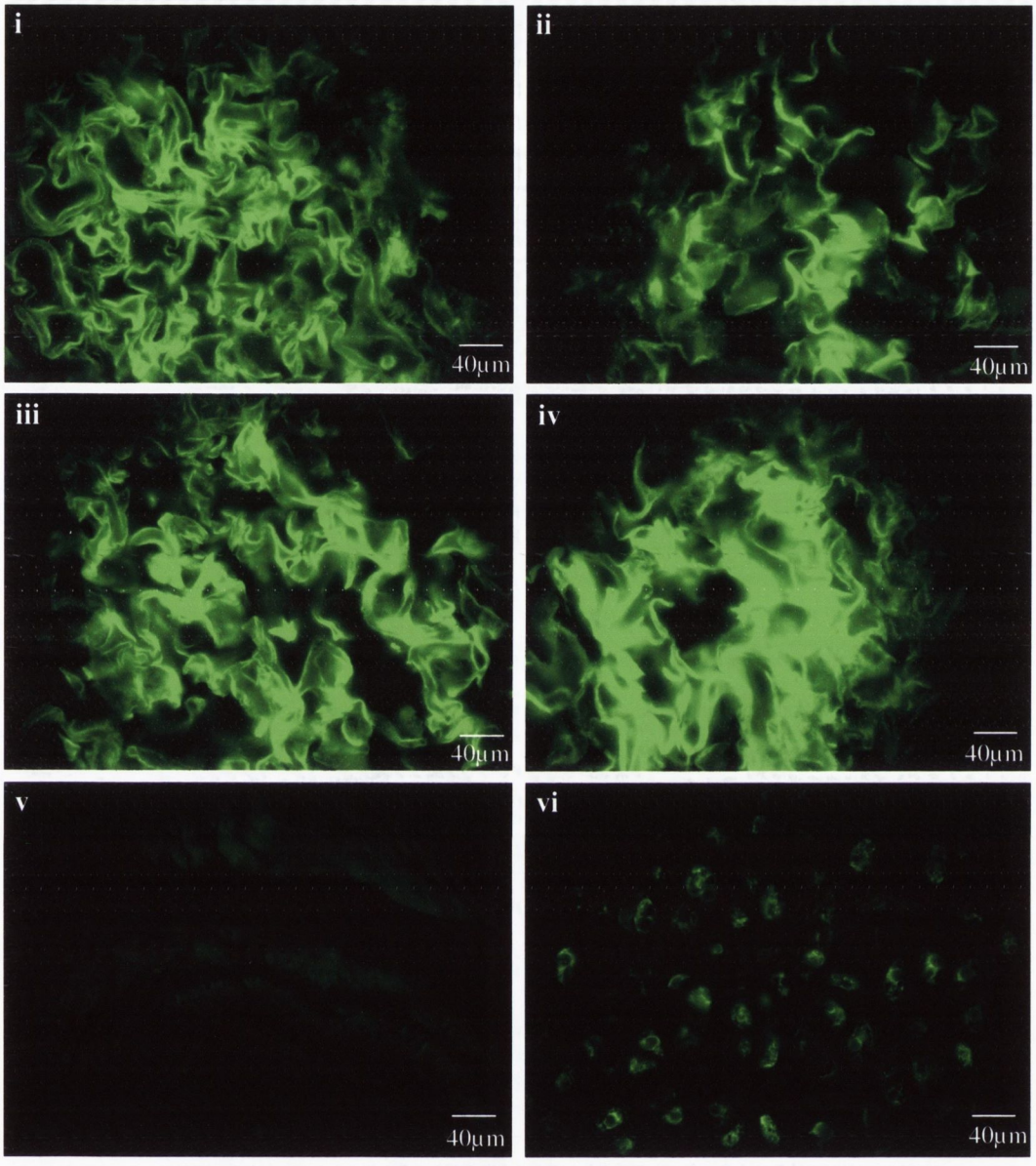


Figure 5.7: Chondrogenesis was inhibited by the PI3 kinase inhibitor LY294002 in 3-D

MSCs were seeded onto collagen GAG scaffolds for 21 days ($n = 4$) in the presence (i-iv) of chondroinductive factors (dexamethasone, 6.8nM; TGFβ-1, 10ng/ml). The inhibitors LY294002 (ii), SB203580 (iii) and U0126 (iv) were used to determine the role of PI3 kinase, p38 and ERK respectively in chondrogenesis. Collagen II expression was reduced in scaffolds cultured with chondroinductive factors and LY294002 (50µM, ii). A piece of oesophagus was used as a negative control (v) and a section of trachea was used as a positive control (vi) to verify specific staining of collagen type II.

5.3 Discussion

The aim of this chapter was to determine the suitability of a collagen GAG scaffold to support differentiation of MSCs down the chondrogenic lineage and to attempt to understand some of the signalling mechanisms behind the process. The results demonstrate the capability of the collagen GAG scaffold support chondrogenesis of adult MSCs. Interestingly the scaffold supported chondrogenesis even in the absence of chondroinductive factors. Toluidine staining showed that cells took up to 21 days to fully penetrate the scaffold in both control and chondroinductive factor-treated samples. In those scaffolds cultured in the presence of chondroinductive factors for 14 days, collagen type II staining was also localised at the outer regions of the scaffolds where the cells were located. This again shows that differentiation had taken place before migration. Neither ERK nor p38 appeared to be involved in the differentiation process. As a result of this finding, and a review of the available literature, it was decided to investigate the possible role of phosphoinositide 3-kinase (PI3K) in this process. Inhibition of PI3K led to the prevention of collagen type II expression after 7 days culture in collagen GAG both in untreated control and chondroinductive factor-treated scaffolds.

An unexpected result was the spontaneous differentiation of cells cultured in the absence of chondroinductive factors. Unfortunately the mechanism by which this occurred is not clear. Perhaps the presence of collagen and chondroitin-6-sulphate was enough to induce differentiation. Bosnakovski *et al.* (2004) also observed spontaneous chondrogenesis in bovine chondrocyte pellet cultures without any bioactive factors. They observed higher levels of type II collagen in pellets cultured without TGFβ-1 compared to those cultured in the presence of 10ng/ml TGFβ-1. Unfortunately, they were also unable to offer an explanation for this occurrence. They hypothesised that the close cellular contact in the pellet led to cytokine release and autocrine/paracrine activity. The authors suggest that the activity of TGFβ-1 is dependent on the timing and duration of exposure, sometimes leading to downregulation of collagen II expression. This might explain the lack of increase in collagen II expression under chondroinductive conditions. De Bari *et al.* (2001) also observed spontaneous chondrogenesis in human periosteum derived cells but only up to passage 2 and only in cells from donors under the age of 30. Fortier *et al.*, (1998) observed

chondrogenesis of equine MSCs in monolayer in the absence of growth factors too. *In vivo*, spontaneous chondrogenesis has been demonstrated Jung *et al.,* 2005). However, the effects of intrinsic factors could not be ruled out in that instance. Perhaps, to a certain extent, spontaneous chondrogenesis is a natural phenomenon. The 3-D environment of the collagen GAG scaffold could not be ruled out as the possible stimulus for the spontaneous differentiation of MSCs along the chondrogenic lineage. In an interesting study, Zanetti *et al.* (1984) demonstrated that disruption of the cytoskeletal elements of chick limb bud mesenchymal cells cultured in monolayer induced chondrogenesis. The authors observed that when cells were treated with cytochalisin D, a cytoskeleton disrupting agent, flattened cells took on a rounded appearance and started producing collagen type II. Perhaps the attachment of the cells to the struts of the scaffold induced a conformational change in the cells, inducing chondrogenic differentiation. Whatever the mechanism causing this process is, it appears to do so without ERK or p38 activity.

The finding that p38 was not involved in chondrogenic differentiation was a surprising one. Recent reports have implicated p38 as a downstream target of TGF-β1, BMP-2 and growth and differentiation factor-5 (GDF-5) in the chondrogenic differentiation of the mouse cell line ATDC5 (Nakamura *et al.,* 1999). The AP-2 transcription factor is a target of TGF-β1 induced chondrogenesis mediated by p38. p38 inhibits AP-2 DNA binding resulting in increased expression of the aggrecan gene (Tuli *et al.,* 2002) by preventing its transcriptional regression. The expected role of ERK was less certain. In 1998, Chang *et al.* showed that protein kinase C (PKC) inhibition prevented chondrogenesis in chick embryonic mesenchyme. Furthermore, the PKC-dependent regulation of chondrogenesis was found to be due to a PKC-induced inhibition of ERK signalling. The downstream consequences of this are a downregulation of integrin α5β1 and fibronectin to initiate the progression of the differentiation process beyond the initial aggregation stage. Other studies also observed a positive role for p38 and a negative one for ERK in chondrogenesis. In 2000, Oh *et al.* showed, that during chondrogenesis of chick limb bud mesenchymal cells, p38 phosphorylation was increased and ERK phosphorylation was decreased and inhibition of each prevented and enhanced chondrogenesis respectively. In 2001 they further demonstrated the necessity for p38 in chondrogenic differentiation with prevention of chondrogenesis by rapamycin, an immunosuppressant, with a

concomitant downregulation of p38 activity, with no observed effect on ERK activity. Furthermore, inhibition of chondrogenesis by epidermal growth factor (EGF) has been shown to involve upregulation of ERK phosphorylation and inhibition of p38 phosphorylation in chick limb bud mesenchymal cells (Yoon *et al.,* 2000). It also inhibited PKCα which presumably led to the activation of ERK. Lee *et al.* (2004) also found that p38 and PKCα upregulated chondrogenesis and ERK inhibited it in embryonic chick mesenchymal cells. In addition, they showed that some of the major proteins involved in chondrogenesis, collagen type II, matrilin-1, PAPS synthetase-2 and CA-II were also affected in the same manner, with expression increasing during ERK inhibition and decreasing when p38 or PKCα activity were inhibited. Bobick and Kulyk (2004) also showed that the MEK/ERK signalling pathway is a negative regulator of chondrogenesis, again in embryonic chick mesenchyme.

Despite all of these findings that ERK is a negative regulator of chondrogenesis, there have been many findings to the contrary. p38 is always shown to be positively involved but the role of ERK is less certain. Lee *et al.* (2004) demonstrated chondrogenesis in adult rabbit MSCs seeded in alginate beads. They observed complete downregulation of collagen II expression with MEK inhibition using U0126, suggestive of a role for ERK in TGF-β3 mediated chondrogenesis in rabbit MSCs. They detected phosphorylation of ERK 1/2 after an hour of treatment with TGF-β3 which peaked after 10 hours and was maintained for 14 days. In contrast, aggrecan expression was not affected by U0126 treatment. It had recently been demonstrated that aggrecan gene expression was regulated by cross-talk between Smad, ERK 1/2 and p38 (Watanabe *et al.,* 2001) in ATDC5 cells, which might suggest why aggrecan expression was unaffected. Murakami *et al.* (2000) demonstrated the requirement for MEK/ERK activation for the induction of sox9 by FGF signalling in mouse primary chondrocytes and C3H10T1/2 cells. FGF signalling has been shown to stimulate ERK and p38 phosphorylation in chondrogenic cells on more than one occasion (Rozenblatt-Rozen *et al.,* 2002, Shimoaka *et al.,* 2002, Legeai-Mallet *et al.,* 1998). Nakajima *et al.* (2004) have not only demonstrated the activation of both ERK and p38 in insulin driven chondrogenesis of ATDC5 cells, they have also shown that the necessary upregulation of these signalling molecules leads to activation of $p21^{Cip\text{-}1/SD\text{-}1/WAF\text{-}1}$. This cyclin-dependent kinase inhibitor was previously demonstrated to be essential for chondrogenesis in ATDC5 cells (Negishi

et al., 2001). Furthermore, Nakamura *et al.* (1999) showed activation of both ERK and p38 with chondrogenic stimulation of ATDC5 cells with GDF-5. They also demonstrated that JNK phosphorylation is not affected during chondrogenesis. Interestingly, most of the research that has found a positive effect of ERK on chondrogenesis has been in ATDC5 cells, and the negative work has been mainly in embryonic chick mesenchymal cells.

As discussed the role of ERK in chondrogenesis was somewhat uncertain and as such it was not surprising that its inhibition did not affect chondrogenesis in this study. However, it was expected that p38 inhibition would lead to the prevention of chondrogenic activity, but this was not observed. As a result, it was decided to determine whether PI3K was involved in the chondrogenic process in adult rat MSCs. According to Vincent *et al.* (2002), the best defined pathway of the prevention of apoptosis by IGF-IR signalling is via PI3K activation and downstream activation of Akt/PKB. With such an important role in chondrogenesis for IGF it makes sense that PI3K might also be involved. Loeser (2000) also demonstrate the importance of IGF in chondrocyte survival. Some previous studies suggested a possible role for PI3K in the chondrogenesis of chick limb bud mesenchyme and ATDC5 cells. In 2001, Hidaka *et al.* demonstrated that PI3K, and its downstream target Akt, were involved in insulin induced chondrogenesis in the ATDC5 cell line. Oh *et al.* (2003) subsequently demonstrated that PI3K was required for the activation of PKC and p38 and the subsequent inhibition of ERK in chick limb bud mesenchymal cells, having previously shown that PKC is necessary for chondrogenesis (Oh *et al.*, 2000) and that activated PKC inhibits ERK activity (Oh *et al.*, 2001). Interestingly, Fujita *et al.*, (2004) found that dexamethasone inhibits insulin induced chondrogenesis in ATDC5 cells by preventing PI3K-Akt signalling and DNA binding of Runx 2/cbfα-1. Runx1/cbfα-2, and runx2/cbfα-1 to a lesser extent, are both highly expressed during chondrogenesis (Wang *et al.*, 2005). Fujita *et al.* observed that insulin increased ERK phosphorylation but dexamethasone didn't affect its activity. PI3K was also shown to be required for BMP induced expression of early osteoblast genes in human MSCs (Osyczka and Leboy, 2005). Clearly, these studies suggested the need for further study. In this chapter, it was shown that, following seven days culture with the PI3K inhibitor LY294002, chondrogenesis was prevented in both untreated control and

chondroinductive factor-treated scaffolds. Further work needs to be carried out to identify the downstream targets of PI3K-Akt in this system.

The field of tissue engineering is advancing at an amazing rate. In 1998, Johnstone *et al.* first described a reproducible method for the differentiation of MSCs into cartilage. Since then many studies have focused on different carrier systems (Donati *et al.,* 2005) and growth factor combinations (Zhou *et al.,* 2005, Indrawattana *et al.,* 2004). Several new approaches to the issue of cartilage tissue engineering are also underway. Palmer *et al.* (2005) have demonstrated gene induced chondrogenesis of adult MSCs using adenoviral vectors encoding chondroinductive factors TGF β-3 BMP-2 and IGF-1. The use of serum free media is much more common (Yates *et al.,* 2005, Fitzsimmons *et al.,* 2004, Mandl *et al.,* 2002). Dennis *et al.* (2004) painted cells with antibodies to matrix molecules in order to promote adherence of stem cells to a cartilage injury site. Direct intra-articular injection of FGF-18 has been shown to stimulate repair of damaged cartilage (Moore *et al.,* 2005). Shirasawa *et al.,*(2005) demonstrated that synovium derived MSCs have a greater chondrogenesis potential than bone marrow-derived MSCs and Yokoyama *et al.,* (2005) have also demonstrated the potential for synovium derived MSCs in 3-D chondrogenic differentiation.

Despite all of these new techniques in use, the mechanisms behind the chondrogenic differentiation process remain largely unknown. What has been examined has largely been contradicted by other studies using slightly different growth factor cocktails, culture periods or cell types. Stanton *et al.* (2003) state, that while not all published data agree on the roles of the MAPK family members in chondrogenesis, particularly ERK, several parameters have to be taken into account including the nature, intensity and duration of upstream signals and crosstalk with other signalling pathways. They also mention that nearly all of the current research has made use of pharmacological inhibitors and there is a need to complement these results with genetic strategies such as targeted gene activiation and siRNA technology, to verify the results obtained at the protein level down at the genetic level. Although it is known that p38 appears to play a critical role in chondrogenesis, its upstream activators and downstream substrates have yet to be conclusively identified. For example, does p38 lead to activation of sox9 or either of the Runx genes? Much work has yet to be done in the

elucidation of the part the MAP kinase family, protein kinases A, B and C and PI3K have to play in chondrogenic differentiation.

The results from this chapter demonstrate the truly biocompatible nature of the collagen GAG scaffold. Not only was chondrogenesis observed in chondroinductive factor-treated samples, but it was also seen in untreated control scaffolds. This is the first scaffold developed that has been shown to support both osteogenesis and chondrogenesis of adult MSCs. While neither ERK nor p38 were involved in this process, PI3K activity was shown to be necessary for the process. Future work will involve analysis of the activity of these three signalling molecules using phospho-specific antibodies and confocal microscopy.

Chapter 6

Investigation of the effects of uniaxial tension on the osteogenic differentiation capacity of MSCs

6.1 Introduction

There are many stimuli to which cells of all phenotypes will respond, including chemical, mechanical and magnetic stimulation (Prina-Mello *et al.,* 2005, Jagodzinski *et al.,* 2004, Aaron *et al.,* 2002, Tsuchida *et al.,* 2003, Herbertson and Aubin, 1997, Cassiede *et al.,* 1996). Since the introduction of Wolff's "Law of Bone remodelling" in 1892, it has been repeatedly shown that bone responds to mechanical stimuli by remodelling itself to cope most efficiently with the strains imposed upon it. Several studies have shown that the internal structure of a piece of bone will reflect the strongest stresses to which it was subjected (Jee and Li, 1990, Rubin *et al.,* 1990, Bertram and Swartz 1991). Additionally it has been demonstrated that disuse will lead to bone wasting and osteoporosis (Weinreb *et al.,* 1989, Van der Wiel *et al.,* 1991). Smith-Adaline *et al.* (2004) have clearly demonstrated the efficacy of strain imposition during fracture repair in the rat model by mechanically loading a fractured rat femur using external fixators. Obviously, some strain sensing cells within the bone tissue play a vital role in bone maintentence and repair. While it is still not known what bone cells are responsible for strain responses that lead to bone remodelling, it is quite likely the osteocyte (Burger and Klein-Nulend, 1999). This cell is involved in bone maintenance and is evenly spread through all bone tissues. However, no matter which bone cell is involved in strain sensing, the question is; does the progenitor cell from which these cells are derived also possess the same strain sensing capabilities? The use of bioreactors in the field of bioengineering is helping to answer this question. Bioreactors alter the physical, chemical and thermal environment in which cells are cultured in order to achieve a certain goal. That goal is usually one of two things; to increase cell numbers or to alter the phenotype of the cell. Recently the use of bioreactors in stem cell biology has yielded promising results, helping to scale up the quantities of haemopoietic and neural stem cells (Cabrita *et al.,* 2003, Sen *et al.,* 2002), both notoriously difficult cells to exploit. In the field of bone tissue engineering, bioreactors are used to provide a mechanical stimulus to cells, generally osteoblasts, to increase the rate of mineralisation *in vitro*. The other factors, pH, temperature and gas supply, are usually controlled by an incubator into which the bioreactor is placed. As described by Pei *et al.* (2002), bioreactors can also be useful in improving cell seeding into 3-D scaffolds.

Numerous studies have been carried out on osteoblasts to characterise their responses to different types of loading (Tang *et al.,* 2004, Kaspar *et al.,* 2002, Klein-Nulend *et al.,* 1999) However, with a few exceptions (Jagodzinski *et al.,* 2004, Simmons *et al.,* 2003) there are practically no reports on the effects of mechanical strain on MSCs. With regard to research into the effects of mechanical strain on the responses of osteoblasts, the results depend on the strain type, magnitude, frequency and time course (Harter *et al.,* 1995, Buckley *et al.,* 1990, Hasegawa *et al.,* 1985) and this unfortunately makes inter-study comparisons very difficult. Strain magnitudes range from physiological strains of 500με (Kaspar *et al.,* 2002, Stanford *et al.,* 2000), up to 20% elongation, as is the case in the study by Danciu *et al.* (2004), which investigated the effects of strain on gingival fibroblasts. 20% elongation could correspond to up to and above 20,000με depending on the original length of the substrate. Strain rates used in these studies vary from 0.1Hz to 20Hz (Stanford *et al.,* 2000) and the loading regimes applied include, hydrostatic pressure (Stanford *et al.,* 1995), biaxial stretching (Neidlinger-Wilke *et al.,* 1994), bending resulting in uniaxial stretching (Kaspar *et al.,* 2002) and fluid shear stress (Klein-Nulend *et al.,* 1995). While all of these studies analyse different osteogenic markers and report different results, there is a general consensus within the literature, that bone cells will respond positively to a mechanical stimulus. Therefore, these cells obviously sense mechanical strain. Suggested possible mechanisms by which this mechano-reception occurs include stretch-activated ion channels, membrane tension, strain related potentials and the primary cilium (Jones *et al.,* 1995). The lipid bilayer and mechanosensitive ion channels embedded within it have also recently been shown to be actively involved in mechanosensation (Kung, 2005, Lin and Corey, 2005, Pederson *et al.,* 2005, Hamill and Martinac, 2001).

If any of these cellular components are responsible for the mechanotransduction that leads to cellular responses in bone cells, it is equally possible that the same response could occur in MSCs. While little is known of the effects of mechanical strain on MSCs, two recent studies have shed some light on the issue. In 2003, Simmons *et al.* reported that 3% cyclic strain at a rate of 0.25Hz enhanced matrix mineralisation by adult human MSCs. In that study, MSCs were cultured in the presence of osteogenic media, with or without mechanical strain. There is a possibility that the mechanical strain simply augmented a response that was already underway as a result of the osteoinductive medium. Simmons *et al.* also showed that ERK was involved in the

strain related increase in mineralisation. Furthermore, p38 was found to play an inhibitory role in the osteogenic process in strained cells, but did not attenuate the response when cells were cultured with osteogenic factors without strain. While there was an increase in mineralisation as a result of mechanical strain, it should be noted that the levels of mineralisation in static cultures were very low for 16 days in culture with osteoinductive factors. In the second study by Jagodzinski *et al.* (2004), it was reported that a stretching regime of 8% at 1Hz for 6 hours a day for 3 days is a stronger differentiation factor than dexamethasone. Unfortunately, very large errors in several experiments made interpretation of results difficult. In addition, in that study a very high concentration of dexamethasone (2.55μM) was used, yet the effect of dexamethasone on the expression of osteocalcin, collagen type I and cbfα-1 was negligible. Indeed, there were no significant differences in the expression of these markers in samples cultured with and without dexamethasone. Since this is such an unexpected result, it is difficult to say that stretching is a stronger stimulus than dexamethasone for osteogenic differentiation of adult MSCs. Clearly there is a need for further research into the possibility of using mechanical strain to induce osteogenic differentiation of adult MSCs.

