



Provided by the author(s) and University of Galway in accordance with publisher policies. Please cite the published version when available.

Title	Chondrogenic and vascular priming: an endochondral ossification approach to bone tissue regeneration
Author(s)	Freeman, Fiona
Publication Date	2015-11-30
Item record	http://hdl.handle.net/10379/5359

Downloaded 2024-04-26T22:10:28Z

Some rights reserved. For more information, please see the item record link above.



Chondrogenic and Vascular Priming: An Endochondral Ossification Approach to Bone Tissue Regeneration

Fiona E. Freeman B.E. (2011)



A Thesis submitted to the National University of Ireland as fulfilment of the
requirements for the degree of Doctor of Philosophy

May 2015

Discipline of Biomedical Engineering,

College of Engineering and Informatics,

National University of Ireland, Galway.

Supervisor of Research: Dr. Laoise M. McNamara

Abstract

Tissue engineering and regenerative medicine have significant potential to treat bone pathologies by exploiting the capacity for bone progenitors to grow and produce tissue constituents under specific biochemical and physical conditions. However, these approaches are limited and as such are not yet widely used for clinical treatment of large bone defects. The main limitations include degradation of the tissue engineered constructs, due to lack of vascularisation, and a lack of mechanical integrity to fulfil loading bearing functions. Recent studies have suggested that in vitro approaches, which mimic certain aspects of bone formation during embryogenesis (i.e. the endochondral ossification process), can promote mineralisation and vascularisation to a certain extent both in vitro and in vivo. However, in vivo endochondral ossification relies on the production of a cartilage template and the invasion of a vascular network into this template, and both events must occur before bone tissue can be produced. Although researchers have demonstrated the separate effectiveness of chondrogenic priming and prevascularisation, to date no tissue engineering strategy has sought to incorporate both of these crucial events. The global objectives of this Thesis are to investigate whether endochondral priming of stem cells in vitro can enhance (a) the osteogenic and vasculogenic potential in vitro and (b) ultimately enhance the angiogenic and mineralisation potential once implanted in vivo.

The first study sought to determine the optimum period for chondrogenic priming of Mesenchymal Stem Cells (MSCs) in vitro that could enhance osteogenic differentiation. The results from this study found that chondrogenic priming of MSCs

in vitro for specific amounts of time (14 days, 21 days) can have an optimum influence on their mineralization capacity and can produce an aggregate that is mineralised throughout the core. The findings of this study provided vital information in determining the optimum time (14-21 days) for chondrogenic priming to produce a fully mineralised bone tissue engineered construct in vitro.

The second study of this Thesis sought to develop an in vitro bone regeneration strategy that mimics critical aspects of the endochondral ossification process, specifically (1) the formation of a cartilage template and (2) subsequent vascularization of this template. The results showed that chondrogenic priming provides a cartilage-like template that provides a suitable platform for Human Umbilical Vein Endothelial Cells (HUVECs) and MSC cells to attach, proliferate, and infiltrate for up to 3 weeks. More importantly this study showed that when both MSCs and HUVECs are added to the already formed cartilage template, rudimentary vessels were formed within this cartilage template and this strategy enhanced the mineralization potential of MSCs.

The third study of this Thesis aimed to investigate whether mimicking both the chondrogenic and vascularisation aspects of the endochondral ossification process could induce osteogenesis, even without the use of any osteogenic supplements. The results from this study showed that the co-culture methodology enhanced both osteogenesis and vasculogenesis compared to osteogenic differentiation alone, whilst allowing for the formation of rudimentary vessels in vitro.

Taken together, the results from Chapters 3-5 showed that the application of both chondrogenic and vascular priming of priming of human MSCs enhanced the mineralization potential of MSCs in vitro whilst also allowing the formation of

immature vessels and can even obviate the need for osteogenic growth factors to induce osteogenesis by human MSCs in vitro. The final study of this Thesis investigated the in vivo potential of the endochondrally primed aggregates developed in Chapter 5. The results from this study found that in aggregates that were both chondrogenically primed and prevascularised viable human MSCs were identified 21 days after subcutaneous implantation. Most importantly these aggregates had mature endogenous vessels and mineralisation nodules, after 4 weeks subcutaneous implantation. In contrast aggregates that were not prevascularised had no vessels or mineralisation within the aggregate interior and human MSCs did not remain viable beyond 14 days.

Taken together, the results from this Thesis provide a novel understanding of the optimum conditions needed to create a bone tissue engineered construct that when implanted in vivo may drive bone formation via an endochondral ossification-like process. Future bone tissue engineered constructs could be designed with these conditions in mind for the repair of non-union bone defects.