To that end, this study aims to investigate the osteoinductive potential of mechanical strain alone, without the use of osteoinductive factors. One benefit of using mechanical strain in bone tissue engineering would be the replacement of dexamethasone and other chemicals with an appropriate strain to induce a similar or better response. Not only is dexamethasone a synthetic glucocorticoid, it is also used at non-physiological concentrations for the induction of osteogenesis. This also applies to the TGF-β superfamily members. The generation of viable constructs for human implantation requires, not only a structurally sound product, but also one devoid of pharmacological complications and side effects. This study will evaluate the osteogenic potential of adult MSCs under a variety of strain parameters with a view to using this knowledge in the 3 dimensional setting of the collagen GAG scaffold.

6.2.1: A uniaxial tension rig is used for mechanical stimulation of MSCs

Figure 6.1 (i and ii) illustrate the uniaxial tension device designed in the Trinity Centre for Bioengineering by Matteo Moretti, Adriele Prina-Mello and Alan Reid (Moretti *et al.,* 2004). This device was designed to hold a cell-seeded silicone strip between 40mm and 60mm in length. A motor driven cam cyclically stretches the strip at a percentage of the original length of the strip up to 10% depending on the cam size. The cycle frequency was controllable, by altering the current being supplied to the motor, over a wide range (0.1Hz-10Hz). The strip was submerged in a petri dish full of medium. The design ensured that only a small amount of medium was covering the strip to prevent any turbulent shear stress from influencing the results.

6.2.2: Cells adhere to silicone strips following two days in culture

Figure 6.2 shows that, following two days in culture on silicone strips MSCs were observed to adhere and spread processes, as occurs on normal plastic culture dishes. 125,000 cells were seeded onto each strip and allowed to adhere to the silicone for a period of 30 min before the culture dish was flooded with medium. Cells were observed to form a confluent layer within 48 hours of plating.

6.2.3: 2.5% strain at 0.17 Hz for 3 days does not lead to significant apoptosis

Figure 6.3 shows that, cyclic mechanical strain of 2.5% of the original length of the seeded silicone strip at 0.17 Hz did not lead to significant cell death by apoptosis, as determined by TUNEL staining, compared to unstrained controls. Results are expressed as a mean ± S.E.M. for 5 individual cell culture preparations. While there was a minor increase in apoptotic cell numbers, from 5.41 ± 1.68% in controls to 8.562 ± , 2.08% in strained cells, this did not reach significant levels (Mean ± SEM; p=0.0738, students paired *t*-test). Representative photomicrographs illustrate cells under control (Figure 6.3B i) and strained (Figure 6.3B ii) conditions.

6.2.4 5% strain at 0.17 Hz for 3 days does not lead to significant apoptosis

Figure 6.4 shows that, cyclic mechanical strain of 5% of the original length of the seeded silicone strip at 0.17 Hz did not lead to significant cell death by apoptosis, as determined by TUNEL staining, compared to unstrained controls. Apoptosis occurring in unstrained control cells was 7.87 ± 1.62% and was 5.51 ± 1.01% in strained cells (Mean ± SEM; p=0.2787, students paired *t*-test). Representative photomicrographs illustrate cells under control (Figure 6.4B i) and strained (Figure 6.4B ii) conditions.

6.2.5: Cyclic strain at 10% and 0.17Hz for 3 days led to significant cellular apoptosis

Despite the preliminary result from the pilot study, Figure 6.5 demonstrates that, cyclic mechanical strain of 10% of the original length of the seeded silicone strip at 0.17 Hz led to significant cell death by apoptosis as evidenced by TUNEL staining. While apoptosis occurred in 11.76 ± 8.06% of unstrained control cells, 47.09 ± 13.9% of strained cells were apoptotic. (Mean ± SEM; P=0.035, students paired *t*-test). Results are expressed as a mean ± S.E.M. for 5 individual cell culture preparations. Representative photomicrographs illustrate cells under control (Figure 6.5B i) and strained conditions (Figure 6.5B ii). This result demonstrates that there is an upper limit to the level of strain imposable on MSCs *in vitro*.

6.2.6: Collagen I but not osteocalcin was evident in strained cells following 2.5% strain at a frequency of 0.17Hz for 3 days

Figure 6.6 demonstrates that, following 3 days strain at 2.5% and a frequency of 0.17Hz, collagen type I expression was observed in cuboidal shaped cells on strained silicone strips (ii) in the absence of osteoinductive factors, but not in unstrained control samples (i). Osteocalcin expression was not observed in either unstrained control (Figure 6.6 iii) or strained samples (Figure 6.6 iv). Results are representative of 5 individual cell culture preparations for the analysis of collagen I production and for 1 cell culture preparation for the analysis of osteocalcin expression.

6.2.7: Collagen I but not osteocalcin was evident in strained cells following 5% strain at a frequency of 0.17Hz for 3 days

Figure 6.7 shows that, following 3 days strain at 5% and a frequency of 0.17Hz, collagen type I was again observed in cuboidal shaped cells (ii) but not in control unstrained samples (i). Osteocalcin expression was not observed in either control (Figure 6.7 iii) or strained samples (Figure 6.7 iv). Results are representative of 6 individual cell culture preparations for the analysis of both collagen I and osteocalcin expression.

6.2.8: Collagen I but not osteocalcin was evident in strained cells following 10% strain at a frequency of 0.17Hz for 3 days

Although almost 50% of strained cells were undergoing apoptosis, collagen type I was still evident in strained cells (Figure 6.8 ii) but not in unstrained control samples (Figure 6.8 i), following 3 days strain at 10% and a frequency of 0.17Hz,. Osteocalcin expression was not observed in either control (Figure 6.8 iii) or strained samples (Figure 6.8 iv). Results are representative of 6 individual cell culture preparations for the analysis of both collagen I and osteocalcin expression.

6.2.9: ERK activity was not observed following 3 days stretching at 2.5% and 0.17Hz

Figure 6.9 shows that, following 3 days strain at 2.5% and a frequency of 0.17Hz, no ERK activity was observed in strained (i) or unstrained control samples (i) as evidenced by immunofluorescent analysis for phosphorylated ERK expression. This suggests that if ERK is involved in the early phase of strain induced osteogenic differentiation, it is activated at an earlier timepoint. Results are representative of 3 individual cell culture preparations.

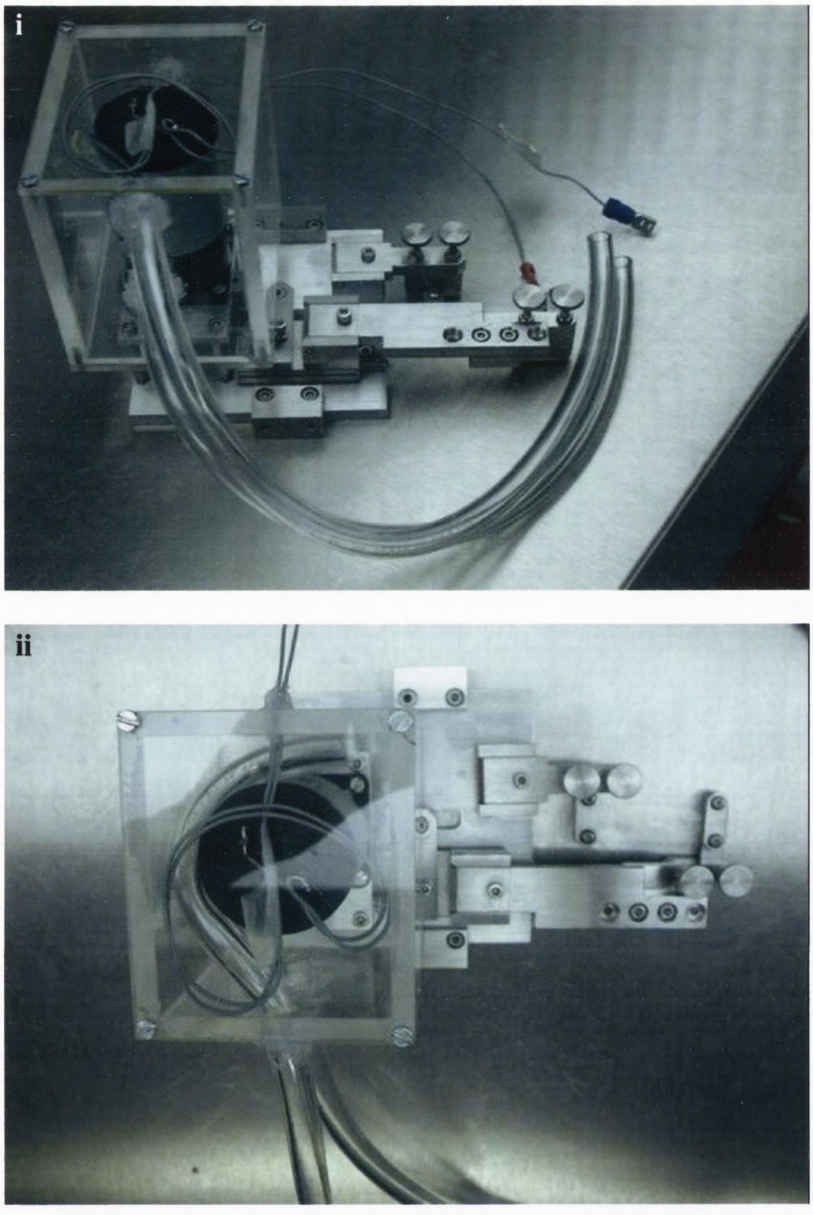


Figure 6.1: Uniaxial tension rig

Figure 6.1 shows the uniaxial tension rig designed in the Trinity Centre for Bioengineering in oblique (i) and plan (ii) views. A motor driven cam cyclically stretched a clamped silicone strip up to 10% its original length. The entire device was kept in the incubator under normal cell culture conditions.

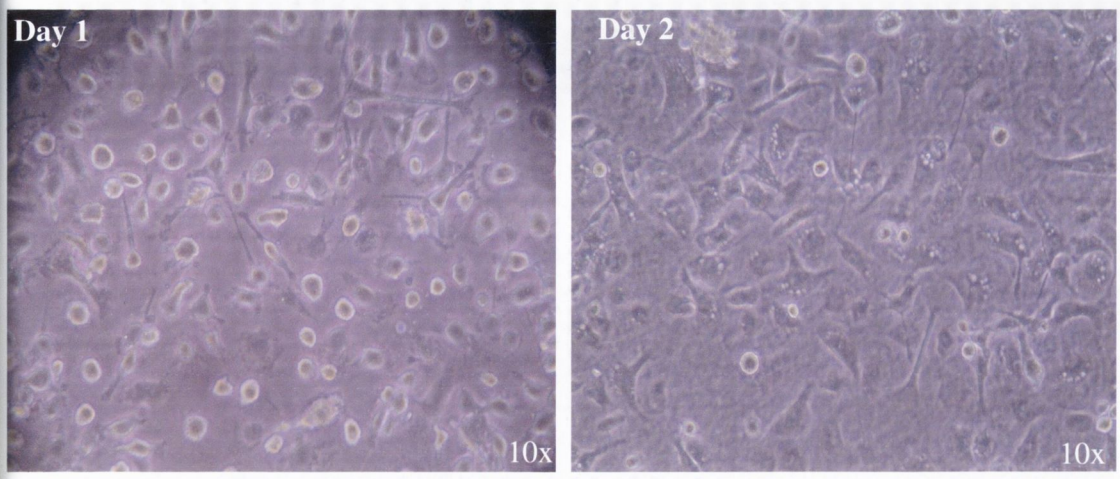


Figure 6.2: Cells adhere to silicone strips following two days in culture

Following two days in culture on silicone strips MSCs were observed to adhere to the silicone and spread processes as occurs on normal plastic culture dishes. Results are representative of 6 individual cell culture preparations.

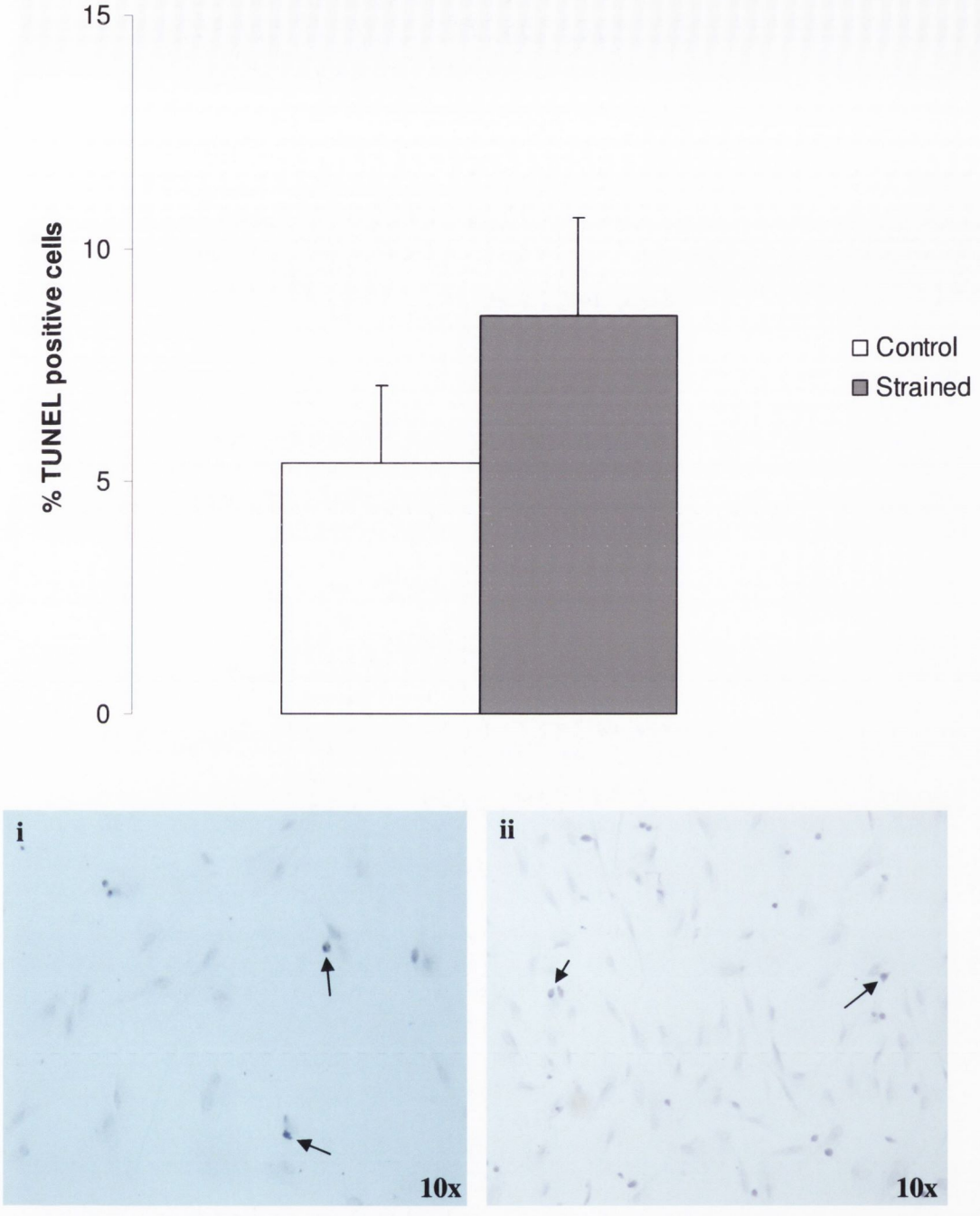


Figure 6.3: 2.5% strain at 0.17 Hz for 3 days does not induce MSC apoptosis

A; Cyclic mechanical strain of 2.5% of the original length of the seeded silicone strip at 0.17 Hz did not lead to significant cell death by apoptosis as determined by TUNEL staining compared to unstrained controls. Results are expressed as a mean ± S.E.M. for 5 individual cell culture preperations. **B;** Representative photomicrographs illustrate cells under control (i) and strained (ii) conditions. Arrows indicate TUNEL positive cells.

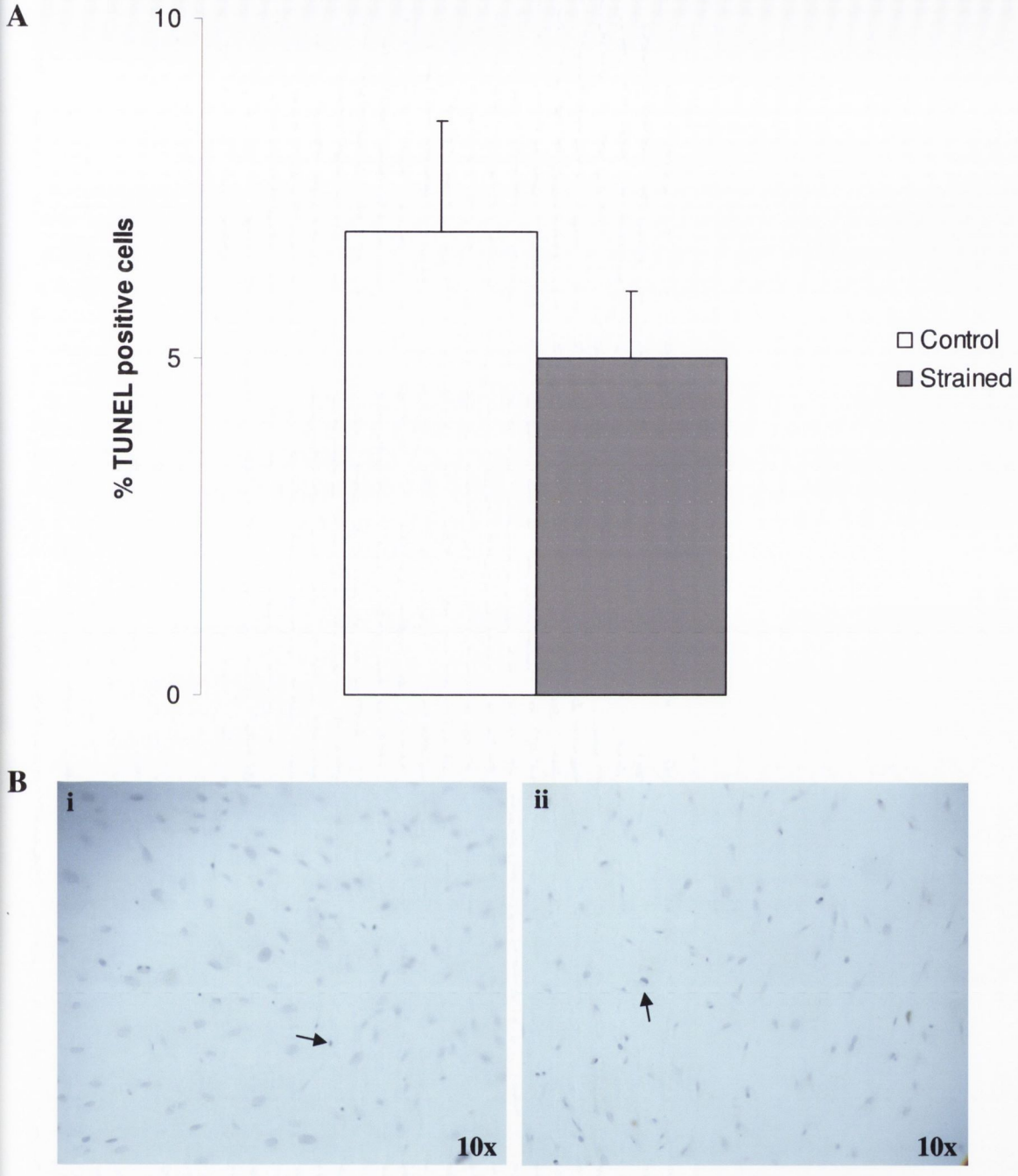


Figure 6.4: 5% strain at 0.17 Hz for 3 days does not induce MSC apoptosis

A; Cyclic mechanical strain of 5% of the original length of the seeded silicone strip at 0.17 Hz did not lead to significant cell death by apoptosis as determined by TUNEL staining compared to unstrained controls. Results are expressed as a mean ± S.E.M. for 5 individual cell culture preparations. **B;** Representative photomicrographs illustrate cells under control (i) and strained (ii) conditions. Arrows indicate TUNEL positive cells.

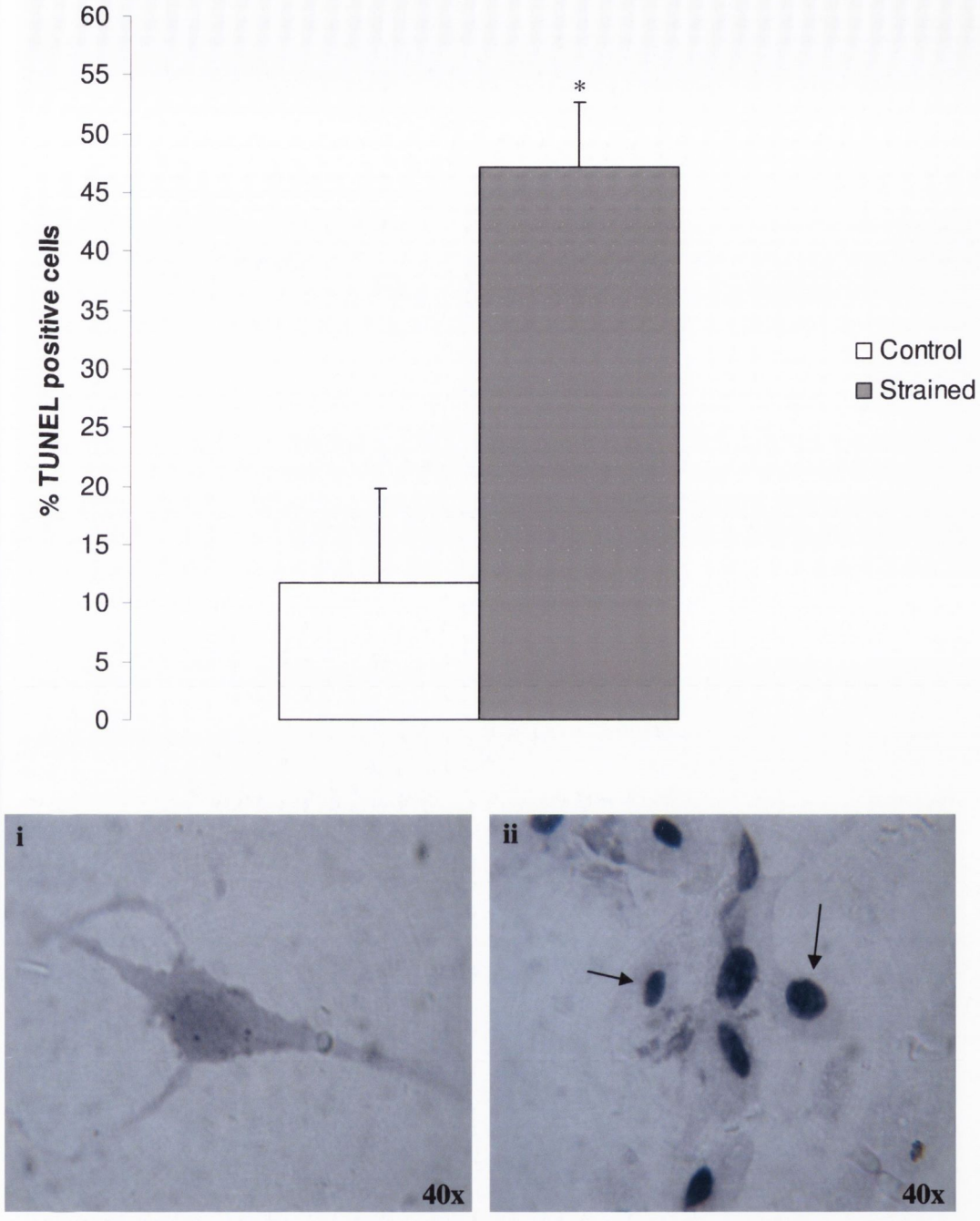


Figure 6.5: Cyclic strain at 10% and 0.17Hz for 3 days led to MSC apoptosis

A; Cyclic mechanical strain of 10% of the original length of the seeded silicone strip at 0.17 Hz led to significant cell death by apoptosis as determined by TUNEL staining compared to unstrained controls. Results are expressed as a mean ± S.E.M. for 5 individual cell culture preparations ($P<0.05$). **B;** Representative photomicrographs illustrate cells under control (i) and strained (ii) conditions. Arrows indicate TUNEL positive cells.

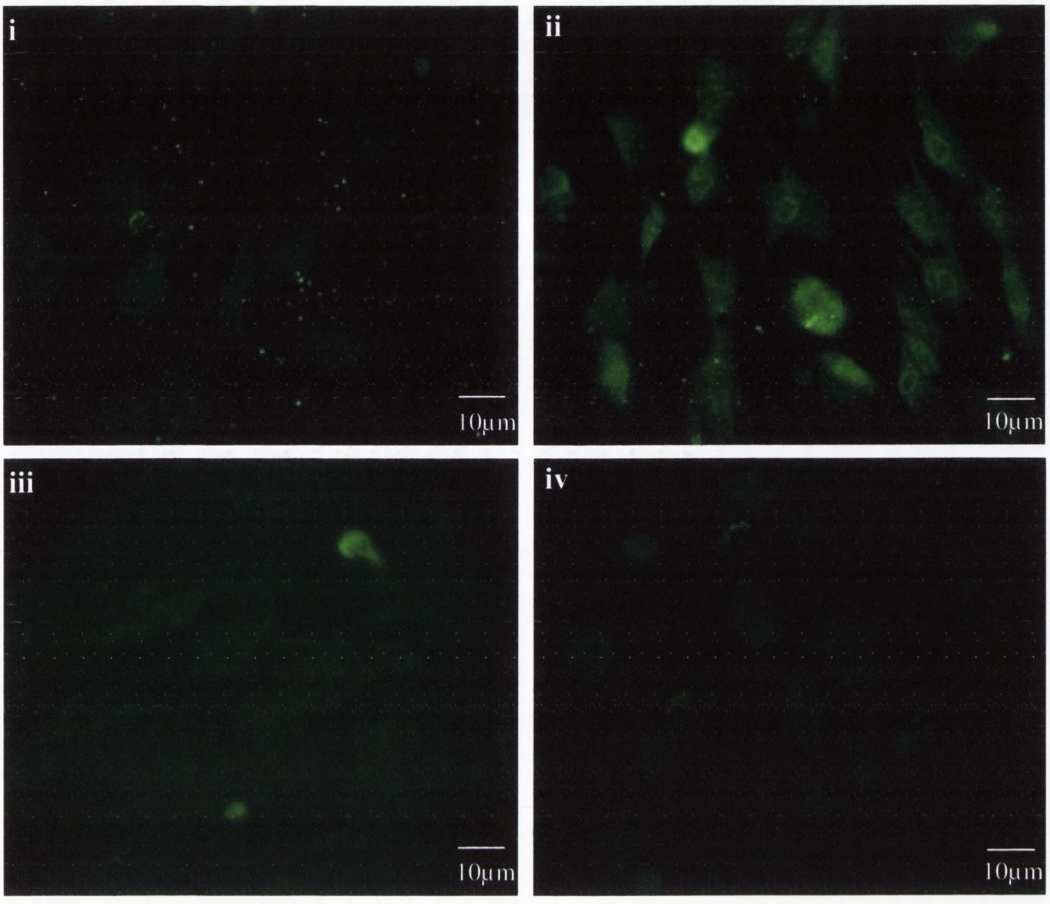


Figure 6.6: Collagen I but not osteocalcin was evident in strained cells following 2.5% strain at a frequency of 0.17Hz for 3 days

Following 3 days strain at 2.5% and a frequency of 0.17Hz, collagen type I was observed in cuboidal shaped cells (ii) but not in control unstrained samples (i). Osteocalcin expression was not observed in either control (iii) or strained samples (iv). Results are representative of 5 individual cell culture preparations for the analysis of collagen I production and for 1 cell culture preparation for the analysis of osteocalcin expression.

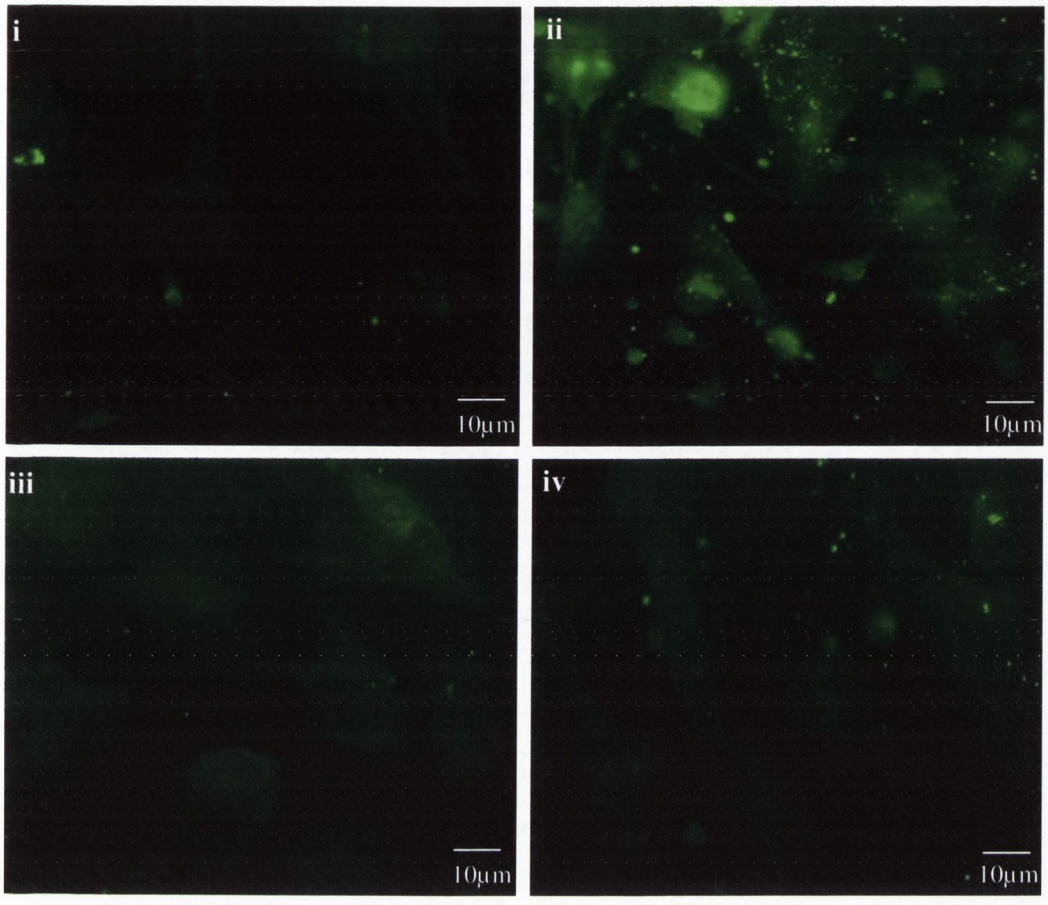


Figure 6.7: Collagen I but not osteocalcin was evident in strained cells following 5% strain at a frequency of 0.17Hz for 3 days

Following 3 days strain at 5% and a frequency of 0.17Hz, collagen type I was observed in cuboidal shaped cells (ii) but not in control unstrained samples (i). Osteocalcin expression was not observed in either control (iii) or strained samples (iv). Results are representative of 6 individual cell culture preparations for the analysis of both collagen I and osteocalcin expression.

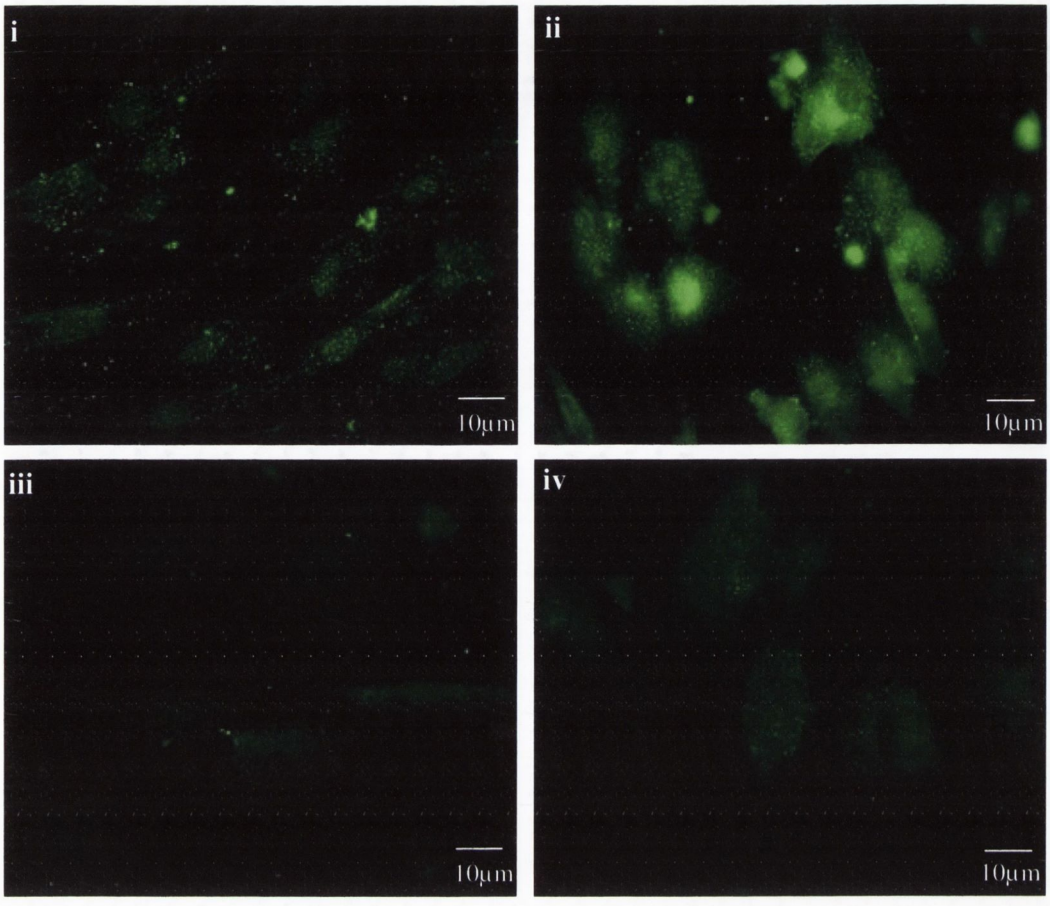


Figure 6.8: Collagen I but not osteocalcin was evident in strained cells following 10% strain at a frequency of 0.17Hz for 3 days

Although almost 50% of strained cells were undergoing apoptosis, collagen type I was still evident in strained cells (ii) but not in control unstrained samples (i), following 3 days strain at 10% and a frequency of 0.17Hz,. Osteocalcin expression was not observed in either control (iii) or strained samples (iv). Results are representative of 6 individual cell culture preparations for the analysis of both collagen I and osteocalcin expression.

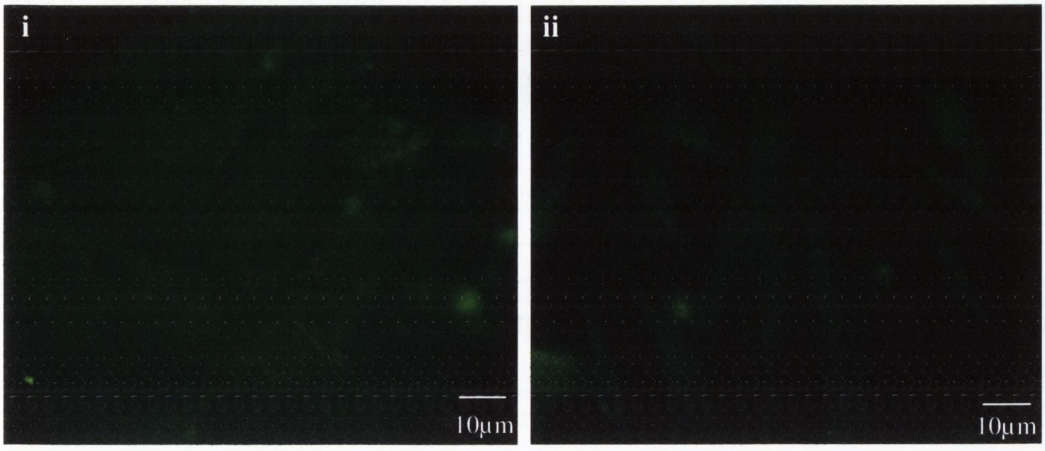


Figure 6.9: Following 3 days at 2.5% strain and 0.17Hz, no ERK activity was observed

Following 3 days strain at 2.5% and a frequency of 0.17Hz, no ERK activity was observed in strained samples (ii) or in unstrained controls (i) as evidenced by immunofluorescent analysis for phosphorylated ERK expression. Results are representative of 3 individual cell culture preparations.

6.3 Discussion

The aim of this study was to investigate the effect of uniaxial strain on MSCs, specifically in relation to their osteogenic potential and viability. The effect of strain magnitudes of 2.5%, 5% and 10% at a rate of 0.17Hz on cell viability was assessed by TUNEL staining for apoptotic cells. At 10% strain, almost 50% of cells on the silicone strip were apoptotic after 3 days, while at the lower magnitudes apoptosis was consistent with normal cellular turnover in culture (Crocker *et al.*, 2001, Vogel *et al.*, 1997). At all three strain magnitudes collagen I production was observed in strained cells but not in unstrained controls. Furthermore, collagen I positive cells were cuboidal in shape consistent with osteogenic cells as opposed to a more satellite fibroblastic morphology. Osteocalcin expression was not observed in any samples at any magnitude, as would be expected at such an early timepoint. This fact further demonstrates that cells were in an early osteoblastic phase of differentiation.

Recent studies by Simmons *et al.* (2003) and Jagodzinski *et al.* (2004) have focused on the effects of mechanical strain on the differentiation of adult MSCs. Those studies used MSCs of human origin and also used osteoinductive factors in their attempt to induce osteogenesis under mechanical strain. Despite these differences, both studies used similar strain rates and magnitudes. In the study by Jagodzinski *et al.* a consistent osteogenic response was only observed when cells were subjected to an 8% strain in the presence of high concentrations of dexamethasone. No effect of strain alone was observed at either 2% or 8% strain. Simmons *et al.* carried out all of their experiments in the presence of standard osteoinductive factors and observed significant increases in the levels of matrix mineralisation in cultures exposed to a 3% cyclic strain at 0.25 Hz. This result is somewhat consistent with this study whereby an early osteogenic response was observed when cells were strained at 2.5%, 5% and 10% at a frequency of 0.17Hz for 3 days in the absence of osteoinductive factors. Simmons *et al.* also demonstrated that ERK played a central role in this differentiation process, with ERK activity occurring as early as 8 minutes after the induction of mechanical strain. In the present study, ERK activity was not observed following three days strain at 2.5% and 0.17Hz. It is possible that ERK activation had occurred at an earlier timepoint and phosphorylated ERK levels had returned to basal levels by day 3. While it has been demonstrated that tensile strain will cause the expression of

collagen I in MSCs, the mechanisms by which this occurs is unknown. Despite this, the phenomenon of mechanotransduction is not a new one, and mechanically gated channels and mechanosensitive transmitter release have been implicated in a huge number of physiological processes, including touch and pain sensation, blood pressure control, tissue growth and micturition (Hamill and Martinac, 2001). The lipid bilayer, making up the cell membrane, has mechanosensitive properties, as do membrane channels, such as alamethicin and gramicidin found in prokaryotes, embedded in the membrane itself (Hamill and Martinac, 2001). Either of these structures, the lipid bilayer or channels, could be involved in the mechanosenstation leading to the cellular differentiation of MSCs. These channels identified as MscS and MscL (Mechanosensitive channel small/large) were originally identified in bacteria, but no eukaryotic counterpart has ever been identified (Lin and Corey 2005, Hamill and Martinac 2001). Recently, mechanically gated channels have also been identified in animal cells. One group of channels, the transient receptor potential (TRP) channels, have been shown to have mechanosensitive properties (Lin and Corey, 2005). TRP channels are comprised of at least 7 subgroups. They are activated by a large number of external and internal stimuli including intracellular and extracellular messengers, chemicals and mechanical strain (Pederson *et al.,* 2005) and have particular importance in sensory function (Lin and Corey, 2005). In general TRP channels are non-selective calcium permeable cation channels though there is some variation in their selective permeabilities within certain subgroups. Different studies have shown these channels to be sensitive to osmotic pressure, direct pressure, stretch and fluid flow (Kung 2005, Lin and Corey, 2005). The expression of TRP channels on the surface of MSCs is a distinct possibility, particularly when the tissues of the mesenchymal lineage, bone, cartilage, muscle, are affected so much by a variety of mechanical strains. Mechanical stimulation has also been shown to increase intracellular calcium concentrations in osteoblast cells via integrin receptors (Pommerenke *et al.,* 1996). Alterations in the concentrations of this ion have wide ranging effects on cellular processes. Interestingly, transmitter release has also been shown to occur in response to mechanical stimuli (Burnstock, 1999) and Chen and Grinell (1997) have demonstrated stretch-induced neurotransmitter release in frog muscle cells. The exact mechanism involved in the present study is as yet unknown but, each of those mentioned above represent possible means by which the stretch induced expression of collagen type I could occur.

In the present study, it was observed that 10% strain at a frequency of 0.17Hz was damaging to cells. In contrast, strains of 2.5% and 5% did not lead to significant increases in cellular apoptosis. Despite the observation that these strain levels are not detrimental to cell survival and even lead to osteogenic differentiation, these strain levels are supra physiological. In fact, it has been shown that these levels of strain (1-3%) are required in order to elicit a cellular response *in vitro* (Murray and Rushton 1990, Burger and Veldhuijzen, 1993). For example unidirectional cell stretching of 1% is required *in vitro* to produce a well described proliferative response in human bone cell cultures (Neidlinger-Wilke *et al.,* 1995). However, *in vivo* a 0.15% bending strain will produce recruitment of osteoblasts to the bone surface (Turner *et al.,* 1991; Forwood 1996). Rubin and Lanyon (1984) demonstrated that maximal strains occurring in bones of galloping horses did not exceed 0.3% and Burr *et al.* (1996) found the maximum physiological loads undergone by athletes to be of the order of 3000με. Even these levels of strain are above those seen in the normal *in vivo* setting. Lanyon *et al.* (1975) recorded strains of only 400με during normal walking. Frost (1992) described a window of mechanical usage; above 1500με bone will undergo remodelling and below 50με bone will be resorbed. As a result of these findings and in an attempt to reconcile the differences found between the effects of *in vitro* and *in vivo* straining, Cowin *et al.,* (1991) proposed the canalicular fluid flow hypothesis. This hypothesis states that the strains imposed on cells *in vivo* are not as a result of the direct strains on the bone tissue but from a local force on the cells. This local force is caused by the displacement of fluid in the canaliculi resulting from compression of the bone matrix (Weinbaum *et al.,* 1994). When these local strains are taken into account, the forces imposed on cells *in vivo* correlate more with the *in vitro* findings of a required strain magnitude of above 1% to illicit a cellular response. In accordance with this theory, Klein-Nulend *et al.* (1995) found a much greater effect of fluid shear stress than hydrostatic pressure osteocytes and osteoblasts. In a study by Kaspar *et al.* (2000), it was found that a low cyclic tensile strain magnitude of 1000 με led to increased proliferation and matrix synthesis but not mineralisation. In that study, while the strain used was tensile, it was suggested that shear stress caused by hydrostatic pressure from the weight of the medium might have caused the cellular response and not the tensile strain. This statement was due to the findings of Klein-Nulend *et al.* (1995) and Owan *et al.* (1997), suggesting that osteoblasts are more

sensitive to shear stresses than strain deformation. However other studies have demonstrated effects of mechanical strain on osteoblasts without fluid flow (Fermor *et al.,* 1998; Murray and Rushton, 1990). Simmons *et al.,* (2003) found that strain slowed proliferation, with significant inhibiton over a 10 day period. Strain did not have an effect on AP activity but did lead to a significant increase in matrix deposition. In this present study, the rig used to strain the cells was designed to reduce any fluid shear stresses or hydrostatic pressures by having the silicone substrate, on which the cells were located, just below the surface of the culture medium. However, it is possible that fluid shear stresses were still introduced into the strained samples, caused by the oscillatory movement of the fluid despite efforts to minimise the effect. Taking these facts into account, and also the fact that the production of collagen type I occurred in the absence of any osteoinductive factors, it is clear that mechanical strain is an inducer of the expression an early marker of osteogenic differentiation in adult rat MSCs.

To summarise, a mechanical uniaxial strain of 2.5% and 5% run cyclically at a rate of 0.17Hz for 3 days leads to an induction of collagen type I expression not seen in unstrained controls. Neither of those stretching regimes led to any significant increase in cell death by apoptosis, as evidenced by TUNEL staining. In contrast, osteocalcin, a late stage marker of osteogenesis was not observed in any samples. This study illustrates the potential for the use of mechanical strain as a replacement for non-endogenous growth factors as a stimulus for osteogenic differentiation of adult rat MSCs. This study was also carried out as a viability study to begin to determine optimum strain rates and magnitudes in a 3-D scaffold as part of a larger project that will also employ finite element modelling to determine suitable straining parameters. Recently, Ignatius *et al.* (2005) demonstrated the promising possibilities in this field. It was found that, cyclic stretching of collagen type I scaffolds seeded with osteoblasts at a frequency of 1Hz and a magnitude of 1% increased cell proliferation and the expression of several bone related genes over a 3 week period. It is noteworthy that these experiments were also carried out in the absence of osteoinductive factors. Taking the findings of Ignatius *et al.* with the results from this chapter, it is hoped that induction of osteogenic differentiation of adult MSCs within the 3-D collagen GAG scaffold driven by mechanical stimulation will soon be a reality.