Publications

Journal Articles:

The following publications have arisen from the work presented in this Thesis:

- Fiona E. Freeman, Matthew G. Haugh, and Laoise M. McNamara “Investigation of Optimal Timing for Chondrogenic Priming of MSCs to Enhance Osteogenic Differentiation In Vitro as a Bone Tissue Engineering Strategy” (2013) Journal of Tissue Engineering and Regenerative Medicine doi: 10.1002/term.1793.
- Fiona E. Freeman, Matthew G. Haugh, and Laoise M. McNamara “An in vitro bone tissue regeneration strategy combining chondrogenic and vascular priming enhances the mineralisation potential of MSCs in vitro whilst also allowing for vessel formation.” (2015) Journal of Tissue Engineering Part A 21(7-8): 1320-1332.
- Fiona, E. Freeman, Ashley, B. Allen, Hazel, Y. Stevens, Robert, E. Guldberg, and Laoise, M. McNamara. “Effects of in vitro endochondral priming and pre-vascularization of human MSC cellular aggregates in vivo.” Journal of Stem Cell Research and Therapy 6(218) doi: 10.1186/s13287-015-0210-2.
- Fiona, E. Freeman, Peter Owens, Hazel, Y. Stevens, Robert, E. Guldberg, and Laoise, M. McNamara. “Endochondrally priming of human MSCs in vitro enhances their mineralisation potential without the addition of osteogenic growth factors” *In Review in Journal of Tissue Engineering Part A*.
- Meadhbh A. Brennan, Fiona, E. Freeman, Laoise, M. McNamara, and Pierre Layrolle. “Role of chondrogenic priming and prevascularisation, of MSC seeded PCL scaffolds, on bone healing in a large mice calvarial defect” *In preparation*.

Conference Presentations

Peer Reviewed International Conferences

- Poster Presentation at the Proceedings of International Mesenchymal Stem Cell Conference, Galway, Ireland. July, 2012.
- Poster Presentation at the Proceedings of Orthopaedic Research Society, Texas, USA. January, 2013.
- Podium Presentation at the Anatomical Society Meeting, RCSI, Dublin, Ireland. July, 2013.
- Podium and poster presentation at the TERMIS EU Meeting, Istanbul, Turkey. June 2013.
- Poster presentation at the TERMIS-Americas, Atlanta, Georgia, USA. November 2013.
- Poster presentation at the Proceedings of Orthopaedic Research Society, New Orleans, Louisiana, USA. March, 2014.
- Podium Presentation at the Proceedings of World congress of Biomechanics, Boston, Massachusetts, USA. July, 2014.
- Poster presentation at the Proceedings of Orthopaedic Research Society, Las Vegas, Nevada, USA. March 2015.
- Two poster presentations at TERMIS World Congress Boston, Massachusetts, USA. September, 2015.
- Podium presentation at the Proceedings of Orthopaedic Research Society, Orlando, Florida, USA. March, 2016.

Peer Reviewed National Conferences

- Podium presentation for the Engineers Ireland Research Medal at the 20th Annual Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland, Limerick. January 2014.

Acknowledgements

I would like to express my greatest gratitude to my supervisor, Dr. Laoise McNamara, for her time, support and encouragement throughout my studies. Over the four years Laoise always challenged me to strive for more, whether it be high quality research or research grants. Her attention to detail and unwavering patience are what finally taught me how to write (then vs. than, foras vs. whereas etc.), and to give a presentation (speak with inflection). Her drive, work ethic and love for bioengineering research are a constant source of inspiration. Thank you for all your time and effort, you have taught me so much!

I was lucky enough to be privy to some amazing postdoctoral researchers (Matt, Muriel, Ted) during my four years. Ted thank you for all presentation tips, chapters/abstracts read, and amazing career advice you may not have wished for it but by the end I made you a “tissue engineering friend”! Muriel thank you for all the time and help you gave me, without which this thesis would be lacking some very pretty pictures. Matt thank you for always being so generous with your time and wisdom those first few years, it was invaluable.

I was fortunate to be part of a fantastic group of research students (past and present) throughout my time in the department. Thank you for providing me with endless gossip, much slagging and many welcome distractions over the four years. Special thanks to the mean girl crew (Paul, Eimear, and Stefaan), Noel, Dave and my 2nd floor peeps whose dedication to having the craic, causing mischief and general misbehaving made the four years fly by. Particularly big thanks to my sarcastic partner crime, Miss Lizanne (grammar nazi) Gallagher. My sincerest thanks also goes out to

the brave souls (Lizanne, Eimear, and Ted) that took the time to help proof read my thesis. I would like to thank everybody in Professor Guldberg's Research group for being so welcoming and knowledgeable and enduring my endless questions. I would also like to thank Professor Layrolle research group especially Meadhbh for all her help and her collaboration during this PhD - cannot wait to celebrate in August.