Chapter 7

Analysis of static magnetic field- mediated ERK and JNK activation

7.1 Introduction

Strong electromagnetic fields of the Tesla (T; 1T=10,000 Gauss) order are rarely encountered in everyday life. The most common reason for a person to come into contact with field strengths of this magnitude would be when undergoing a magnetic resonance imaging scan (MRI). By comparison, the earths magnetic field only corresponds to 0.7 Gauss or 70μT. Anything below 0.5T is considered safe for humans and, according to Villa *et al.* (1991), the effects of strong magnetic fields on whole organisms have been found to be either transient or absent. At the level of the individual cell, the responses have been found to be somewhat varied. A host of studies have observed no effects of static magnetic fields on a variety of cell types, including human breast cancer cells (Pacini *et al.,* 1999a), moto-neurons in chick embryos (Yip *et al.,* 1995, Yip *et al.,* 1994), lymphocytes, monocytes and T cells (Aldinucci *et al.,* 2003, Norimura *et al.,* 1993). It should be noted that the field strength in these studies ranged from 0.2T to 6.3T. Despite these findings, there has been some contrary evidence to show an effect of static magnetic fields on human neurons (Pacini *et al.,* 1999b) and human cancer cells (Raylman *et al.,* 1996). Denegre *et al.* (1998) even observed malformation of frog eggs caused by alteration of the direction of the cleavage planes caused by magnetic fields. It is clear that under certain circumstances, some cell types will respond to a strong static electromagnetic field *in vitro*.

To date, the effects of static magnetic fields on intracellular signalling mechanisms have not been studied. The purpose of this study was to investigate the effects of a range of static magnetic fields on two intracellular signalling cascades, in an attempt to understand the effects of these fields on cell survival and possible differentiation capacity. The mitogen activated protein kinase ERK is closely coupled to a host of cell functions related to survival and differentiation (Pearson *et al.,* 2001), while JNK has been implicated in the apoptotic pathway and is responsive to oxidative stress, growth factor withdrawal and radiation (Yoshizumi *et al.,* 2002, Eilers *et al.,* 2001, Pearson *et al.,* 2001). The activity of both of these molecules was assayed at a variety of magnetic field strengths. This study is also the first step in assessing the potential of static magnetic fields for tissue engineering purposes. If an increase in ERK activity is observed, pulsing stem cells for a short period of time might prove to be

another successful stimulus to be used in the induction of differentiation or matrix production, both *in vitro* and *in vivo*. The reason for using neurons in this study instead of MSCs stems from their ready availability in the laboratory for use in such a preliminary set of experiments.

7.2: Results

7.2.1: Apparatus used for culturing neurons in static magnetic field

Figure 7.1 illustrates the apparatus used for the stimulation of neurons with a range of static magnetic fields ranging from 0.1T to 5T. Magnetic field exposure was carried out using a superconducting solenoid with a wide, open circular bore (cryogenic Ltd, London, UK), with dedicated software for real-time measurement of the magnetic field. The maximum field strength was 5T with a 0.01% linear variation in the field strength over the length of the culture volume. Cells were cultured on coverslips and placed into Petri dishes containing CO_2 independent medium for the duration of the stimulation period. They were maintained in a humid atmosphere at 37°C. Control cultures were maintained in the same conditions as the magnetically stimulated cells without the magnetic field for the duration of the experiment.

7.2.2: Static magnetic fields affect ERK activity in cultured cortical neurons

To study the effects of magnetic fields on ERK activation, cultured cortical neurons from neonatal rats were exposed to static magnetic fields of several intensities (0.1, 0.5, 1, 2, 5T) for one hour. pERK immunoreactivity was assessed by immunocytochemistry and expressed as a percentage pERK positive cells of the total number counted. 400 cells per coverslip were counted for 10 individual preparations at each field strength. ERK activity was expressed as a percentage difference vs control. Figure 7.2 demonstrates that, at a field strength of 0.75T ERK activity was significantly elevated compared to unstimulated controls (10.4 ± 1.8% $p<0.01$, Student's t test). At no other stimulation intensity was ERK activity significantly different from unstimulated control cells. ERK activity appeared to increase to a maximum of 0.75T and then decrease at higher field intensities in a dose responsive manner. This would suggest an optimum field strength of 0.75T for the induction of ERK phosphorylation in neurons *in vitro*. Representative photomicrographs demonstrate positive pERK staining in control (Figure 7.2 (i)) and magnetically stimulated cells (Figure 7.2 (ii)) at a field strength of 0.1T.

7.2.3: Static magnetic fields affect JNK activity in cultured cortical neurons

To study the effects of magnetic fields on JNK activation, cultured cortical neurons from neonatal rats were exposed to static magnetic fields of several intensities (0.1, 0.5, 1, 2, 5T) for one hour. pJNK immunoreactivity was assessed by immunocytochemistry and expressed as a percentage pJNK positive cells of the total number counted. 400 cells per coverslip were counted for 10 individual preparations at each field strength. JNK activity was expressed as a percentage difference vs control. Figure 7.2 demonstrates that, at a field strength of 2T and 5T, JNK activation was significantly higher than in unstimulated control cells (9.8 ± 1.6% at 2T, $p<0.05$; 11.0 ± 1.7% at 5T, $p<0.01$, Student's t-test). JNK activity appeared to be in apposition to ERK activity. When pERK levels were elevated, JNK levels were lowest and at higher field strengths, when ERK activity was lowest, JNK activity was significantly elevated. It appears that low static magnetic fields will induce cell survival signals, whereas higher intensities will induce JNK signalling and possibly the induction of apoptotic pathways. Representative photomicrographs demonstrate positive pJNK staining in control (Figure 7.3 (i)) and magnetically stimulated cells (Figure 7.3 (ii)) at a field strength of 5T.

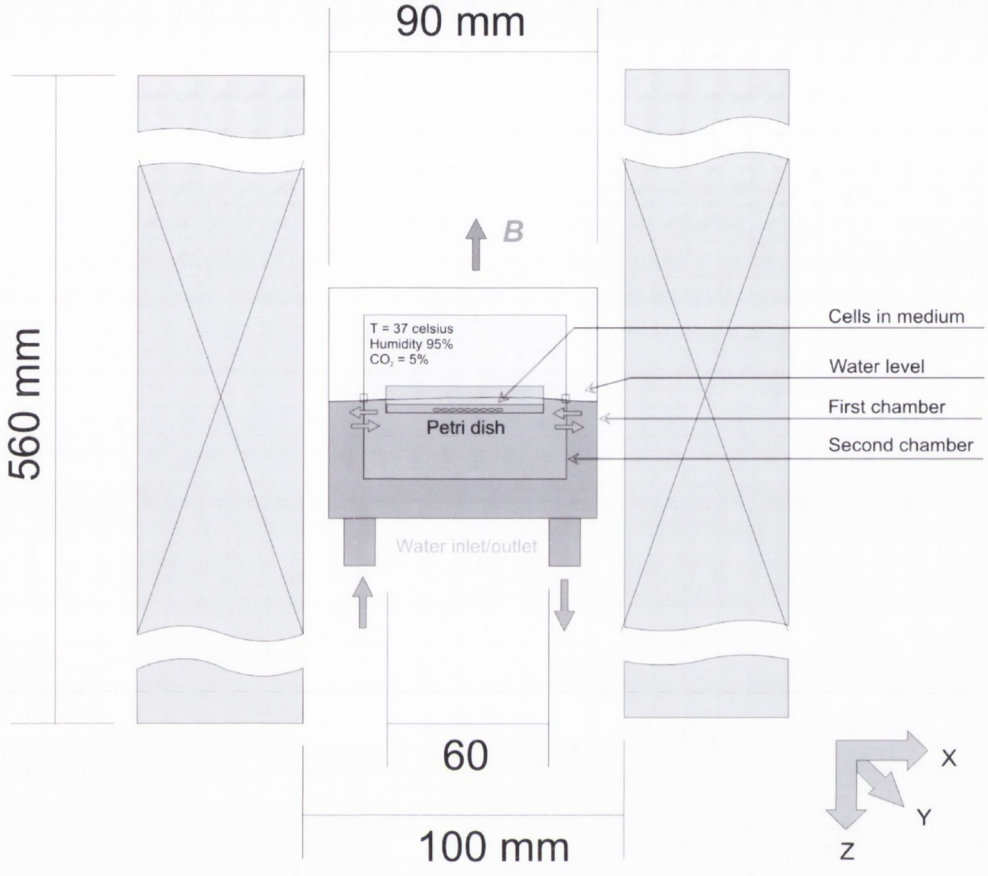


Figure 7.1: Apparatus used for culturing neurons in static magnetic field

Cortical neurons were cultured on coverslips in Petri dishes and maintained in CO_2 independent medium for the duration of the stimulation period at 37°C and 95% humidity. The strength of the magnetic field varied from 0.1T to 5T. The field in the $\phi =$ 100 mm open bore is vertical.

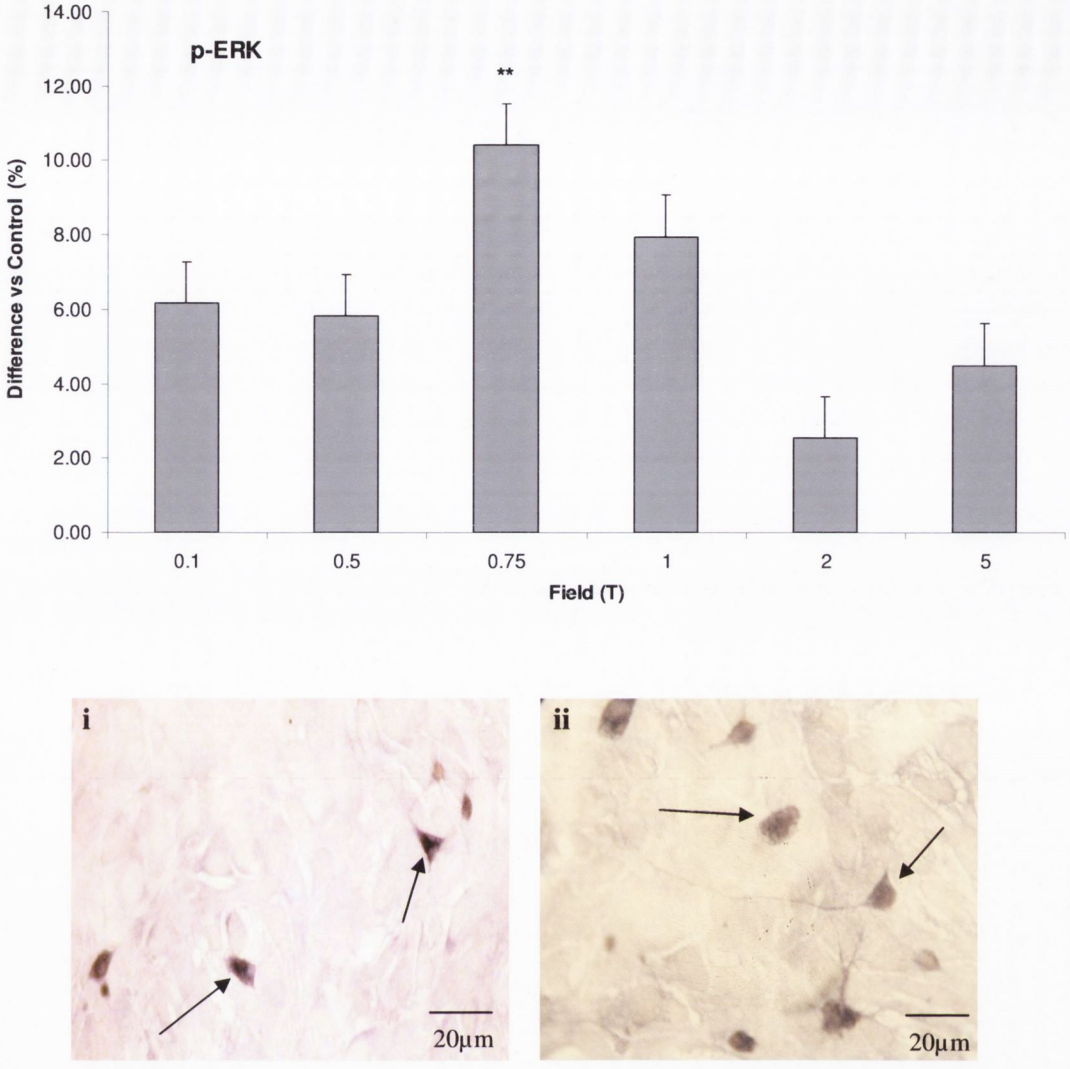
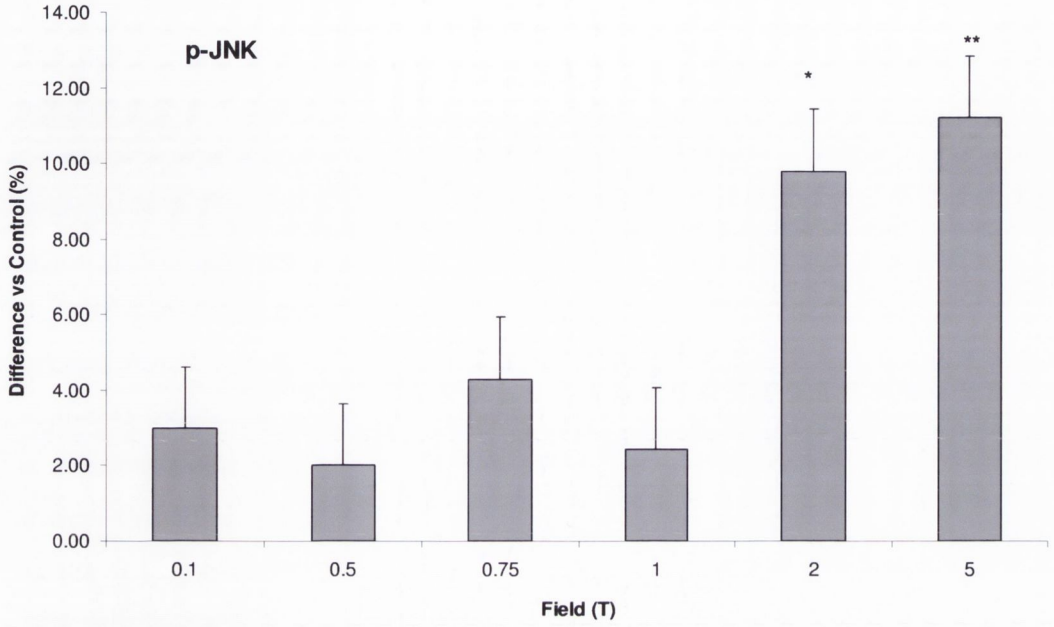


Figure 7.2: Static magnetic fields affect ERK activity in cultured cortical neurons

Neonatal rat cortical neurons were exposed to static magnetic fields of varying strength, from 0.1T to 5T for a period of 1 hour. Phosphorylated ERK expression was analysed by immunocytochemistry and expressed as a percentage of total cells counted per coverslip (400). At a magnetic field strength of 0.75T, ERK activity in cells was significantly higher than in controls not exposed to any magnetic field ($p<0.01$, Student's t-test). Results are representative of 10 individual cell culture preparations at each magnetic field intensity. Images (i) and (ii) show representative control and stimulated cells at 0.1T respectively. Arrows indicate pERK positive cells.



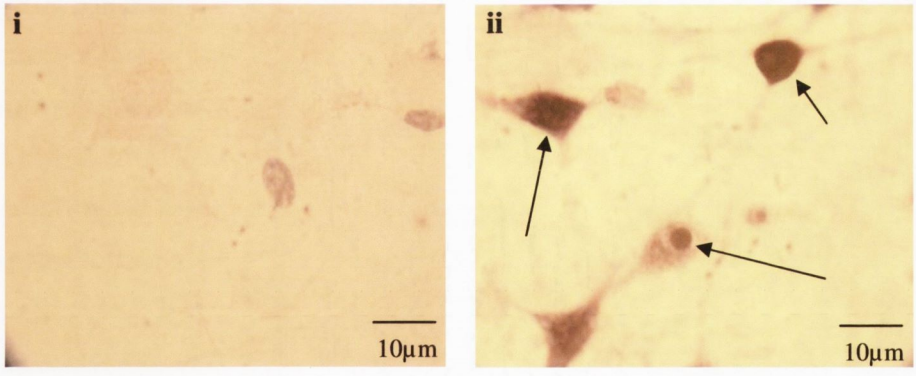


Figure 7.3: Static magnetic fields affect JNK activity in cultured cortical neurons

Neonatal rat cortical neurons were exposed to static magnetic fields of varying strength, from 0.1T to 5T for a period of 1 hour. Phosphorylated JNK expression was analysed by immunocytochemistry and expressed as a percentage JNK positive cells of total cells counted per coverslip (400). At a magnetic field strength of 2T and 5T, JNK activity in cells was significantly higher than in controls not exposed to any magnetic field ($p<0.05$ at 2T,$p<0.01$ at 5T Student's t-test). Results are representative of 10 individual cell culture preparations at each magnetic field intensity. Images (i) and (ii) show representative control and stimulated cells at 5T respectively. Arrows indicate pJNK positive cells.

7.3: Discussion

The aim of this study was to investigate the effects of static magnetic fields on mitogen activated protein kinase family members ERK and JNK. ERK activity was shown to be significantly elevated in magnetically stimulated cells at a magnetic field strength of 0.75T compared to unstimulated controls, while JNK activation was significantly higher than controls at the higher field intensities of 2T and 5T. While both ERK and JNK have been shown to be activated by a large number of external stimuli (Pearson *et al.,* 2001), this is the first time static magnetic fields have been shown to induce the phosphorylation of these signalling molecules. Although, only one step in a complex pathway has been elucidated in this study, it is reasonable to assume that ERK and JNK activation in this system will lead to similar downstream events as occurs in other modes of stimulation. ERK activity is generally associated with cellular survival and differentiation (Cobb *et al.,* 1995), however, in the case of the neuronal cells, differentiation is unlikely and this signal is more likely to be protective in nature. JNK on the other hand is usually related to cell stress and apoptosis (Xia *et al.,* 1995). While the potential benefits of inducing ERK phosphorylation using static magnetic fields are obvious, such as the possibility for recovery of necrotic/apoptotic cells after ischaemic insult, the knowledge of what levels of field intensity will induce JNK activity would also be useful. Not only is it important to know what level will induce apoptosis or stress pathway activation in cells of interest, it might also prove to be a useful tool in destroying unwanted cells such as cancerous ones.

Clearly, different magnetic fields can lead to different signal responses and kinase activation *in vitro*. However, the mechanisms behind this signal transduction are unknown, as is the timecourse of activity. Only one stimulus duration was used and it is not known whether longer, shorter or pulsed stimuli will elicit a different response. Although the mechanisms of activation of these pathways are unknown, there are several possibilities. Membrane potential across a cell is very important for signalling and homeostasis. It is conceivable that the magnetic field induced could cause alterations in the resting potential of the neurons. This could lead to the opening of voltage gated ion channels causing a series of intracellular responses (Rogachefsky *et al.,* 2004, Malagoli *et al.,* 2003). Kotani *et al.,* (2002) observed orientation responses

to static magnetic fields in MC3T3 cells. Perhaps the field is inducing some type of mechanotransduction event by altering the alignment of cytoskeletal elements or elements of the lipid bilayer (Hamill *et al.,* 2001) leading to the intracellular signalling cascade. At the physiological level there are three proposed mechanisms by which magnetic fields are sensed in some animals; 1) electromagnetic induction which possibly occurs in the elasmobranch fish, 2)chemical megnetoreception, whereby the spin state of electrons in paired ions is altered by the earths magnetic field and 3) magnetite-based magnetoreception. In this third mechanism, minute crystals of magnetite (Fe_3O_4) are present in cells and their orientation to the geomagnetic field can exert pressure or other strains on receptors, ion channels and presumably other cellular organelles (Johnsen *et al.,* 2005). It is possible that magnetite or some other magnetic field sensitive substance is present in neurons, causing ion transport alterations and leading to activation of intracellular signalling cascades. To date however, there is little evidence that magnetite functions in magnetoreception and no distinct magnetoreceptor has been identified in any animal (Johnsen *et al.,* 2005)

The data presented in this chapter suggests a possible neuroprotective role of "lower" intensity static magnetic fields and a damaging effect of field strengths above 2T. Further work will be required to verify this hypothesis. However, if this is indeed the case, this method of stimulating increased ERK activity in cells might be successfully transferred to the tissue engineering setting. In this thesis ERK has been repeatedly shown to be necessary for the osteogenic differentiation of MSCs. As stated, the use of growth factors would preferably occur, if alternatives were available. Perhaps a short pulse of magnetic stimulation coupled with mechanical strain could lead to improved osteogenic differentiation. Obviously more work is needed to understand the timing of this signalling mechanism and its effects on a different cell system. However, Kotani *et al.* (2002) have succeeded in increasing cell differentiation and matrix synthesis in MC3T3 cells using an 8T static magnetic field for a period of 60 hours. In the same study, they demonstrated ectopic bone formation in mice using the same stimulus in the presence of BMP. The magnetic field not only induced matrix deposition and mineralisation, but also altered the orientation of the bone, parallel to the field, a possibly useful effect for tailored bone growth. The results presented here,

while preliminary, demonstrate the potential benefits of static magnetic fields in tissue engineering and other areas of research.

Chapter 8

Final discussion

8.1 Discussion

In 1966, Friedenstein *et al.* first demonstrated the presence of MSCs in the adult marrow. Since then, the significant potential for the treatment of many debilitating diseases using this multipotential cell type has been recognised. Given that MSCs can give rise to bone, cartilage, fat, tendon, muscle and marrow stroma (Pittenger *et al.,* 1999), there are great prospects for new treatments of many age related musculoskeletal disorders. The future demographic trends towards an increasingly aged population, susceptible to many musculoskeletal disorders and the current inadequacies in some of the treatments of these diseases necessitate the need for new approaches to these problems. Tissue engineering, if successful, offers great potential for the treatment of a number of diseases. The aim of this project was to induce differentiation of MSCs derived from young adult wistar rats along the osteogenic and chondrogenic lineages in both 2-D and 3-D environments. It should be noted however, as discussed in chapters 1 and 3, that the MSC cultures used in this study were not a 100% pure population, nor is it possible at this time to achieve such a culture. Endoglin immunostaining and morphological characterisation demonstrated the presence of a high proportion of MSCs in culture. The use of monolayer cultures can be very useful in providing information about cellular morphology and signalling mechanisms in a variety of scenarios. However, the culture of MSCs in 2-D does not accurately mimic the *in vivo* environment, nor does it provide the possibility for the regeneration of 3-D tissues as a possible treatment of diseases. It was therefore necessary to move to the 3-D environment of the biomimetic scaffold in order to provide an appropriate substrate that would support osteogenesis and chondrogenesis.

The initial phase of this study focused on the establishment and optimisation of procedures for the isolation and culture of MSCs in this laboratory, so that they may be induced to differentiate along the osteogenic or chondrogenic lineage. Immunoreactivity for Endoglin, a putative stem cell marker (Lodie *et al.,* 2002), verified the presence of MSCs in culture after 3 days and these cells were shown to proliferate for up to 5 weeks in culture, an important requirement given the low number of MSCs present in the bone marrow. In cells treated with osteoinductive factors, the presence of osteocalcin, a low weight protein expressed at the post proliferative osteoblastic phase of differentiation (Bilezikian *et al.,* 2001), definitively demonstrated that osteogenesis had taken place. As shown in adult human stem cell models, (Jaiswal *et al.,* 2000, Simmons *et al.,* 2003) ERK

is involved in the induction of differentiation along the osteogenic lineage and I have demonstrated this same mechanism to be involved in osteogenesis in the rodent model, since the induction of osteocalcin expression was blocked by the ERK inhibitor U0126. As Jaiswal *et al.* (2000) demonstrated, ERK activation peaked between 7 and 11 days in culture. Those results correlate well with the findings of this study, which showed that ERK activation was necessary for osteocalcin expression although ERK activity was not demonstrated at day 14. It is likely that once the activated ERK signal had been propagated to the nucleus, ERK activation had returned to basal levels. This would explain how ERK activation was not observed despite the finding that U0126 prevented the induction of osteocalcin expression. This hypothesis was verified by the observation that ERK activity peaked at day 10. A timecourse of collagen type I and osteocalcin expression and matrix mineralisation in 2-D MSC cultures was also carried out. The timecourse of expression of these markers of osteogenesis mirrored the findings of others (Bilezikian, 2001, Aubin *et al.,* 1995). Collagen type I was strongly expressed earlier than osteocalcin and potent expression of osteocalcin coincided with matrix mineralisation as evidenced by Alizarin Red S staining. Since osteocalcin is involved in matrix mineralisation, it was expected that its expression would closely relate to calcium deposition. ERK activity was observed to increase above control levels at day 10 of culture. It was also observed that its inhibition prevented osteocalcin expression. I therefore conclude that ERK is involved in the later stages of osteogenic differentiation of rodent MSCs. The activity of p38 was shown to be elevated at day 7 of culture, suggesting it plays a slightly earlier, and as yet unknown, role in the osteogenic process.

The results from Chapter 4, demonstrate the ability of adult rat MSCs to be sustained in a 3-D collagen GAG scaffold. The culture of cells in biodegradable scaffolds appears to require longer culture periods in order for differentiation to occur, partly due to the need for the cells to migrate through the structure as has been shown by others (Laurencin *et al.,* 1996, Freed *et al.,* 1998). Following 14 days in culture, cells were still located to the outer regions of the scaffold. By 21 days complete scaffold penetration had occurred. Despite this requirement for a longer culture period in 3-D, I have clearly demonstrated that osteogenesis, as evidenced by immunoreactivity for osteocalcin, has begun to take place. Since osteocalcin expression has been shown experimentally to occur between days 11 and 16 in 2-D following exposure of MSCs were to osteoinductive factors (Jaiswal *et al.,* 1997, Pittenger *et al.,* 1999) and the response of cells to their environment is slower in

3-D (Laurencin *et al.,* 1996) it is not surprising that matrix mineralisation had not occurred by day 14. Debate exists as to whether the expression of osteocalcin (a late stage marker in osteogenesis) alone is indicative of full osteoblast differentiation, and a number of research groups classify mineralization of the extracellular matrix as a more definitive marker of osteogenesis (Bilezikian *et al.,* 2001). Matrix mineralization is the final stage in the three-stage process of osteoblast differentiation, which are; 1) osteoprogenitor proliferation, 2) differentiation and 3) matrix mineralization. The presence of calcium in these scaffolds definitively shows that osteogenesis has taken place after 21 days in culture.

Despite the occurrence of complete mineralisation in 21 days in some samples, there was some variability in the levels of mineralisation. Quite often, mineralisation only occurred at one surface of the scaffold and spread around the edges. I hypothesised that this was not as a result of cell necrosis in the centre of the scaffold caused by insufficient nutrient delivery, but rather by insufficient time allowed for complete mineralisation to occur. In order to confirm this hypothesis, scaffolds were seeded with MSCs for up to 7 weeks. At 4, 5, 6 and 7 weeks, scaffolds seeded with cells from the same culture preparation were harvested and histologically analysed for mineralisation. Although at each timepoint the scaffold sample was different the cell source was the same to rule out inter-animal variation. There was an observed time related progression of mineralisation through the material until almost complete mineralisation was observed at 6 weeks. Since the cells were from the same preparation and were seeded and treated together, this suggests that at the earlier timepoint of 4 weeks, the lack of complete mineralisation was not due to inability of the cells to completely mineralise, but due to insufficient time in culture. The differences in mineralisation levels at 3 weeks between samples could thus be attributed to inter-animal variation. As was observed in the compression analysis, this phenomenon is very important and can have large effects.

In keeping with the results from the previous chapter, ERK was shown to be necessary for effective osteogenesis to occur in 3-D. Osteocalcin expression was abolished and matrix mineralisation, while not completely prevented, was reduced. Interestingly, p38 inhibition completely prevented osteocalcin expression in collagen GAG scaffolds without having any effect on mineralisation. Although this finding was very unexpected, it did correlate with the findings of Jaiswal *et al.* (2000) and Simmons *et al.* (2003). Jaiswal *et al.* also

observed no effect of p38 inhibition on mineralisation. Unfortunately, in that study osteocalcin expression was not assessed. The precise role for p38 in osteogenesis remains unclear.

The role of dexamethasone in osteogenic differentiation of MSCs is a confusing one. Traditionally, glucocorticoids are known to cause osteoporosis and atrophy of a number of other tissues when administered systemically and even topically (Schaecke *et al.,* 2002). Patients subjected to long term glucocorticoid treatment develop an increased risk of fracture as a result (Schaecke *et al.,* 2002). Despite this, dexamethasone is commonly used in the induction of osteogenesis *in vitro* and has been shown to act at both early and late stages of this process (Porter *et al.,* 2003). Studies have also shown dexamethasone decreases Cbfα-1 expression and osteocalcin promoter activity and increases the expression of PPARγ, suggestive of inhibition of the osteogenic process and induction of adipogenesis (Li *et al.,* 2005), while Igarashi *et al.* (2004) found that dexamethasone administration in foetal rat calvarial cells caused an upregulation of Cbfα-1 and Osterix, a newly identified zinc-finger containing protein, expression. In this thesis, dexamethasone, in combination with ascorbic acid and β-glycerophosphate, was shown to induce osteogenesis in 2-D and 3-D in and ERK dependent manner.

Clearly some of the findings mentioned above are at odds with each other. The effects of glucocorticoids, including dexamethasone are mediated through two distinct nuclear receptors, the glucocorticoid receptor and the mineralocorticoid receptor, although the expression of mineralocorticoid receptor is restricted to epithelial cells, the brain and the heart. Upon binding of glucocorticoid ligands to the glucocorticoid receptor the complex translocates from the cytoplasm to the nucleus (Schaeke *et al.,* 2002). Shur *et al.,* (2005) have demonstrated activation of cFos by this glucocorticoid response complex and this is presumably how Cbfα-1 and Osterix are also activated. cFos is an early response gene that belongs to the AP-1 transcription complex and is necessary for osteoprogenitor differentiation (Shur *et al.,* 2005). Although definitive proof as to whether dexamethasone induces or inhibits osteogenesis is lacking, it is clear from the results in chapters 3 and 4 in this thesis that it forms part of a cocktail vital for successful osteogenic differentiation of adult rodent MSCs. As stated, this process occurs in an ERK dependent manner. Interestingly, the effects of dexamethasone are not mediated via this signalling pathway as discussed above and recent findings suggest that dexamethasone actually inhibits ERK

activity. Engelbrecht *et al.* (2003) and Kassel *et al.* (2001) have both demonstrated that dexamethasone inhibited ERK activity by upregulation of mitogen activated protein kinase phosphatase activity, leading to dephosphorylation of ERK in osteoblast cell lines and mast cells respectively. Not only does dexamethasone appear to inhibit ERK activity, but Plotkin *et al.* (1999) demonstrated that dexamethasone induced apoptosis in murine osteoblastic cells was reversed by bisphosphanates via ERK activation. The precise interplay between dexamethasone and ERK activation in this study is not known, but it is obvious that dexamethasone does not inhibit the activity of ERK nor the expression of bone specific proteins under the culture conditions used in this study.

It has also been shown that dexamethasone has positive and negative effects on chondrogenesis (Awad *et al.,* 2003, Miyazaki *et al.,* 2000 ,Quarto *et al.,* 1992). The variety in response in this instance has been related to the cell type being studied. Kim *et al.* (2005) suggest that dexamethasone will enhance proteoglycan synthesis in chondrocytes but will inhibit chondrogenesis in prechondrogenic cells. However, dexamethasone has long been a standard ingredient in chondroinductive factor cocktails (Shirasawa *et al.,* 2005, Johnstone *et al.,* 1998, Mackay *et al.,* 1998). As discussed in chapter 5, the role of ERK in chondrogenesis is also somewhat controversial. Perhaps there is some relation between the inductive and inhibitory roles of ERK and dexamethasone in osteogenesis and chondrogenesis depending on cell type, concentration of dexamethasone duration of treatment and several other factors. A clearly standardised set of criteria for the treatment of specific cell types to induce cellular differentiation in a defined way would be a welcome addition to the field of tissue engineering, making inter-study correlations easier.

Chondrogenesis, as evidenced by immunoreactivity with type II collagen, was shown to occur after 21 days culture throughout collagen GAG scaffolds and at the periphery after 14 days. The use of the collagen GAG scaffold is important for the study of chondrogenesis in particular, as the complete differentiation of MSCs along this pathway cannot be induced on the 2-D surface of a tissue culture flask (Johnstone *et al.,* 1998). I have demonstrated here the versatility of this scaffold in the maintenance of MSCs as they have the proclivity to progress along both osteogenic and chondrogenic lineages in this environment. This is in stark contrast to other scaffolds currently in use, such as PLLA, PLGA and alginates, which have been shown only to support differentiation along

one specific lineage (Yang *et al.,* 2001, Yang *et al.,* 2002, Honda *et al.,* 2003, Wang *et al.,* 2003). These results are very promising, primarily since there was no need for the alteration of the scaffold in any way in order to promote cell adhesion and growth. With many of the types of scaffold currently in use, there is a requirement for some type of surface treatment, such as type I collagen coating, before MSCs will adhere (Honda *et al* 2003). With the collagen GAG, this is not necessary, and as shown by Xiao *et al.* (1998), type I collagen actually promotes osteogenesis while glycosaminoglycans increase the integrity of collagenous scaffolds in long term cultures (Pieper *et al.* 2000). These facts coupled with the desired criteria described by O' Brien *et al.,* (2004) that a scaffold should fulfil, namely biocompatibility, appropriate degradation rate, ligand binding sites and appropriate pore size and volume fraction, are further evidence of the compatibility of this collagen GAG material.

An unexpected result in chapter 5 was the ability of the scaffold to support chondrogenic differentiation of MSCs, to the point of expressing collagen type II, in the absence of TGFβ. Normally, TGFβ is considered essential for the chondrogenic differentiation of MSCs (Barry *et al.,* 2001). Clearly some property of the scaffold caused the induction of the chondrogenic differentiation process without the need for this factor. What that property is, remains unknown but could possibly be related to both the chemical and physical makeup of the material. For example, the cytoskeletal arrangement necessary for cell to scaffold interaction might induce chondroinductive signalling processes as occurs following cytoskeletal disruption (Lim *et al.,* 2003, Lim *et al.,* 2000, Zanetti *et al.,* 1984). A study by Veilleux *et al.,* (2004) using a type II collagen GAG scaffold manufactured in the same manner demonstrated the ability to sustain the chondrogenic potential of cultured articular chondrocytes in the absence of TGFβ. While several parameters differed, primarily the collagen type, this study does serve to demonstrate that collagen GAG scaffolds will also support chondrogenesis of chondrocytes in the absence of TGFβ and not allow dedifferentiation, which will happen in the 2-D environment in the absence of chondroinductive factors. Neither p38 nor ERK inhibition prevented chondrogenesis, either in the presence or absence of chondroinductive factors. Again this was unexpected. While the role of ERK in chondrogenesis is somewhat controversial, with stimulatory and inhibitory roles in chondrogenesis being demonstrated (Lee *et al.,* 2004, Oh *et al.,* 2001, 2000, Yoon *et al.,* 2000), p38 has repeatedly been shown to be necessary for chondrogenesis in both chick limb bud mesenchymal cells and ATDC5 cells (Lee *et al.,*

2004, Yoon *et al.,* 2000, Nakamura *et al.,* 1999). In this thesis, p38 inhibition at 7 days and 21 days was shown to have no effect on collagen type II expression. As a result of this finding, it was hypothesised that PI3 kinase might be involved in chondrogenesis of rodent MSCs. Previous studies had proposed a role for PI3 kinase in chondrogenesis in ATDC5 cells and chick limb bud mesenchymal cells (Oh *et al.,* 2003, Hidaka *et al.,* 2001). After 7 days culture PI3 kinase inhibition did appear to reduce the level of collagen type II expression in both control and chondroinductive factor treated cells. The results presented here are the first to demonstrate the ability to culture adult MSCs in 3-D collagen GAG scaffolds and to induce their differentiation along both osteogenic and chondrogenic lineages to the end points of matrix mineralization in the case of osteogenesis and collagen type II production in the case of chondrogenesis.

Unlike dexamethasone, the requirement for TGFβ for chondrogenesis is much clearer and is not disputed (Barry *et al.,* 2001). There are several TGFβs isoforms however, and some have been shown to be more potent inducers of chondrogenesis than others. TGFβ-3 is now the preferred family member for inducing chondrogenesis (Shirasawa *et al.,* 2005, Barry *et al.,* 2001). The activity of TGFβ is mediated via the Smad family of intracellular mediators after association with their receptors. The receptor serine/threonine kinase family of receptors are responsible for transducing the TGFβ into the cell. They are composed of type I and type II components. Upon ligand binding of TGFβ to the type II component, the type I receptor is incorporated into a ligand receptor complex. The type I receptor component is subsequently phosphorylated and thereby activated by the type II component which appears to be constitutively active (Shi and Massague, 2003). Activated receptors phosphorylate members of the R-Smad Smad 2 and 3 subfamily of molecules directly causing their accumulation in the nucleus where they activate a variety of transcription factors (Attisano *et al.,* 2002). Signals from the BMPs are mediated through the same pathway via Smads 1, 5 and 8. Once again ERK is involved in this process, in two ways. Once Smad has been activated and is translocated to the nucleus, it can be phosphorylated by ERK, which results in its removal from the nucleus where it accumulates. This can cause an inhibition of the TGFβ mediated signal. This would explain the findings of several groups that ERK has an inhibitory role in chondrogenesis (Chang *et al.,* 1998). On the other hand, recent evidence has suggested that several members of the MAPK family including ERK and p38 can be directly activated by TGFβ independent of Smads, as can Akt which is downstream of PI3K (Shi *et al.,* 2003,

Attisano *et al.,* 2002, Yu *et al.,* 2002). This might explain some of the discrepancies in the literature regarding these signalling molecules in chondrogenesis. This applies particularly to ERK where it both excitatory and inhibitory roles via TGFβ exist. Factors such as treatment duration, TGFβ member, concentration and cell type would all affect these signalling pathways and the subsequent role of ERK.

Mechanical stimulation of cells is becoming an increasingly popular method of inducing cellular responses (Jagodzinski *et al.,* 2004, Simmons *et al.,* 2003, Kaspar *et al.,* 2002). Chapter 6 demonstrated the strain induced expression of collagen type I after 3 days uniaxial strain at 2.5%, 5% and 10% elongation at a rate of 0.17Hz. As expected, the later marker of osteogenesis, namely osteocalcin, was not expressed at this timepoint. While collagen I is not a definitive marker of osteogenesis, being expressed by other cell types including fibroblasts, this is a promising first step in the mechanical stimulation of MSCs. The morphology of those cells expressing collagen I was also more characteristic of osteoblast like cells than fibroblasts. This finding is one of the first to suggest osteoinductive factor-independent strain mediated differentiation of MSCs. Several possible mechanisms of mechanotransduction leading to this collagen I expression exist. These include sensation by the lipid bilayer itself, (Hamill and Martinac, 2001), ion channels within the cell membrane (Lin and Corey 2005) and strain-induced transmitter release (Burnstock, 1999). TUNEL staining for apoptosis showed that a strain of 10% elongation was seriously detrimental to cell survival, whereas a strain of 2.5% and 5% did not lead to apoptosis levels above those observed during normal cellular turnover (Crocker *et al.,* 2001, Vogel *et al.,* 1997). As discussed in chapter 6, levels of strain used in these mechanical stimulation devices are at least an order of magnitude above that observed *in vivo*. However, taking the canalicular fluid flow hypothesis into account (Cowin *et al.,* 1991), the strains imposed on individual cells are more akin to what is observed *in vivo*. The results from this chapter suggest a possibly useful strain mode, magnitude and frequency for the induction of osteogenic differentiation of MSC *in vitro*. Further strain experiments of longer duration are clearly warranted.

Also of possible use for the priming of differentiation pathways are static magnetic fields. Preliminary work in chapter 7 illustrated a field strength dependent activation of the ERK and JNK signalling cascades in cultured cortical neurons. The relatively lower field strength of 0.75 Tesla caused significantly elevated levels of ERK phosphorylation

compared to unstimulated controls, while field strengths of 2T and 5T caused JNK activation, presumably a stress response. As ERK has been demonstrated to be vital for successful osteogenic differentiation of adult MSCs, a possible use of static magnetic fields in the induction of osteogenesis is proposed. Of great importance, would be the understanding of the mechanisms by which cells respond to these magnetic fields, and how they differentiate between the varying intensities. As demonstrated by Kotani *et al.* (2002), there is potential for the use of this stimulus mode in the bone tissue engineering context.

At present, the treatment methods of cartilage and bone related diseases are unsatisfactory and are often only temporary solutions to the problem, particularly with regard to bone and cartilage replacement. Often, repeated treatment or surgical interventions are required to repair damage and refix prosthetics that have come loose over time. This is because of the lack of integration of current prosthetic implants with the tissue of the recipient. The use of autologous or even allogenic stem cells to grow viable bone or cartilage constructs offers huge potential in revolutionising these treatments. Their use would negate the issue of tissue rejection and the need for the lifelong use of immunosuppressants. Biocompatibility would also cease to be a problem between implant and tissue leading to better integration with the host. There is a lot of work to be done in this area before this becomes a reality, but this study not only demonstrates the possibilities, but also brings that reality one step closer.

8.2 Future Work

The work presented in this thesis clearly shows the promise offered by MSC seeded collagen GAG scaffolds. It is also obvious, however that there is some variation in the cellular response at a given timepoint. There are several contributing factors including, inter animal variation, serum batch variation and possible variations in the structure of the collagen GAG scaffold itself. It will be important in the future to try to standardise the response of these constructs so that they produce a predictable level of mineralisation at a given timepoint. The use of pooled cells for a large number of experiments to standardise the biological response and the removal of serum from the medium are possible avenues of future investigation. Batch to batch variation is a large problem with serum and the development of new serum free media, that have promising results, make culturing in its

absence a promising alternative. This would give control over one of the unknown variables.

In order to induce the differentiation of MSCs along specific lineages, certain signalling cascades must be activated. To date I and others have shown the mitogen activated protein kinase ERK to be involved in the differentiation of MSCs along the osteogenic route (Jaiswal *et al.*, 2000) and the subsequent activation of the gene Cbfα-1 (Ducy *et al.*, 1997, Rodan *et al.*, 1997). The stress activated protein kinase JNK is also thought to play a role, though somewhat later in the process, its expression being associated with extracellular matrix synthesis and calcium deposition (Jaiswal *et al.*, 2000). p38 was also shown to play a role in the osteogenic process in this thesis and by others (Jaiswal *et al* 2000). Its activation at 7 days in 2-D and inhibition of osteocalcin expression in 3-D illustrate this fact. However, a precise role for p38 in the osteogenic process still remains elusive. In order to better understand the osteogenic differentiation process, it will be important to determine exactly where p38 fits in. The upstream activators of these pathways are also not currently known. It would be of great interest to determine the receptors responsible for responding to the inductive factor cocktails and inducing ERK and p38 activity.

The spontaneous chondrogenesis phenomenon obviously warrants much further investigation. As discussed in chapter 5, the mechanism by which this takes place is unclear. To determine whether it is the chemical or structural/mechanical environment of the scaffold that is inducing this response, cells could be cultured on 2-D sheets of the same collagen-GAG material and spontaneous collagen type II expression in 2-D and 3-D could be investigated. This would provide an easy way of determining which of these two most likely causes, if either, is inducing this expression. The outcome of that experiment will then dictate further avenues of investigation. It is my intention to also look further at the inhibition of ERK and p38. The fact that neither ERK nor p38 inhibition led to a reduction on collagen II expression was unexpected. Using laser scanning confocal microscopy, I intend to analyse ERK and p38 phosphorylation in samples cultured with and without chondroinductive factors and the ERK and p38 inhibitors. This will not only verify that the specific inhibitors were effective, but it will also show if inhibition of one signalling molecule led to upregulation of the other as a compensatory mechanism. PI3K activation will also be analysed.

ERK has also been shown to be involved in matrix mineralization that has been induced by cyclic strain (Simmons *et al.,* 2003). Angele *et al.* (2003) also demonstrated increased matrix production in chondrocytes when exposed to cyclic strain. Strain has been shown to aid in the repair of fractures in humans and is often used as a treatment using external fixators and rehabilitation regimes. Despite this, no pharmacological alternative has been developed for the activation of the pathways involved (Yang *et al.,* 2002). This is because the mechanisms behind strain induced differentiation are poorly understood. Using both 2-D and 3-D models, attempts will be made to induce the differentiation of adult rat MSCs along both osteogenic and chondrogenic lineages. MSCs will be strained for longer periods of time initially and will be analysed for later stage markers of osteogenic differentiation. If longer stretching periods can lead to further differentiation, the need for expensive non-endogenous factors will be reduced or at least their use can be augmented by biophysical stimuli to increase the rate of differentiation of MSCs along defined lineages. This could give rise to shorter incubation periods and increased matrix synthesis. Understanding the signalling mechanisms behind this process could also be hugely beneficial. The 3-D models of cyclic strain will employ the already used collagen GAG. These scaffolds will be seeded with MSCs and stretched in the same rig. 2-D and 3-D results will be compared. As well as elucidating the signalling behind strain induced differentiation, it is hoped that induction of osteogenesis and chondrogenesis without the use of inductive factors will be possible.

The purpose of this work was to develop tissue engineered bone and cartilage constructs from adult MSCs *in vitro* as a first step to the development of replacement tissues for use in the clinical setting. While there are several areas that warrant further work, complete mineralisation of the collagen GAG scaffold has been demonstrated. The next step is to assess the repair capabilities of these mineralising scaffolds when implanted into a critical size defect. Given the source of these cells, the most suitable animal model would be the rat. There have been several studies examining the suitability of the rat critical size defect as a model for bone repair. Bosch *et al.* (1998) created 5mm critical size defects in the calvaria and observed no spontaneous bone regeneration after 6 and 12 months post-surgery, demonstrating that a defect of this size could be considered critical. Jager *et al.* (2005) state that the rat is at the lower limit of small animals suitable for such experiments but conclude that the femur is also a suitable site for analysis of critical size

defects of 4mm. The mandible is also a suitable site for this work (Arosarena *et al.*, 2003), with lower load bearing than the femur but higher than the calvarial model. Considering the mechanical properties of the current collagen GAG scaffold, carrying out a study in either a non-load bearing defect or a defect undergoing submaximal loads would be most suitable. This would make either the calvarial or mandibular defect most practical for such a study. The size of the defect is also quite suitable. Owing to the cell mediated contraction of the scaffolds over long culture periods, scaffolds with an original size of 8mm by 8mm can often contract to half that size.