To my close friends, thank you for your constant encouragement, and of course, your endless "support" (I never once felt too big for my boots)! You were a constant source of fun, advice, and reassurance and were always there to celebrate any wins and drown any losses and for that I am extremely grateful.

Last but very much not least I would like to thank my family. Mum and Dad thank you for your love, support, and unwavering confidence in my ability (They are not worthy!). I don't think I would have made it through the last four years without it, that and the amazing roast dinners. Kiddy thank you for always being an amazing role model and best friend - mom and dad didn't do too bad having two doctors in the family!

Acronyms

MSC	Mesenchymal Stem Cell
HUVEC	Human Umbilical Vein Endothelial Cell
sGAG	sulphate Glycosaminoglycan
ALP	Alkaline Phosphatase
BALB/c	Bagg Albino
VEGF	Vascular Endothelial Growth Factor
BLI	Bioluminescence Imaging
PTH	Parathyroid Hormone
IGF	Insulin-like Growth Factor
TGF- β	Transforming Growth Factor – β
BMP	Bone Morphogenic Protein
FGF	Fibroblast Growth Factor
DMP	Dentine Matrix Protein
NO	Nitric Oxide
RANK	Receptor Activator of Nuclear Factor Kappa B
RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
ISCT	International Society for Cellular Therapy
ECM	Extracellular Matrix
HSC	Haematopoietic Stem Cell
TNF- α	Tumour Necrosis Factor – α
IL-6	Interleukin-6
PLA	Polylactic Acid
PCL	Polycaprolactone

PLGA	Poly(lactide-co-Glycolide)
EC	Endothelial Cell
PBS	Phosphate-Buffered Saline
ACREC	Animal Care Research Ethics
DMEM	Dulbecco's Modified Eagle's Medium
α MEM	α Minimum Essential Medium Eagle
FBS	Fetal Bovine Serum
ITS	Insulin-Transferrin-Selenium
EDTA	Ethlenediaminetetraacetic Acid
DMB	Dimethylmethylen Blue
pNPP	p-Nitrophenyl Phosphate
HCL	Hydrochloric Acid
EGM	Endothelial Growth Medium
ELISA	Enzyme Linked Immunosorbent Assay
BSA	Bovine Serum Albumin
NGS	Normal Goat Serum
PI	Propidium Iodide
ANOVA	Analysis of Variance
CP	Chondrogenic Priming
AMP	2-Amino-2 Methyl-1-Propanol
DAPI	4',6-Diamidino-2-Phenylindole
μ CT	Microcomputed Tomography
GFP	Green Fluorescent Protein
Luc	Luciferin
MOI	Multiplicity of Infection

ROI

Region of Interest

3D

Three Dimensional

Table of Contents

Chapter 1: Introduction	1
1.1 Clinical Need for Bone Tissue Engineering for Large Bone Defects	1
1.1.1 Current Treatments for Large Bone Defects	2
1.1.2 Bone Tissue Engineering Applications for Large Bone Defects	3
1.2 Objectives and Hypothesis.....	7
1.3 Thesis Structure	8
Chapter 2: Literary Review	10
2.1 Introduction.....	10
2.1.1 Bone Function and Anatomy	10
2.1.2 Bone Tissue Composition.....	10
2.1.3 Bone Structure and Organisation.....	11
2.2 Bone Cells.....	14
2.2.1 Bone Lining Cells and Osteoblasts.....	14
2.2.2 Osteocytes.....	17
2.2.3 Osteoclasts.....	20
2.2.4 Mesenchymal Stem Cells	22
2.3 Bone Marrow as a Stem Cell Niche.....	24
2.4 Bone Development.....	25
2.4.1 Intramembranous Ossification.....	26
2.4.2 Endochondral Ossification	27
2.5 Bone as an Adaptive material	31
2.5.1 Bone Modelling and Remodelling.....	31
2.5.2 Fracture Healing	33
2.6 Bone Pathology.....	35

2.7	Bone Tissue Engineering	37
2.8	Endochondral Ossification as a Bone Tissue Engineering approach.....	43
2.8.1	Formation of the Cartilage Template.....	45
2.8.2	Vascularisation of the Cartilage Template	49
2.9	Summary	52

Chapter 3: Is there an optimum time for chondrogenic priming of MSCs in vitro that will enhance mineralisation in vitro?54