The work described in this thesis contributes greatly to the field of tissue engineering. The adult rodent MSC model is ideally suited for the study of 3-D osteogenesis and chondrogenesis in novel scaffolds and the use of mechanical devices for the induction of osteogenesis, due to their ready availability and relatively low cost. For the first time, the ability to culture these MSCs in a novel collagen GAG scaffold was demonstrated and this scaffold was also shown to support complete osteogenic and chondrogenic differentiation of these cells. This demonstrates the highly biocompatible nature of this scaffold and its suitability as a tissue engineering material. The role of the MAP kinases ERK and p38 in osteogenesis and chondrogenesis in 3-D were also demonstrated for the first time. In order to better develop optimal culturing conditions for the induction of differentiation of stem cells in the shortest time possible, it is of the utmost importance to understand the mechanisms behind these differentiation processes. Also, we should not confine ourselves to manipulation of the chemical environment of these cells. As has been discussed, it is becoming increasingly clear that cells have mechanosensing capabilities. Researchers are becoming increasingly aware of the potential of this property. I demonstrated the ability of mechanical strain, in the form of uniaxial tension, to induce collagen type I expression in MSCs in the absence of osteoinductive factors, suggestive of the initiation of osteogenesis. While this result is preliminary and warrants further investigation, to analyse more conclusive markers of osteogenesis at later timepoints, it is promising nonetheless. It is my hope that the work presented here will serve to further the field of tissue engineering to the benefit of all.

References

Aaron RK, Wang S & Ciombor DM. (2002). Upregulation of basal TGFbeta1 levels by EMF coincident with chondrogenesis--implications for skeletal repair and tissue engineering. *J Orthop Res* **20,** 233-240.

Adams M, Reginato MJ, Shao D, Lazar MA & Chatterjee VK. (1997). Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *J Biol Chem* **272,** 5128-5132.

Akhyari P, Fedak PW, Weisel RD, Lee TY, Verma S, Mickle DA & Li RK. (2002). Mechanical stretch regimen enhances the formation of bioengineered autologous cardiac muscle grafts. *Circulation* **106,** I137-142.

Aldinucci C, Garcia JB, Palmi M, Sgaragli G, Benocci A, Meini A, Pessina F, Rossi C, Bonechi C & Pessina GP. (2003). The effect of strong static magnetic field on lymphocytes. *Bioelectromagnetics* **24,** 109-117.

Anderson RE, Kemp JW, Jee WS & Woodbury DM. (1984). Ion-transporting ATPases and matrix mineralization in cultured osteoblastlike cells. *In Vitro* **20,** 837-846.

Angele P, Yoo JU, Smith C, Mansour J, Jepsen KJ, Nerlich M & Johnstone B. (2003). Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated in vitro. *J Orthop Res* **21,** 451-457.

Arosarena OA, Falk A, Malmgren L, Bookman L, Allen MJ, Schoonmaker J, Tatum S & Kellman R. (2003). Defect repair in the rat mandible with bone morphogenic proteins and marrow cells. *Arch Facial Plast Surg* **5,** 103-108.

Attisano L & Wrana JL. (2002). Signal transduction by the TGF-beta superfamily. *Science* **296,** 1646-1647.

Aubin JE. (1999). Osteogenic cell differentiation. In *Bone Formation and Healing*, pp. 19-30.

Aubin JE, Liu F, Malaval L & Gupta AK. (1995). Osteoblast and chondroblast differentiation. *Bone* **17,** 77S-83S.

Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R & Pennesi G. (2005). Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* **35,** 1482-1490.

Awad HA, Butler DL, Boivin GP, Smith FN, Malaviya P, Huibregtse B & Caplan AI. (1999). Autologous mesenchymal stem cell-mediated repair of tendon. *Tissue Eng* **5,** 267-277.

Awad HA, Halvorsen YD, Gimble JM & Guilak F. (2003). Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. *Tissue Eng* **9,** 1301-1312.

Azizi SA, Stokes D, Augelli BJ, DiGirolamo C & Prockop DJ. (1998). Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats--similarities to astrocyte grafts. *Proc Natl Acad Sci U S A* **95,** 3908-3913.

Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R & Quarto R. (2000). Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. *Exp Hematol* **28,** 707-715.

Barry F, Boynton RE, Liu B & Murphy JM. (2001). Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. *Exp Cell Res* **268,** 189-200.

Barry FP, Boynton RE, Haynesworth S, Murphy JM & Zaia J. (1999). The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). *Biochem Biophys Res Commun* **265,** 134-139.

Barry FP & Murphy JM. (2004). Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* **36,** 568-584.

Bayliss MT. (1990). Proteoglycan structure and metabolism during maturation and ageing of human articular cartilage. *Biochem Soc Trans* **18,** 799-802.

Bellows CG, Aubin JE, Heersche JN & Antosz ME. (1986). Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *Calcif Tissue Int* **38,** 143-154.

Bertram JE & Swartz SM. (1991). The 'law of bone transformation': a case of crying Wolff? *Biol Rev Camb Philos Soc* **66,** 245-273.

Bianco P, Riminucci M, Gronthos S & Robey PG. (2001). Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* **19,** 180-192.

Bilezikian J, Raisz L & Rodan GA. (2001). *Principles of Bone Biology.*

Bjornson CR, Rietze RL, Reynolds BA, Magli MC & Vescovi AL. (1999). Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* **283,** 534-537.

Blenis J. (1993). Signal transduction via the MAP kinases: proceed at your own RSK. *Proc Natl Acad Sci U S A* **90,** 5889-5892.

Bobick BE & Kulyk WM. (2004). The MEK-ERK signaling pathway is a negative regulator of cartilage-specific gene expression in embryonic limb mesenchyme. *J Biol Chem* **279,** 4588-4595.

Bonassar LJ. (2002). Cartilage Reconstruction. In *Methods of Tissue Engineering*, pp. 1027-1039. Academic Press.

Bongso A, Fong CY, Ng SC & Ratnam S. (1994). Isolation and culture of inner cell mass cells from human blastocysts. *Hum Reprod* **9,** 2110-2117.

Bosch C, Melsen B & Vargervik K. (1998). Importance of the critical-size bone defect in testing bone-regenerating materials. *J Craniofac Surg* **9,** 310-316.

Bosnakovski D, Mizuno M, Kim G, Ishiguro T, Okumura M, Iwanaga T, Kadosawa T & Fujinaga T. (2004). Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. *Exp Hematol* **32,** 502-509.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH & Yancopoulos GD. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to

insulin and NGF. *Cell* **65,** 663-675.

Boulton TG, Yancopoulos GD, Gregory JS, Slaughter C, Moomaw C, Hsu J & Cobb MH. (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* **249,** 64-67.

Bourdeau A, Dumont DJ & Letarte M. (1999). A murine model of hereditary hemorrhagic telangiectasia. *J Clin Invest* **104,** 1343-1351.

Bruder SP, Horowitz MC, Mosca JD & Haynesworth SE. (1997a). Monoclonal antibodies reactive with human osteogenic cell surface antigens. *Bone* **21,** 225-235.

Bruder SP, Jaiswal N & Haynesworth SE. (1997b). Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* **64,** 278-294.

Bruinink A, Siragusano D, Ettel G, Brandsberg T, Brandsberg F, Petitmermet M, Muller B, Mayer J & Wintermantel E. (2001). The stiffness of bone marrow cell-knit composites is increased during mechanical load. *Biomaterials* **22,** 3169-3178.

Buckley MJ, Banes AJ & Jordan RD. (1990). The effects of mechanical strain on osteoblasts in vitro. *J Oral Maxillofac Surg* **48,** 276-282; discussion 282-273.

Buckwalter JA & Mankin HJ. (1998). Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr Course Lect* **47,** 477-486.

Burg KJ, Delnomdedieu M, Beiler RJ, Culberson CR, Greene KG, Halberstadt CR, Holder WD,

Jr., Loebsack AB, Roland WD & Johnson GA. (2002). Application of magnetic resonance microscopy to tissue engineering: a polylactide model. *J Biomed Mater Res* **61,** 380-390.

Burger EH & Klein-Nulen J. (1999). Responses of bone cells to biomechanical forces in vitro. *Adv Dent Res* **13,** 93-98.

Burger EH & Veldhuijzen JP. (1993). *Bone*, vol. 7.

Burke JF, Yannas IV, Quinby WC, Jr., Bondoc CC & Jung WK. (1981). Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury. *Ann Surg* **194,** 413-428.

Burnstock G. (1999). Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J Anat* **194 (Pt 3),** 335-342.

Burr DB, Milgrom C, Fyhrie D, Forwood M, Nyska M, Finestone A, Hoshaw S, Saiag E & Simkin A. (1996). In vivo measurement of human tibial strains during vigorous activity. *Bone* **18,** 405-410.

Butler DL, Goldstein SA & Guilak F. (2000). Functional tissue engineering: the role of biomechanics. *J Biomech Eng* **122,** 570-575.

Cabrita GJ, Ferreira BS, da Silva CL, Goncalves R, Almeida-Porada G & Cabral JM. (2003). Hematopoietic stem cells: from the bone to the bioreactor. *Trends Biotechnol* **21,** 233-240.

Cancedda R, Descalzi Cancedda F & Castagnola P. (1995). Chondrocyte differentiation. *Int Rev Cytol* **159,** 265-358.

Cano E & Mahadevan LC. (1995). Parallel signal processing among mammalian MAPKs. *Trends Biochem Sci* **20,** 117-122.

Caplan AI. (1991). Mesenchymal stem cells. *J Orthop Res* **9,** 641-650.

Caplan AI. (1994). The mesengenic process. *Clin Plast Surg* **21,** 429-435.

Caplan AI & Bruder SP. (2001). Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med* **7,** 259-264.

Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R, Freed LE & Vunjak-Novakovic G. (1999). Cardiac tissue engineering: cell seeding, cultivation parameters, and tissue construct characterization. *Biotechnol Bioeng* **64,** 580-589.

Carter DR & Giori NJ. (1991). Effect of mechanical stress on tissue differentiation in the bony implant bed. In *The Bone-Biomaterial Interface*, ed. Davies JE, pp. 367-376. University of Toronto Press, Toronto.

Cassiede P, Dennis JE, Ma F & Caplan AI. (1996). Osteochondrogenic potential of marrow mesenchymal progenitor cells exposed to TGF-beta 1 or PDGF-BB as assayed in vivo and in vitro. *J Bone Miner Res* **11,** 1264-1273.

Chamberlain LJ, Yannas IV, Hsu HP, Strichartz G & Spector M. (1998). Collagen-GAG substrate enhances the quality of nerve regeneration through collagen tubes up to level of

autograft. *Exp Neurol* **154,** 315-329.

Chang SH, Oh CD, Yang MS, Kang SS, Lee YS, Sonn JK & Chun JS. (1998). Protein kinase C regulates chondrogenesis of mesenchymes via mitogen-activated protein kinase signaling. *J Biol Chem* **273,** 19213-19219.

Chapekar MS. (2000). Tissue engineering: challenges and opportunities. *J Biomed Mater Res* **53,** 617-620.

Charbord P, Oostendorp R, Pang W, Herault O, Noel F, Tsuji T, Dzierzak E & Peault B. (2002). Comparative study of stromal cell lines derived from embryonic, fetal, and postnatal mouse blood-forming tissues. *Exp Hematol* **30,** 1202-1210.

Chen BM & Grinnell AD. (1997). Kinetics, Ca2+ dependence, and biophysical properties of integrin-mediated mechanical modulation of transmitter release from frog motor nerve terminals. *J Neurosci* **17,** 904-916.

Cobb MH & Goldsmith EJ. (1995). How MAP kinases are regulated. *J Biol Chem* **270,** 14843-14846.

Coelho MJ & Fernandes MH. (2000). Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteoblastic differentiation. *Biomaterials* **21,** 1095-1102.

Cohen NP, Foster RJ & Mow VC. (1998). Composition and dynamics of articular cartilage: structure, function, and maintaining healthy state. *J Orthop Sports Phys Ther* **28,** 203-215.

Conrad C, Gottgens B, Kinston S, Ellwart J & Huss R. (2002). GATA transcription in a small rhodamine 123(low)CD34(+) subpopulation of a peripheral blood-derived CD34(-)CD105(+) mesenchymal cell line. *Exp Hematol* **30,** 887-895.

Corvol MT. (2000). The chondrocyte: from cell aging to osteoarthritis. *Joint Bone Spine* **67,** 557-560.

Cowin SC, Moss-Salentijn L & Moss ML. (1991). Candidates for the mechanosensory system in bone. *J Biomech Eng* **113,** 191-197.

Crews CM, Alessandrini A & Erikson RL. (1992). The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* **258,** 478-480.

Crocker IP, Barratt S, Kaur M & Baker PN. (2001). The in-vitro characterization of induced apoptosis in placental cytotrophoblasts and syncytiotrophoblasts. *Placenta* **22,** 822-830.

Daamen WF, van Moerkerk HT, Hafmans T, Buttafoco L, Poot AA, Veerkamp JH & van Kuppevelt TH. (2003). Preparation and evaluation of molecularly-defined collagen-elastin-glycosaminoglycan scaffolds for tissue engineering. *Biomaterials* **24,** 4001-4009.

Danciu TE, Gagari E, Adam RM, Damoulis PD & Freeman MR. (2004). Mechanical strain delivers anti-apoptotic and proliferative signals to gingival fibroblasts. *J Dent Res* **83,** 596-601.

Datta SR, Brunet A & Greenberg ME. (1999). Cellular survival: a play in three Akts. *Genes Dev* **13,** 2905-2927.

Davisson T, Kunig S, Chen A, Sah R & Ratcliffe A. (2002). Static and dynamic compression modulate matrix metabolism in tissue engineered cartilage. *J Orthop Res* **20,** 842-848.

De Bari C, Dell'Accio F & Luyten FP. (2001). Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. *Arthritis Rheum* **44,** 85-95.

Deak M, Clifton AD, Lucocq LM & Alessi DR. (1998). Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *Embo J* **17,** 4426-4441.

Deans RJ & Moseley AB. (2000). Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol* **28,** 875-884.

DeLise AM, Fischer L & Tuan RS. (2000). Cellular interactions and signaling in cartilage development. *Osteoarthritis Cartilage* **8,** 309-334.

Denegre JM, Valles JM, Jr., Lin K, Jordan WB & Mowry KL. (1998). Cleavage planes in frog eggs are altered by strong magnetic fields. *Proc Natl Acad Sci U S A* **95,** 14729-14732.

Dennis JE, Cohen N, Goldberg VM & Caplan AI. (2004). Targeted delivery of progenitor cells for cartilage repair. *J Orthop Res* **22,** 735-741.

Dent P, Haser W, Haystead TA, Vincent LA, Roberts TM & Sturgill TW. (1992). Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. *Science* **257,** 1404-1407.

Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M & Davis RJ. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76,** 1025-1037.

Devine SM. (2002). Mesenchymal stem cells: will they have a role in the clinic? *J Cell Biochem Suppl* **38,** 73-79.

Donati I, Stredanska S, Silvestrini G, Vetere A, Marcon P, Marsich E, Mozetic P, Gamini A, Paoletti S & Vittur F. (2005). The aggregation of pig articular chondrocyte and synthesis of extracellular matrix by a lactose-modified chitosan. *Biomaterials* **26,** 987-998.

Ducy P, Zhang R, Geoffroy V, Ridall AL & Karsenty G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89,** 747-754.

Duncia JV, Santella JB, 3rd, Higley CA, Pitts WJ, Wityak J, Frietze WE, Rankin FW, Sun JH, Earl RA, Tabaka AC, Teleha CA, Blom KF, Favata MF, Manos EJ, Daulerio AJ, Stradley DA, Horiuchi K, Copeland RA, Scherle PA, Trzaskos JM, Magolda RL, Trainor GL, Wexler RR, Hobbs FW & Olson RE. (1998). MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorg Med Chem Lett* **8,** 2839-2844.

Eilers A, Whitfield J, Shah B, Spadoni C, Desmond H & Ham J. (2001). Direct inhibition of c-Jun N-terminal kinase in sympathetic neurones prevents c-jun promoter activation and NGF withdrawal-induced death. *J Neurochem* **76,** 1439-1454.

Engelbrecht Y, de Wet H, Horsch K, Langeveldt CR, Hough FS & Hulley PA. (2003).

Glucocorticoids induce rapid up-regulation of mitogen-activated protein kinase phosphatase-1 and dephosphorylation of extracellular signal-regulated kinase and impair proliferation in human and mouse osteoblast cell lines. *Endocrinology* **144,** 412-422.

Evans MJ & Kaufman MH. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154-156.

Fedewa MM, Oegema TR, Jr., Schwartz MH, MacLeod A & Lewis JL. (1998). Chondrocytes in culture produce a mechanically functional tissue. *J Orthop Res* **16,** 227-236.

Fitzsimmons JS, Sanyal A, Gonzalez C, Fukumoto T, Clemens VR, O'Driscoll SW & Reinholz GG. (2004). Serum-free media for periosteal chondrogenesis in vitro. *J Orthop Res* **22,** 716-725.

Fortier LA, Nixon AJ, Williams J & Cable CS. (1998). Isolation and chondrocytic differentiation of equine bone marrow-derived mesenchymal stem cells. *Am J Vet Res* **59,** 1182-1187.

Forwood MR. (1996). Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. *J Bone Miner Res* **11,** 1688-1693.

Frank EH & Grodzinsky AJ. (1987). Cartilage electromechanics--I. Electrokinetic transduction and the effects of electrolyte pH and ionic strength. *J Biomech* **20,** 615-627.

Freed LE, Hollander AP, Martin I, Barry JR, Langer R & Vunjak-Novakovic G. (1998). Chondrogenesis in a cell-polymer-bioreactor system. *Exp Cell Res* **240,** 58-65.

Freed LE, Langer R, Martin I, Pellis NR & Vunjak-Novakovic G. (1997). Tissue engineering of

cartilage in space. *Proc Natl Acad Sci U S A* **94,** 13885-13890.

Friedenstein AJ, Petrakova KV, Kurolesova AI & Frolova GP. (1968). Heterotopic of bone marrow.Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* **6,** 230-247.

Friedenstein AJ, Piatetzky S, II & Petrakova KV. (1966). Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* **16,** 381-390.

Frost HM. (1992). Perspectives: bone's mechanical usage windows. *Bone Miner* **19,** 257-271.

Frost JA, Steen H, Shapiro P, Lewis T, Ahn N, Shaw PE & Cobb MH. (1997). Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. *Embo J* **16,** 6426-6438.

Fujita T, Fukuyama R, Enomoto H & Komori T. (2004). Dexamethasone inhibits insulin-induced chondrogenesis of ATDC5 cells by preventing PI3K-Akt signaling and DNA binding of Runx2. *J Cell Biochem* **93,** 374-383.

Galli R, Borello U, Gritti A, Minasi MG, Bjornson C, Coletta M, Mora M, De Angelis MG, Fiocco R, Cossu G & Vescovi AL. (2000). Skeletal myogenic potential of human and mouse neural stem cells. *Nat Neurosci* **3,** 986-991.

Gerber I & ap Gwynn I. (2002). Differentiation of rat osteoblast-like cells in monolayer and micromass cultures. *Eur Cell Mater* **3,** 19-30.

Goedert M, Cuenda A, Craxton M, Jakes R & Cohen P. (1997). Activation of the novel stress-

activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *Embo J* **16,** 3563-3571.

Golden LH & Insogna KL. (2004). The expanding role of PI3-kinase in bone. *Bone* **34,** 3-12.

Goldstein AS. (2001). Effect of seeding osteoprogenitor cells as dense clusters on cell growth and differentiation. *Tissue Eng* **7,** 817-827.

Gooch KJ, Kwon JH, Blunk T, Langer R, Freed LE & Vunjak-Novakovic G. (2001). Effects of mixing intensity on tissue-engineered cartilage. *Biotechnol Bioeng* **72,** 402-407.

Gredinger E, Gerber AN, Tamir Y, Tapscott SJ & Bengal E. (1998). Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells. *J Biol Chem* **273,** 10436-10444.

Guicheux J, Lemonnier J, Ghayor C, Suzuki A, Palmer G & Caverzasio J. (2003). Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. *J Bone Miner Res* **18,** 2060-2068.

Hamill OP & Martinac B. (2001). Molecular basis of mechanotransduction in living cells. *Physiol Rev* **81,** 685-740.

Han J, Lee JD, Bibbs L & Ulevitch RJ. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265,** 808-811.

Hanada K, Solchaga LA, Caplan AI, Hering TM, Goldberg VM, Yoo JU & Johnstone B. (2001). BMP-2 induction and TGF-beta 1 modulation of rat periosteal cell chondrogenesis. *J Cell Biochem* **81,** 284-294.

Hannigan M, Zhan L, Ai Y & Huang CK. (1998). The role of p38 MAP kinase in TGF-beta1-induced signal transduction in human neutrophils. *Biochem Biophys Res Commun* **246,** 55-58.

Harter LV, Hruska KA & Duncan RL. (1995). Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation. *Endocrinology* **136,** 528-535.

Hasegawa S, Sato S, Saito S, Suzuki Y & Brunette DM. (1985). Mechanical stretching increases the number of cultured bone cells synthesizing DNA and alters their pattern of protein synthesis. *Calcif Tissue Int* **37,** 431-436.

Hauschka PV, Frenkel J, DeMuth R & Gundberg CM. (1983). Presence of osteocalcin and related higher molecular weight 4-carboxyglutamic acid-containing proteins in developing bone. *J Biol Chem* **258,** 176-182.

Haynesworth SE, Baber MA & Caplan AI. (1992). Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone* **13,** 69-80.

Herbertson A & Aubin JE. (1997). Cell sorting enriches osteogenic populations in rat bone marrow stromal cell cultures. *Bone* **21,** 491-500.

Herr I & Debatin KM. (2001). Cellular stress response and apoptosis in cancer therapy. *Blood*

98, 2603-2614.

Herzog EL, Chai L & Krause DS. (2003). Plasticity of marrow-derived stem cells. *Blood* **102,** 3483-3493.

Hidaka K, Kanematsu T, Takeuchi H, Nakata M, Kikkawa U & Hirata M. (2001). Involvement of the phosphoinositide 3-kinase/protein kinase B signaling pathway in insulin/IGF-I-induced chondrogenesis of the mouse embryonal carcinoma-derived cell line ATDC5. *Int J Biochem Cell Biol* **33,** 1094-1103.

Hirota Y, Tsukazaki T, Yonekura A, Miyazaki Y, Osaki M, Shindo H & Yamashita S. (2000). Activation of specific MEK-ERK cascade is necessary for TGFbeta signaling and crosstalk with PKA and PKC pathways in cultured rat articular chondrocytes. *Osteoarthritis Cartilage* **8,** 241-247.

Holy CE, Shoichet MS & Davies JE. (2000). Engineering three-dimensional bone tissue in vitro using biodegradable scaffolds: investigating initial cell-seeding density and culture period. *J Biomed Mater Res* **51,** 376-382.

Honda M, Morikawa N, Hata K, Yada T, Morita S, Ueda M & Kimata K. (2003). Rat costochondral cell characteristics on poly (L-lactide-co-epsilon-caprolactone) scaffolds. *Biomaterials* **24,** 3511-3519.

Hu E, Kim JB, Sarraf P & Spiegelman BM. (1996). Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. *Science* **274,** 2100-2103.

Huang CY, Hagar KL, Frost LE, Sun Y & Cheung HS. (2004). Effects of cyclic compressive

loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells. *Stem Cells* **22,** 313-323.

Igarashi M, Kamiya N, Hasegawa M, Kasuya T, Takahashi T & Takagi M. (2004). Inductive effects of dexamethasone on the gene expression of Cbfa1, Osterix and bone matrix proteins during differentiation of cultured primary rat osteoblasts. *J Mol Histol* **35,** 3-10.

Ignatius A, Blessing H, Liedert A, Schmidt C, Neidlinger-Wilke C, Kaspar D, Friemert B & Claes L. (2005). Tissue engineering of bone: effects of mechanical strain on osteoblastic cells in type I collagen matrices. *Biomaterials* **26,** 311-318.

Indrawattana N, Chen G, Tadokoro M, Shann LH, Ohgushi H, Tateishi T, Tanaka J & Bunyaratvej A. (2004). Growth factor combination for chondrogenic induction from human mesenchymal stem cell. *Biochem Biophys Res Commun* **320,** 914-919.

Ishaug-Riley SL, Crane-Kruger GM, Yaszemski MJ & Mikos AG. (1998). Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* **19,** 1405-1412.

Ishaug SL, Crane GM, Miller MJ, Yasko AW, Yaszemski MJ & Mikos AG. (1997). Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J Biomed Mater Res* **36,** 17-28.

Ishida H, Bellows CG, Aubin JE & Heersche JN. (1995). Tri-iodothyronine (T3) and dexamethasone interact to modulate osteoprogenitor cell differentiation in fetal rat calvaria cell cultures. *Bone* **16,** 545-549.

Jager M, Sager M, Lensing-Hohn S & Krauspe R. (2005). The critical size bony defect in a small animal for bone healing studies (II): implant evolution and surgical technique on a rat's femur. *Biomed Tech (Berl)* **50,** 137-142.

Jagodzinski M, Drescher M, Zeichen J, Hankemeier S, Krettek C, Bosch U & van Griensven M. (2004). Effects of cyclic longitudinal mechanical strain and dexamethasone on osteogenic differentiation of human bone marrow stromal cells. *Eur Cell Mater* **7,** 35-41; discussion 41.

Jaiswal N, Haynesworth SE, Caplan AI & Bruder SP. (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem* **64,** 295-312.

Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR & Pittenger MF. (2000). Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. *J Biol Chem* **275,** 9645-9652.

Jee WS & Li XJ. (1990). Adaptation of cancellous bone to overloading in the adult rat: a single photon absorptiometry and histomorphometry study. *Anat Rec* **227,** 418-426.

Jenkins DD, Yang GP, Lorenz HP, Longaker MT & Sylvester KG. (2003). Tissue engineering and regenerative medicine. *Clin Plast Surg* **30,** 581-588.

Jessop HL, Rawlinson SC, Pitsillides AA & Lanyon LE. (2002). Mechanical strain and fluid movement both activate extracellular regulated kinase (ERK) in osteoblast-like cells but via different signaling pathways. *Bone* **31,** 186-194.

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA & Verfaillie CM. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418,** 41-49.

Johnsen S & Lohmann KJ. (2005). The physics and neurobiology of magnetoreception. *Nat Rev Neurosci* **6,** 703-712.

Johnstone B, Hering TM, Caplan AI, Goldberg VM & Yoo JU. (1998). In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* **238,** 265-272.

Jones D, Leivseth G & Tenbosch J. (1995). Mechano-reception in osteoblast-like cells. *Biochem Cell Biol* **73,** 525-534.

Jung M, Gotterbarm T, Gruettgen A, Vilei SB, Breusch S & Richter W. (2005). Molecular characterization of spontaneous and growth-factor-augmented chondrogenesis in periosteum-bone tissue transferred into a joint. *Histochem Cell Biol* **123,** 447-456.

Kadiyala S, Young RG, Thiede MA & Bruder SP. (1997). Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro. *Cell Transplant* **6,** 125-134.

Kapeller R & Cantley LC. (1994). Phosphatidylinositol 3-kinase. *BioEssays* **16,** 565-576.

Kaspar D, Seidl W, Neidlinger-Wilke C, Beck A, Claes L & Ignatius A. (2002). Proliferation of human-derived osteoblast-like cells depends on the cycle number and frequency of uniaxial strain. *J Biomech* **35,** 873-880.

Kaspar D, Seidl W, Neidlinger-Wilke C, Ignatius A & Claes L. (2000). Dynamic cell stretching increases human osteoblast proliferation and CICP synthesis but decreases osteocalcin synthesis and alkaline phosphatase activity. *J Biomech* **33,** 45-51.

Kassel O, Sancono A, Kratzschmar J, Kreft B, Stassen M & Cato AC. (2001). Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *Embo J* **20,** 7108-7116.

Kasugai S, Todescan R, Jr., Nagata T, Yao KL, Butler WT & Sodek J. (1991). Expression of bone matrix proteins associated with mineralized tissue formation by adult rat bone marrow cells in vitro: inductive effects of dexamethasone on the osteoblastic phenotype. *J Cell Physiol* **147,** 111-120.

Keller R. (2002). Stem cells on the way to restorative medicine. *Immunol Lett* **83,** 1-12.

Kelly DJ & Prendergast PJ. (2004). Effect of a degraded core on the mechanical behaviour of tissue-engineered cartilage constructs: a poro-elastic finite element analysis. *Med Biol Eng Comput* **42,** 9-13.

Kelly DJ, Prendergast PJ & Blayney AW. (2003). The effect of prosthesis design on vibration of the reconstructed ossicular chain: a comparative finite element analysis of four prostheses. *Otol Neurotol* **24,** 11-19.

Kim BS, Putnam AJ, Kulik TJ & Mooney DJ. (1998). Optimizing seeding and culture methods to engineer smooth muscle tissue on biodegradable polymer matrices. *Biotechnol Bioeng* **57,** 46-54.

Kim H, Suh H, Jo SA, Kim HW, Lee JM, Kim EH, Reinwald Y, Park SH, Min BH & Jo I. (2005). In vivo bone formation by human marrow stromal cells in biodegradable scaffolds that release dexamethasone and ascorbate-2-phosphate. *Biochem Biophys Res Commun* **332,** 1053-1060.

Kirschtein R. (2001). Stem Cells: Scientific progress and future research directions, ed. health Rpbtnio.

Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ & Burger EH. (1995). Sensitivity of osteocytes to biomechanical stress in vitro. *Faseb J* **9,** 441-445.

Kobayashi N, Yasu T, Ueba H, Sata M, Hashimoto S, Kuroki M, Saito M & Kawakami M. (2004). Mechanical stress promotes the expression of smooth muscle-like properties in marrow stromal cells. *Exp Hematol* **32,** 1238-1245.

Kosako H, Gotoh Y, Matsuda S, Ishikawa M & Nishida E. (1992). Xenopus MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. *Embo J* **11,** 2903-2908.

Kotani H, Kawaguchi H, Shimoaka T, Iwasaka M, Ueno S, Ozawa H, Nakamura K & Hoshi K. (2002). Strong static magnetic field stimulates bone formation to a definite orientation in vitro and in vivo. *J Bone Miner Res* **17,** 1814-1821.

Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC & Young PR. (1997). Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem Biophys Res Commun* **235,** 533-538.

Kummer JL, Rao PK & Heidenreich KA. (1997). Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. *J Biol Chem* **272,** 20490-20494.

Kung C. (2005). A possible unifying principle for mechanosensation. *Nature* **436,** 647-654.

Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR & Avruch J. (1992). Raf-1 activates MAP kinase-kinase. *Nature* **358,** 417-421.

Kyriakis JM & Avruch J. (1990). pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J Biol Chem* **265,** 17355-17363.

Kyriakis JM & Avruch J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* **81,** 807-869.

Langer RS & Vacanti JP. (1999). Tissue engineering: the challenges ahead. *Sci Am* **280,** 86-89.

Lanyon LE, Hampson WG, Goodship AE & Shah JS. (1975). Bone deformation recorded in vivo from strain gauges attached to the human tibial shaft. *Acta Orthop Scand* **46,** 256-268.

Laurencin CT, Attawia MA, Elgendy HE & Herbert KM. (1996). Tissue engineered bone-regeneration using degradable polymers: the formation of mineralized matrices. *Bone* **19,** 93S-99S.

Lee AJ, Hodges S & Eastell R. (2000). Measurement of osteocalcin. *Ann Clin Biochem* **37 (Pt 4),** 432-446.

Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P & Karsenty G. (1997). Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat Genet* **16,** 307-310.

Lee JW, Kim YH, Kim SH, Han SH & Hahn SB. (2004a). Chondrogenic differentiation of mesenchymal stem cells and its clinical applications. *Yonsei Med J* **45 Suppl,** 41-47.

Lee SJ, Jeon HB, Lee JH, Yoo JS, Chun JS & Yoo YJ. (2004b). Identification of proteins differentially expressed during chondrogenesis of mesenchymal cells. *FEBS Lett* **563,** 35-40.

Legeai-Mallet L, Benoist-Lasselin C, Delezoide AL, Munnich A & Bonaventure J. (1998). Fibroblast growth factor receptor 3 mutations promote apoptosis but do not alter chondrocyte proliferation in thanatophoric dysplasia. *J Biol Chem* **273,** 13007-13014.

LeGeros RZ. (2002). Properties of osteoconductive biomaterials: calcium phosphates. *Clin Orthop Relat Res*, 81-98.

Li X, Jin L, Cui Q, Wang GJ & Balian G. (2005). Steroid effects on osteogenesis through mesenchymal cell gene expression. *Osteoporos Int* **16,** 101-108.

Li Y, Ma T, Kniss DA, Lasky LC & Yang ST. (2001). Effects of filtration seeding on cell density, spatial distribution, and proliferation in nonwoven fibrous matrices. *Biotechnol*

Prog **17,** 935-944.

Lim YB, Kang SS, An WG, Lee YS, Chun JS & Sonn JK. (2003). Chondrogenesis induced by actin cytoskeleton disruption is regulated via protein kinase C-dependent p38 mitogen-activated protein kinase signaling. *J Cell Biochem* **88,** 713-718.

Lim YB, Kang SS, Park TK, Lee YS, Chun JS & Sonn JK. (2000). Disruption of actin cytoskeleton induces chondrogenesis of mesenchymal cells by activating protein kinase C-alpha signaling. *Biochem Biophys Res Commun* **273,** 609-613.

Lin SY & Corey DP. (2005). TRP channels in mechanosensation. *Curr Opin Neurobiol* **15,** 350-357.

Liu F, Aubin JE & Malaval L. (2002). Expression of leukemia inhibitory factor (LIF)/interleukin-6 family cytokines and receptors during in vitro osteogenesis: differential regulation by dexamethasone and LIF. *Bone* **31,** 212-219.

Locklin RM, Oreffo RO & Triffitt JT. (1999). Effects of TGFbeta and bFGF on the differentiation of human bone marrow stromal fibroblasts. *Cell Biol Int* **23,** 185-194.

Lodie TA, Blickarz CE, Devarakonda TJ, He C, Dash AB, Clarke J, Gleneck K, Shihabuddin L & Tubo R. (2002). Systematic analysis of reportedly distinct populations of multipotent bone marrow-derived stem cells reveals a lack of distinction. *Tissue Eng* **8,** 739-751.

Lodish H, Berk A & Zipursky SL. (2000). *Molecular Cell Biology*. W.H. Freeman and Co., New York.

Loeser RF & Shanker G. (2000). Autocrine stimulation by insulin-like growth factor 1 and insulin-like growth factor 2 mediates chondrocyte survival in vitro. *Arthritis Rheum* **43,** 1552-1559.

Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO & Pittenger MF. (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* **4,** 415-428.

Malagoli D, Gobba F & Ottaviani E. (2003). Effects of 50-Hz magnetic fields on the signalling pathways of fMLP-induced shape changes in invertebrate immunocytes: the activation of an alternative "stress pathway". *Biochimica et Biophysica Acta (BBA) - General Subjects* **1620,** 185.

Mandl EW, van der Veen SW, Verhaar JA & van Osch GJ. (2002). Serum-free medium supplemented with high-concentration FGF2 for cell expansion culture of human ear chondrocytes promotes redifferentiation capacity. *Tissue Eng* **8,** 573-580.

Maniatopoulos C, Sodek J & Melcher AH. (1988). Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* **254,** 317-330.

Mansour SJ, Resing KA, Candi JM, Hermann AS, Gloor JW, Herskind KR, Wartmann M, Davis RJ & Ahn NG. (1994). Mitogen-activated protein (MAP) kinase phosphorylation of MAP kinase kinase: determination of phosphorylation sites by mass spectrometry and site-directed mutagenesis. *J Biochem (Tokyo)* **116,** 304-314.

Martin GR. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* **78,** 7634-7638.

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

Martin I, Padera RF, Vunjak-Novakovic G & Freed LE. (1998). In vitro differentiation of chick embryo bone marrow stromal cells into cartilaginous and bone-like tissues. *J Orthop Res* **16,** 181-189.

Martin I, Wendt D & Heberer M. (2004). The role of bioreactors in tissue engineering. *Trends Biotechnol* **22,** 80-86.

Mauck RL, Soltz MA, Wang CC, Wong DD, Chao PH, Valhmu WB, Hung CT & Ateshian GA. (2000). Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. *J Biomech Eng* **122,** 252-260.

McKay R. (1997). Stem cells in the central nervous system. *Science* **276,** 66-71.

Meier R, Rouse J, Cuenda A, Nebreda AR & Cohen P. (1996). Cellular stresses and cytokines activate multiple mitogen-activated-protein kinase kinase homologues in PC12 and KB cells. *Eur J Biochem* **236,** 796-805.

Meunier P, Aaron J, Edouard C & Vignon G. (1971). Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. A quantitative study of 84 iliac bone biopsies. *Clin Orthop Relat Res* **80,** 147-154.

Miyazaki Y, Tsukazaki T, Hirota Y, Yonekura A, Osaki M, Shindo H & Yamashita S. (2000).

Dexamethasone inhibition of TGF beta-induced cell growth and type II collagen mRNA expression through ERK-integrated AP-1 activity in cultured rat articular chondrocytes. *Osteoarthritis Cartilage* **8,** 378-385.

Moore EE, Bendele AM, Thompson DL, Littau A, Waggie KS, Reardon B & Ellsworth JL. (2005). Fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair in a rat model of injury-induced osteoarthritis. *Osteoarthritis Cartilage* **13,** 623-631.

Moretti M, Prina-Mello A, Reid AJ, Barron V & Prendergast PJ. (2004). Endothelial cell alignment on cyclically-stretched silicone surfaces. *J Mater Sci Mater Med* **15,** 1159-1164.

Muraglia A, Cancedda R & Quarto R. (2000). Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J Cell Sci* **113 (Pt 7),** 1161-1166.

Murakami S, Kan M, McKeehan WL & de Crombrugghe B. (2000). Up-regulation of the chondrogenic Sox9 gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. *Proc Natl Acad Sci U S A* **97,** 1113-1118.

Murray DW & Rushton N. (1990). The effect of strain on bone cell prostaglandin E2 release: a new experimental method. *Calcif Tissue Int* **47,** 35-39.

Nakajima M, Negishi Y, Tanaka H & Kawashima K. (2004). p21(Cip-1/SDI-1/WAF-1) expression via the mitogen-activated protein kinase signaling pathway in insulin-induced chondrogenic differentiation of ATDC5 cells. *Biochem Biophys Res Commun* **320,** 1069-1075.

Nakamura K, Shirai T, Morishita S, Uchida S, Saeki-Miura K & Makishima F. (1999). p38 mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells. *Exp Cell Res* **250,** 351-363.

Negishi Y, Ui N, Nakajima M, Kawashima K, Maruyama K, Takizawa T & Endo H. (2001). p21Cip-1/SDI-1/WAF-1 gene is involved in chondrogenic differentiation of ATDC5 cells in vitro. *J Biol Chem* **276,** 33249-33256.

Nehrer S, Breinan HA, Ramappa A, Young G, Shortkroff S, Louie LK, Sledge CB, Yannas IV & Spector M. (1997). Matrix collagen type and pore size influence behaviour of seeded canine chondrocytes. *Biomaterials* **18,** 769-776.

Neidlinger-Wilke C, Stalla I, Claes L, Brand R, Hoellen I, Rubenacker S, Arand M & Kinzl L. (1995). Human osteoblasts from younger normal and osteoporotic donors show differences in proliferation and TGF beta-release in response to cyclic strain. *J Biomech* **28,** 1411-1418.

Neidlinger-Wilke C, Wilke HJ & Claes L. (1994). Cyclic stretching of human osteoblasts affects proliferation and metabolism: a new experimental method and its application. *J Orthop Res* **12,** 70-78.

Nerem R. (2000). In *Principles of Tissue Engineering*, ed. Lanza RP, Langer R & Vacanti JP, pp. 9-15. Academic press, New York.

Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R & Langer R. (1999). Functional arteries grown in vitro. *Science* **284,** 489-493.

Norimura T, Imada H, Kunugita N, Yoshida N & Nikaido M. (1993). Effects of strong magnetic fields on cell growth and radiation response of human T-lymphocytes in culture. *J Uoeh* **15,** 103-112.

Nuttall ME, Patton AJ, Olivera DL, Nadeau DP & Gowen M. (1998). Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: implications for osteopenic disorders. *J Bone Miner Res* **13,** 371-382.

O'Brien FJ, Harley BA, Yannas IV & Gibson L. (2004). Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds. *Biomaterials* **25,** 1077-1086.

O'Brien FJ, Harley BA, Yannas IV & Gibson LJ. (2005). The effect of pore size on cell adhesion in collagen-GAG scaffolds. *Biomaterials* **26,** 433-441.

O'Driscoll SW. (1998). The healing and regeneration of articular cartilage. *J Bone Joint Surg Am* **80,** 1795-1812.

Oh CD, Chang SH, Yoon YM, Lee SJ, Lee YS, Kang SS & Chun JS. (2000). Opposing role of mitogen-activated protein kinase subtypes, erk-1/2 and p38, in the regulation of chondrogenesis of mesenchymes. *J Biol Chem* **275,** 5613-5619.

Oh CD & Chun JS. (2003). Signaling mechanisms leading to the regulation of differentiation and apoptosis of articular chondrocytes by insulin-like growth factor-1. *J Biol Chem* **278,** 36563-36571.

Oh CD, Kim SJ, Ju JW, Song WK, Kim JH, Yoo YJ & Chun JS. (2001). Immunosuppressant rapamycin inhibits protein kinase C alpha and p38 mitogen-activated protein kinase

leading to the inhibition of chondrogenesis. *Eur J Pharmacol* **427,** 175-185.

Ohgushi H, Dohi Y, Katuda T, Tamai S, Tabata S & Suwa Y. (1996). In vitro bone formation by rat marrow cell culture. *J Biomed Mater Res* **32,** 333-340.

Osyczka AM & Leboy PS. (2005). Bone morphogenetic protein regulation of early osteoblast genes in human marrow stromal cells is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase signaling. *Endocrinology* **146,** 3428-3437.

Owan I, Burr DB, Turner CH, Qiu J, Tu Y, Onyia JE & Duncan RL. (1997). Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain. *Am J Physiol* **273,** C810-815.

Owen M. (1985). Lineage of osteogenic cells and their relationship to the stromal system. *Bone and Mineral Research* **3**.

Pacini S, Aterini S, Pacini P, Ruggiero C, Gulisano M & Ruggiero M. (1999a). Influence of static magnetic field on the antiproliferative effects of vitamin D on human breast cancer cells. *Oncol Res* **11,** 265-271.

Pacini S, Vannelli GB, Barni T, Ruggiero M, Sardi I, Pacini P & Gulisano M. (1999b). Effect of 0.2 T static magnetic field on human neurons: remodeling and inhibition of signal transduction without genome instability. *Neurosci Lett* **267,** 185-188.

Palmer GD, Steinert A, Pascher A, Gouze E, Gouze JN, Betz O, Johnstone B, Evans CH & Ghivizzani SC. (2005). Gene-induced chondrogenesis of primary mesenchymal stem cells in vitro. *Mol Ther* **12,** 219-228.

Park J, Kim I, Oh YJ, Lee K, Han PL & Choi EJ. (1997). Activation of c-Jun N-terminal kinase antagonizes an anti-apoptotic action of Bcl-2. *J Biol Chem* **272,** 16725-16728.

Pauwels F. (1980). A new theory concerning the influence of mechanical stimuli on the differentiation of the supporting tissues. In *Biomechanics of the locomotor apparatus*, pp. 375-407.

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K & Cobb MH. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* **22,** 153-183.

Pedersen SF, Owsianik G & Nilius B. (2005). TRP channels: An overview. *Cell Calcium* **38,** 233-252.

Pederson R. (1999). Embryonic stem cells for medicine. *Sci Am* **280,** 44.

Pei M, Solchaga LA, Seidel J, Zeng L, Vunjak-Novakovic G, Caplan AI & Freed LE. (2002). Bioreactors mediate the effectiveness of tissue engineering scaffolds. *Faseb J* **16,** 1691-1694.

Perry D. (2000). Patients' voices: the powerful sound in the stem cell debate. *Science* **287,** 1423.

Petrakova KV, Tolmacheva AA & AIa F. (1963). [Bone Formation Occurring In Bone Marrow Transplantation In Diffusion Chambers.]. *Biull Eksp Biol Med* **56,** 87-91.

Pieper JS, van Wachem PB, van Luyn MJA, Brouwer LA, Hafmans T, Veerkamp JH & van Kuppevelt TH. (2000). Attachment of glycosaminoglycans to collagenous matrices modulates the tissue response in rats. *Biomaterials* **21,** 1689-1699.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S & Marshak DR. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* **284,** 143-147.

Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC & Bellido T. (1999). Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. *J Clin Invest* **104,** 1363-1374.

Pommerenke H, Schreiber E, Durr F, Nebe B, Hahnel C, Moller W & Rychly J. (1996). Stimulation of integrin receptors using a magnetic drag force device induces an intracellular free calcium response. *Eur J Cell Biol* **70,** 157-164.

Port FK. (2002). OPTN/SRTS annual report. In *Scientific Registry of Transplant Recipients and Organ Procurement Transplantation Network.* Washington, D.C.

Porter RM, Huckle WR & Goldstein AS. (2003). Effect of dexamethasone withdrawal on osteoblastic differentiation of bone marrow stromal cells. *J Cell Biochem* **90,** 13-22.

Powell CA, Smiley BL, Mills J & Vandenburgh HH. (2002). Mechanical stimulation improves tissue-engineered human skeletal muscle. *Am J Physiol Cell Physiol* **283,** C1557-1565.