3.1	Introduction.....	54
3.2	Methods.....	57
3.2.1	Isolation and Characterisation of BALB/c and Human bone marrow derived Mesenchymal Stem Cells (MSCs).....	57
3.2.2	Aggregate Formation.....	58
3.2.3	Aggregate Harvesting.....	60
3.2.4	Quantitative Biochemical Analysis	61
3.2.4.1	DNA Content	61
3.2.4.2	sGAG Content.....	62
3.2.4.3	ALP Production	62
3.2.4.4	Calcium Content	62
3.2.5	Histology	63
3.2.6	Immunohistochemical Analysis	63
3.2.7	Statistical Analysis	64
3.3	Results.....	64
3.3.1	Chondrogenic priming of BALB/c MSC's.....	64
3.3.1.1	DNA Content	64
3.3.1.2	Production of the Cartilage Template	65
3.3.1.3	Mineralisation of the Cartilage Template	68
3.3.2	Chondrogenic priming of Human MSC's	76

3.3.2.1	Mineralisation of the Cartilage Template	76
3.4	Discussion.....	78
3.5	Conclusion	83
Chapter 4:	Does Chondrogenic and Vascular priming enhance mineralisation whilst allowing for vessel formation in vitro?.....	84
4.1	Introduction.....	84
4.2	Methods.....	87
4.2.1	Cell Isolation and Characterisation.....	87
4.2.1.1	Human Donor MSC: Isolation and Characterisation	87
4.2.1.2	HUVECs Culture	89
4.2.2	Aggregate Formation.....	89
4.2.3	Histochemical analysis and Biochemical Analysis	91
4.2.3.1	Quantitative Biochemical Analysis.....	92
4.2.3.2	Histology	93
4.2.3.3	CD31 and Collagen Type X Immunohistochemical Analysis	94
4.2.4	Statistical Analysis	95
4.3	Results.....	95
4.3.1	Characterisation of Human Donor MSCs.....	95
4.3.2	Cell Number	97
4.3.3	Production of the Cartilage Template.....	98
4.3.3.1	Alcian Blue Staining	98
4.3.4	Mineralisation of the Cartilage Template	99
4.3.4.1	ALP Production	99
4.3.4.2	Calcium Content	100
4.3.4.3	Alizarin Red	101
4.3.5	Vascularisation of the Cartilage Template	103
4.3.5.1	VEGF Production.....	103

4.3.5.2	CD31+ Staining	104
4.3.6	Production of Hypertrophic Cartilage Template	107
4.3.6.1	Collagen Type X Staining.....	107
4.4	Discussion	108
4.5	Conclusions.....	114

Chapter 5: Will both Chondrogenic and Vessel Priming obviate the need for osteogenic supplements to induce osteogenesis in vitro?116

5.1	Introduction.....	116
5.2	Methods.....	120
5.2.1	Cell Culture	120
5.2.1.1	Human MSC Culture	120
5.2.1.2	HUVECs Culture	120
5.2.2	Aggregate Formation.....	120
5.2.3	Histochemical analysis and Biochemical Analysis	122
5.2.3.1	Quantitative Biochemical Analysis.....	122
5.2.3.2	Histology.....	124
5.2.3.3	CD31 Immunohistochemical Analysis	124
5.2.4	Statistical Analysis	125
5.3	Results.....	125
5.3.1	DNA Content.....	125
5.3.2	Production of the Cartilage Template.....	127
5.3.2.1	Alcian Blue Staining	127
5.3.3	Mineralisation of the Cartilage Template	129
5.3.3.1	ALP Production	129
5.3.3.2	Calcium Content	132
5.3.3.3	Alizarin Red	134
5.3.4	Vascularisation of the Cartilage Template	136

5.3.4.1	VEGF Production.....	136
5.3.4.2	CD31+ Staining	137
5.4	Discussion	141
5.5	Conclusions.....	149

**Chapter 6: What effect will the in vitro endochondral priming and
prevascularisation of cellular aggregates have in vivo?150**

6.1	Introduction.....	150
6.2	Methods.....	153
6.2.1	Cell Culture	153
6.2.1.1	Human Donor MSCs.....	153
6.2.1.2	Cell Labelling.....	154
6.2.1.3	HUVECs Culture	154
6.2.2	Aggregate Formation.....	155
6.2.3	Construct Preparation	156
6.2.4	Surgical Procedures	157
6.2.5	Bioluminescent imaging (BLI).....	158
6.2.6	Microcomputed Tomography Imaging.....	158
6.2.7	Histochemical analysis	160
6.2.8	Immunohistochemical Analysis	160
6.2.9	Statistical Analysis	161
6.3	Results.....	161
6.3.1	Construct Morphology.....	161
6.3.2	Mineral Formation.....	164
6.3.3	Cell Viability	166
6.3.4	Vessel Infiltration	168
6.4	Discussion	173
6.5	Conclusions.....	180