Prendergast PJ, Huiskes R & Soballe K. (1997). ESB Research Award 1996. Biophysical stimuli on cells during tissue differentiation at implant interfaces. *J Biomech* **30,** 539-548.

Pri-Chen S, Pitaru S, Lokiec F & Savion N. (1998). Basic fibroblast growth factor enhances the growth and expression of the osteogenic phenotype of dexamethasone-treated human bone marrow-derived bone-like cells in culture. *Bone* **23,** 111-117.

Prina-Mello A, Farrell E, Prendergast PJ & Campbell VA. (2005). Influence of strong static magnetic fields on primary cortical neurons. *Bioelectromagnetism* **In Press**.

Quarto R, Campanile G, Cancedda R & Dozin B. (1992). Thyroid hormone, insulin, and glucocorticoids are sufficient to support chondrocyte differentiation to hypertrophy: a serum-free analysis. *J Cell Biol* **119,** 989-995.

Raimondi MT, Boschetti F, Falcone L, Fiore GB, Remuzzi A, Marinoni E, Marazzi M & Pietrabissa R. (2002). Mechanobiology of engineered cartilage cultured under a quantified fluid-dynamic environment. *Biomech Model Mechanobiol* **1,** 69-82.

Ray LB & Sturgill TW. (1988). Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo. *Proc Natl Acad Sci U S A* **85,** 3753-3757.

Raylman RR, Clavo AC & Wahl RL. (1996). Exposure to strong static magnetic field slows the growth of human cancer cells in vitro. *Bioelectromagnetics* **17,** 358-363.

Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L & Verfaillie CM. (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* **98,** 2615-2625.

Ringe J, Kaps C, Burmester GR & Sittinger M. (2002). Stem cells for regenerative medicine: advances in the engineering of tissues and organs. *Naturwissenschaften* **89,** 338-351.

Robinson MJ, Cheng M, Khokhlatchev A, Ebert D, Ahn N, Guan KL, Stein B, Goldsmith E & Cobb MH. (1996). Contributions of the mitogen-activated protein (MAP) kinase backbone and phosphorylation loop to MEK specificity. *J Biol Chem* **271,** 29734-29739.

Rodan GA & Harada S. (1997). The missing bone. *Cell* **89,** 677-680.

Rogachefsky RA, Altman RD, Markov MS & Cheung HS. (2004). Use of a permanent magnetic field to inhibit the development of canine osteoarthritis. *Bioelectromagnetics* **25,** 260-270.

Rose FR & Oreffo RO. (2002). Bone tissue engineering: hope vs hype. *Biochem Biophys Res Commun* **292,** 1-7.

Rossomando AJ, Dent P, Sturgill TW & Marshak DR. (1994). Mitogen-activated protein kinase kinase 1 (MKK1) is negatively regulated by threonine phosphorylation. *Mol Cell Biol* **14,** 1594-1602.

Rossomando AJ, Payne DM, Weber MJ & Sturgill TW. (1989). Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. *Proc Natl Acad Sci U S A* **86,** 6940-6943.

Rozenblatt-Rosen O, Mosonego-Ornan E, Sadot E, Madar-Shapiro L, Sheinin Y, Ginsberg D & Yayon A. (2002). Induction of chondrocyte growth arrest by FGF: transcriptional and cytoskeletal alterations. *J Cell Sci* **115,** 553-562.

Rubin CT & Lanyon LE. (1984). Regulation of bone formation by applied dynamic loads. *J Bone Joint Surg Am* **66,** 397-402.

Rubin CT, McLeod KJ & Bain SD. (1990). Functional strains and cortical bone adaptation: epigenetic assurance of skeletal integrity. *J Biomech* **23 Suppl 1,** 43-54.

Salgado AJ, Coutinho OP & Reis RL. (2004). Bone tissue engineering: state of the art and future trends. *Macromol Biosci* **4,** 743-765.

Sanchez-Ramos JR. (2002). Neural cells derived from adult bone marrow and umbilical cord blood. *J Neurosci Res* **69,** 880-893.

Sandell LJ & Adler P. (1999). Developmental patterns of cartilage. *Front Biosci* **4,** D731-742.

Satomura K, Hiraiwa K & Nagayama M. (1991). Mineralized nodule formation in rat bone marrow stromal cell culture without beta-glycerophosphate. *Bone Miner* **14,** 41-54.

Schacke H, Docke WD & Asadullah K. (2002). Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* **96,** 23-43.

Schaeffer HJ & Weber MJ. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* **19,** 2435-2444.

Schecroun N & Delloye C. (2003). Bone-like nodules formed by human bone marrow stromal cells: comparative study and characterization. *Bone* **32,** 252-260.

Seger R, Seger D, Lozeman FJ, Ahn NG, Graves LM, Campbell JS, Ericsson L, Harrylock M, Jensen AM & Krebs EG. (1992). Human T-cell mitogen-activated protein kinase kinases are related to yeast signal transduction kinases. *J Biol Chem* **267,** 25628-25631.

Sen A, Kallos MS & Behie LA. (2002). Expansion of mammalian neural stem cells in bioreactors: effect of power input and medium viscosity. *Brain Res Dev Brain Res* **134,** 103-113.

Shi Y & Massague J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113,** 685-700.

Shimoaka T, Ogasawara T, Yonamine A, Chikazu D, Kawano H, Nakamura K, Itoh N & Kawaguchi H. (2002). Regulation of osteoblast, chondrocyte, and osteoclast functions by fibroblast growth factor (FGF)-18 in comparison with FGF-2 and FGF-10. *J Biol Chem* **277,** 7493-7500.

Shirasawa S, Sekiya I, Sakaguchi Y, Yagishita K, Ichinose S & Muneta T. (2005). In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: Optimal condition and comparison with bone marrow-derived cells. *J Cell Biochem.*

Shum L & Nuckolls G. (2002). The life cycle of chondrocytes in the developing skeleton. *Arthritis Res* **4,** 94-106.

Shur I, Socher R & Benayahu D. (2005). Dexamethasone regulation of cFos mRNA in osteoprogenitors. *J Cell Physiol* **202,** 240-245.

Simmons CA, Matlis S, Thornton AJ, Chen S, Wang CY & Mooney DJ. (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,** 1087-1096.

Simmons PJ & Torok-Storb B. (1991). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* **78,** 55-62.

Sittinger M, Bujia J, Minuth WW, Hammer C & Burmester GR. (1994). Engineering of cartilage tissue using bioresorbable polymer carriers in perfusion culture. *Biomaterials* **15,** 451-456.

Smith-Adaline EA, Volkman SK, Ignelzi MA, Jr., Slade J, Platte S & Goldstein SA. (2004). Mechanical environment alters tissue formation patterns during fracture repair. *J Orthop Res* **22,** 1079-1085.

Spitzer RS, Perka C, Lindenhayn K & Zippel H. (2002). Matrix engineering for osteogenic differentiation of rabbit periosteal cells using alpha-tricalcium phosphate particles in a three-dimensional fibrin culture. *J Biomed Mater Res* **59,** 690-696.

Stanford CM, Welsch F, Kastner N, Thomas G, Zaharias R, Holtman K & Brand RA. (2000). Primary human bone cultures from older patients do not respond at continuum levels of in vivo strain magnitudes. *J Biomech* **33,** 63-71.

Stanton LA, Underhill TM & Beier F. (2003). MAP kinases in chondrocyte differentiation. *Dev Biol* **263,** 165-175.

Stein WD & Bronner F. (1989). *Cell shape. Determinants, regulation and regulatory role.* Academic Press Inc., San Diego.

Stevens DA & Williams GR. (1999). Hormone regulation of chondrocyte differentiation and endochondral bone formation. *Mol Cell Endocrinol* **151,** 195-204.

Stokoe D, Campbell DG, Nakielny S, Hidaka H, Leevers SJ, Marshall C & Cohen P. (1992). MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *Embo J* **11,** 3985-3994.

Sun JS, Chang WH, Chen LT, Huang YC, Juang LW & Lin FH. (2004). The influence on gene-expression profiling of osteoblasts behavior following treatment with the ionic products of sintered beta-dicalcium pyrophosphate dissolution. *Biomaterials* **25,** 607-616.

Tang YL, Zhao Q, Zhang YC, Cheng L, Liu M, Shi J, Yang YZ, Pan C, Ge J & Phillips MI. (2004). Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. *Regul Pept* **117,** 3-10.

Temenoff JS & Mikos AG. (2000). Review: tissue engineering for regeneration of articular cartilage. *Biomaterials* **21,** 431-440.

Ter Brugge PJ & Jansen JA. (2002). In vitro osteogenic differentiation of rat bone marrow cells subcultured with and without dexamethasone. *Tissue Eng* **8,** 321-331.

Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR & Miller FD. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* **3,** 778-784.

Tsuchida H, Hashimoto J, Crawford E, Manske P & Lou J. (2003). Engineered allogeneic mesenchymal stem cells repair femoral segmental defect in rats. *J Orthop Res* **21,** 44-53.

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

Tuli R, Tuli S, Nandi S, Huang X, Manner PA, Hozack WJ, Danielson KG, Hall DJ & Tuan RS. (2003). Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. *J Biol Chem* **278,** 41227-41236.

Turner CH, Akhter MP, Raab DM, Kimmel DB & Recker RR. (1991). A noninvasive, in vivo model for studying strain adaptive bone modeling. *Bone* **12,** 73-79.

Ueno A, Kitase Y, Moriyama K & Inoue H. (2001). MC3T3-E1-conditioned medium-induced mineralization by clonal rat dental pulp cells. *Matrix Biol* **20,** 347-355.

Urist MR, DeLange RJ & Finerman GA. (1983). Bone cell differentiation and growth factors. *Science* **220,** 680-686.

Vacanti CA, Bonassar LJ & Vacanti JP. (2000). Structural tissue engineering. In *Principles of Tissue Engineering*, 2nd edn, ed. Lanza RP, Langer R & Vacanti JP. Academic Press, San Diege.

Vacanti CA, Langer R, Schloo B & Vacanti JP. (1991). Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. *Plast Reconstr Surg* **88,**

753-759.

van der Wiel HE, Lips P, Nauta J, Netelenbos JC & Hazenberg GJ. (1991). Biochemical parameters of bone turnover during ten days of bed rest and subsequent mobilization. *Bone Miner* **13,** 123-129.

Veilleux NH, Yannas IV & Spector M. (2004). Effect of passage number and collagen type on the proliferative, biosynthetic, and contractile activity of adult canine articular chondrocytes in type I and II collagen-glycosaminoglycan matrices in vitro. *Tissue Eng* **10,** 119-127.

Verfaillie CM. (2002). Adult stem cells: assessing the case for pluripotency. *Trends Cell Biol* **12,** 502-508.

Villa M, Mustarelli P & Caprotti M. (1991). Biological effects of magnetic fields. *Life Sci* **49,** 85-92.

Vincent AM & Feldman EL. (2002). Control of cell survival by IGF signaling pathways. *Growth Horm IGF Res* **12,** 193-197.

Vogel P, Dux E & Wiessner C. (1997). Evidence of apoptosis in primary neuronal cultures after heat shock. *Brain Res* **764,** 205-213.

Vunjak-Novakovic G & al e. (1996). Effects of mixing on the composition and morphology of tissue-engineered cartilage. *AIChE J* **42,** 850-860.

Vunjak-Novakovic G, Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R & Freed LE.

(1999). Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. *J Orthop Res* **17,** 130-138.

Vunjak-Novakovic G, Obradovic B, Martin I, Bursac PM, Langer R & Freed LE. (1998). Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering. *Biotechnol Prog* **14,** 193-202.

Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI & Goldberg VM. (1994). Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg Am* **76,** 579-592.

Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Hong J & Kandel RA. (2003). Effect of biomechanical conditioning on cartilaginous tissue formation in vitro. *J Bone Joint Surg Am* **85-A Suppl 2,** 101-105.

Wang L, Shelton RM, Cooper PR, Lawson M, Triffitt JT & Barralet JE. (2003). Evaluation of sodium alginate for bone marrow cell tissue engineering. *Biomaterials* **24,** 3475-3481.

Watanabe H, de Caestecker MP & Yamada Y. (2001). Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-beta-induced aggrecan gene expression in chondrogenic ATDC5 cells. *J Biol Chem* **276,** 14466-14473.

Weber M, Steinert A, Jork A, Dimmler A, Thurmer F, Schutze N, Hendrich C & Zimmerman U. (2002). Formation of cartilage matrix proteins by BMP-transfected murine mesenchymal stem cells encapsulated in a novel class of alginates. *Biomaterials* **23,** 2003-2013.

Weinbaum S, Cowin SC & Zeng Y. (1994). A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. *J Biomech* **27,** 339-360.

Weinreb M, Rodan GA & Thompson DD. (1989). Osteopenia in the immobilized rat hind limb is associated with increased bone resorption and decreased bone formation. *Bone* **10,** 187-194.

Wendt D, Marsano A, Jakob M, Heberer M & Martin I. (2003). Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity. *Biotechnol Bioeng* **84,** 205-214.

Williams JT, Southerland SS, Souza J, Calcutt AF & Cartledge RG. (1999). Cells isolated from adult human skeletal muscle capable of differentiating into multiple mesodermal phenotypes. *Am Surg* **65,** 22-26.

Wolff J. (1892). *Das gesetz der transformation der knochen.* Berlin.

Wong M, Siegrist M, Wang X & Hunziker E. (2001). Development of mechanically stable alginate/chondrocyte constructs: effects of guluronic acid content and matrix synthesis. *J Orthop Res* **19,** 493-499.

Xia Z, Dickens M, Raingeaud J, Davis RJ & Greenberg ME. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270,** 1326-1331.

Xiao G, Cui Y, Ducy P, Karsenty G & Franceschi RT. (1997). Ascorbic acid-dependent activation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence. *Mol Endocrinol*

11, 1103-1113.

Xiao G, Wang D, Benson MD, Karsenty G & Franceschi RT. (1998). Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. *J Biol Chem* **273,** 32988-32994.

Xiao Y, Qian H, Young WG & Bartold PM. (2003). Tissue engineering for bone regeneration using differentiated alveolar bone cells in collagen scaffolds. *Tissue Eng* **9,** 1167-1177.

Xiao ZS, Hinson TK & Quarles LD. (1999). Cbfa1 isoform overexpression upregulates osteocalcin gene expression in non-osteoblastic and pre-osteoblastic cells. *J Cell Biochem* **74,** 596-605.

Yang XB, Roach HI, Clarke NM, Howdle SM, Quirk R, Shakesheff KM & Oreffo RO. (2001). Human osteoprogenitor growth and differentiation on synthetic biodegradable structures after surface modification. *Bone* **29,** 523-531.

Yang Y, Magnay JL, Cooling L & El HA. (2002). Development of a 'mechano-active' scaffold for tissue engineering. *Biomaterials* **23,** 2119-2126.

Yaszemski MJ, Oldham JB, Lu L & Currier BL. (1994). In *Bone Engineering*, pp. 541. Em squared, Toronto.

Yates KE, Allemann F & Glowacki J. (2005). Phenotypic analysis of bovine chondrocytes cultured in 3D collagen sponges: effect of serum substitutes. *Cell Tissue Bank* **6,** 45-54.

Ying QL, Nichols J, Evans EP & Smith AG. (2002). Changing potency by spontaneous fusion.

Nature **416,** 545-548.

Yip YP, Capriotti C, Norbash SG, Talagala SL & Yip JW. (1994). Effects of MR exposure on cell proliferation and migration of chick motoneurons. *J Magn Reson Imaging* **4,** 799-804.

Yip YP, Capriotti C & Yip JW. (1995). Effects of MR exposure on axonal outgrowth in the sympathetic nervous system of the chick. *J Magn Reson Imaging* **5,** 457-462.

Yokoyama A, Sekiya I, Miyazaki K, Ichinose S, Hata Y & Muneta T. (2005). In vitro cartilage formation of composites of synovium-derived mesenchymal stem cells with collagen gel. *Cell Tissue Res*, 1-10.

Yoon YM, Oh CD, Kim DY, Lee YS, Park JW, Huh TL, Kang SS & Chun JS. (2000). Epidermal growth factor negatively regulates chondrogenesis of mesenchymal cells by modulating the protein kinase C-alpha, Erk-1, and p38 MAPK signaling pathways. *J Biol Chem* **275,** 12353-12359.

Yoshizumi M, Kogame T, Suzaki Y, Fujita Y, Kyaw M, Kirima K, Ishizawa K, Tsuchiya K, Kagami S & Tamaki T. (2002). Ebselen attenuates oxidative stress-induced apoptosis via the inhibition of the c-Jun N-terminal kinase and activator protein-1 signalling pathway in PC12 cells. *Br J Pharmacol* **136,** 1023-1032.

Young RG, Butler DL, Weber W, Caplan AI, Gordon SL & Fink DJ. (1998). Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. *J Orthop Res* **16,** 406-413.

Younger EM & Chapman MW. (1989). Morbidity at bone graft donor sites. *J Orthop Trauma* **3,** 192-195.

Yu L, Hebert MC & Zhang YE. (2002). TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. *Embo J* **21,** 3749-3759.

Zaleskas JM, Kinner B, Freyman TM, Yannas IV, Gibson LJ & Spector M. (2004). Contractile forces generated by articular chondrocytes in collagen-glycosaminoglycan matrices. *Biomaterials* **25,** 1299-1308.

Zanetti NC & Solursh M. (1984). Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton. *J Cell Biol* **99,** 115-123.

Zheng CF & Guan KL. (1993). Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. *J Biol Chem* **268,** 11435-11439.

Zhou S, Yates KE, Eid K & Glowacki J. (2005). Demineralized bone promotes chondrocyte or osteoblast differentiation of human marrow stromal cells cultured in collagen sponges. *Cell Tissue Bank* **6,** 33-44.

Zimmerma S, Hawkes WG, Hudson JI, Magaziner J, Hebel JR, Towheed T, Gardner J, Provenzano G & Kenzora JE. (2002). Outcomes of surgical management of total HIP replacement in patients aged 65 years and older: cemented versus cementless femoral components and lateral or anterolateral versus posterior anatomical approach. *J Orthop Res* **20,** 182-191.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP & Hedrick MH. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7,** 211-228.

Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA & Maini RN. (2000). Mesenchymal precursor cells in the blood of normal individuals. *Arthritis Res* **2,** 477-488.

Appendix I. Suppliers Addresses

Amersham	Amersham Biosciences Ltd., Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, United Kingdom.
BDH	BDH Laboratory supplies, Poole, Dorset, BH151TD, England.
Becton Dickinson	Becton Dickinson Labware Europe, 1 Rue Aristide Berges, BP4, 38800 Le Pont De Claix, France.
Bio-Rad	Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire HP2 7TD, United Kingdom.
Biosource	Biosource International, 542 Flynn Road, Camarillo, CA 93012, USA.
Calbiochem	Calbiochem-Novabiochem Corp., 10394 Pacifica Centre Court, San Diego, CA 92121, USA.
Chance Propper	Chance Propper Ltd, PO Box 53, Spon Lane South, Smethwick, West Midlands, B661NZ, England.

Chemicon	Chemicon International Inc., 28820 Single Oak Drive, Temecula, CA 92590, USA.
DAKO	DakoCytomation California Inc., 6392 Via Real, Carpintera, CA 93013, USA.
Gibco	Gibco Ltd., 3 Fountain Drive, Inchinnan Drive, Paisley PA4 9RF, Scotland, United Kingdom.
Invitrogen	Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, United Kingdom.
Jencons	Jencons (Scientific) Ltd., Cherrycourt Way, Stanbridge Road, Leighton Buzzard, Bedfordshire, LU7 4UA, United Kingdom.
Lennox	Lennox Laboratory Supplies, John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.
Pall Gelman	Pall Gelman Sciences Inc., 2200 Northern Boulevard. East Hills, New York 11548, USA.

Pierce	Pierce Biotechnologies 3747 N. Meridian Road, P.O. Box 117, Rockford, IL 61105, USA.
Promega	Promega, 2800 Woods Hollow Road, Madison, WI 53711, USA.
Roche Diagnostics	Roche Diagnostics Ltd, Bell Lane, Lewes, East Sussex, BN7 1LG, England.
R&D Systems	R&D Systems, 614 McKinley Place NE, Minneapolis, MN 55413, USA.
Santa Cruz	Santa Cruz Biotechnologies, 2161 Delaware avenue, Santa Cruz, CA 95060, USA.
Sarsdedt	Sarstedt Ltd., Sinnottstown Lane, Drinagh, Wexford, Ireland.
Sigma	Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset BH12 4QH, United Kingdom.

Vector	Vector Laboratories Inc., 30 Ingold Road, Burlingame, CA 94010, USA.
Whatman	Whatman plc., Whatman House, St.Leonard's Road, 20/20 Maidstone, Kent ME16 0LS, United Kingdom.

Appendix II. Solutions

The following solutions were used:

Electrode running buffer

Tris base, 25mM

Glycine, 192mM

SDS, 0.1% (w/v)

Sample Buffer

Tris-HCl, 0.05M, pH6.8

Glycerol 10% (v/v)

SDS 4% (w/v)

β-Mercaptoethanol 5% (v/v)

Bromophenol blue 0.002% (w/v)

Stacking gel (4%), pH6.8

Acrylamide/bis acrylamide (30% stock), 13%(v/v)

dH_2O, 60% (v/v)

Tris-HCl, 0.05M, pH6.8, 25% (v/v)

SDS (10%w/v stock), 1% (v/v)

APS (10% w/v stock), 0.5% (v/v)

TEMED, 0.05% (v/v)

Separating gel (12%), pH 8.8

Acrylamide/bis acrylamide (30% stock), 40%(v/v)

dH_2O, 33% (v/v)

Tris-HCl, 0.05M, pH8.8, 25% (v/v)

SDS (10%w/v stock), 1% (v/v)

APS (10% w/v stock), 0.5% (v/v)

TEMED, 0.05% (v/v)

Separating gel (10%), pH 8.8

Acrylamide/bis acrylamide (30% stock), 33%(v/v)

dH_2O, 40% (v/v)

Tris-HCl, 0.05M, pH8.8, 25% (v/v)

SDS (10%w/v stock), 1% (v/v)

APS (10% w/v stock), 0.5% (v/v)

TEMED, 0.05% (v/v)

Transfer buffer, pH8.3

Tris base, 25mM

Glycine, 192mM

MeOH, 20%

SDS, 0.05% (w/v)

Tris-buffered saline (TBS), pH7.4

Tris-HCl, 20mM

NaCl, 150nM

Tris-buffered saline tween (TBS), pH7.4

TBS with 0.05% Tween

Lysis buffer pH 7.4

25mM HEPES

5mM $MgCl_2$

5mM dithiothreitol

5mM EDTA

2mM PMSF

5µg/ml aprotinin

Supplemented Dulbecco's Modified Eagles Medium

2% penicillin/streptomycin 100U/ml

10% Foetal Bovine Serum

0.5% l-Glutamine

0.5% Glutamax

1% non essential amino acids

Phosphate Buffered Saline pH 7.4

100mM NaCl

80mM Na_2HPO_4

20mM $Na_2H_2PO_4$

Ethanol fixative

50mM glycine in 70ml absolute alcohol to give 100ml fixative, pH 2.0