Chapter 7:	Discussion and Conclusion	182
7.1	Introduction.....	182
7.2	Main Findings of the Thesis.....	182
7.3	Implication for the Bone Tissue Engineering Field	186
7.4	Recommendations for Future Work.....	192
7.4.1	Experimental characterisation of the signalling pathways as a results of the co-culture of MSCs and HUVECs.....	193
7.4.2	Understanding the effect of long term subcutaneous implantation of prevascularised cartilaginous cellular aggregates.....	193
7.4.3	Understanding the effects the application of mechanical stimulation in vitro will have on the cellular aggregates when implanted in vivo.....	194
7.4.4	Applying the optimum priming conditions in vitro and investigating the regeneration potential of aggregates in a large bone defect model.....	197
7.5	Conclusions.....	197
References	199

Table of Figures

Figure 2.1: Hierarchical Structure of bone demonstrating the composite structure from the nanoscale to the macroscale (a-e) (Vaughan et al., 2012).....	12
Figure 2.2: (a) Macrostructure of the Long bone showing (b) micro structure of the Epiphysis and (c) macrostructure of the Diaphysis (Marieb and Hoen, 2006).....	13
Figure 2.3: Differentiation of cell types along the two lineages: (A) the osteogenic lineage and (B) the osteoclastic lineage (Wang et al., 2012b).....	14
Figure 2.4: TEM image of an osteocyte, osteoblast and osteoclast, with ruffled border of the osteoclast indicated (red arrow). Reproduced with permission of Dr. L.M. McNamara.....	15
Figure 2.5: Confocal scans performed from the surface of a cut rat femur allowing for visualisation of the pericellular space, osteocytes and osteoblasts (Verbruggen et al., 2015).	19
Figure 2.6: Multinucleated Osteoclast in its resorption cavity or Howship's Lacunae denoted by the letter (A) (Cormack, 2001).....	21
Figure 2.7: Mesenchymal Cell Lineages - MSCs have the capacity to differentiate into bone, cartilage, muscle, marrow stroma, tendon/ligament, fat and other connective tissues (Caplan, 2005).....	23
Figure 2.8: Schematic of the co-localisation of the osteoblastic niche and vascular niche present within bone marrow (Ross and Christiano, 2006).	25
Figure 2.9: The Intramembranous Ossification process: (A) MSCs condense form ossification centre, (B) the deposited osteoid entombs osteoblasts, which then become osteocytes, (C) periosteum and trabeculae form and vessels invade, (D) compact bone develops superficial to the trabecular bone (Marieb and Hoen, 2006).	27

Figure 2.10: Schematic of the different zones present in the cartilage template (Zuniga, 2015).....	29
Figure 2.11: Endochondral Ossification: (A) Formation of the cartilage template, (B) Chondrocytes become hypertrophic and vessels begin to invade, (C) osteoid and mineral is formed upon the cartilage template and the primary ossification centre is formed, (D) Marrow, secondary ossification centre and epiphyseal plate are formed (McNamara, 2011).	31
Figure 2.12: Diagram showing the phases of the bone remodelling cycle: Activation Phase, Resorption Phase, Reverse Phase, and Formation Phase. OCL denote osteoclasts and OBLs denote osteoblasts.....	33
Figure 2.13: The healing process of bone fracture (1) Hematoma formation, (2) Fibrocartilagenous callus formation, (3) Bony callus formation, and (4) Bone Remodelling (Marieb and Hoen, 2006).	34
Figure 2.14: Schematic of the three stages of the bone tissue engineering triad: (1) Cells, (2) Scaffold and (3) Growth Factors.....	37
Figure 2.15: Example of Core degradation in a rat calvarial defect treated with tissue engineered collagen-calcium phosphate scaffold for 4 weeks post implantation. (a) Shows full defect site and (b) shows higher magnification of the same image. Red arrows show the fibrous tissue capsule and black arrow shows the acellular core (O'Brien, 2011).....	42
Figure 2.16: Three-dimensional μ CT reconstructions (A-D) and quantitative histomorphometric data ($n = 4$) of mineral volume (E-F) and density of the constructs chondrogenically primed for 14 days (early hypertrophic) versus constructs that were chondrogenically primed for 21 days followed by 2 weeks in hypertrophic medium	

(late hypertrophic) (* indicates significant differences; $p < 0.01$) (Scotti et al., 2010).

.....46

Figure 2.17: Micro-CT images of the mineralised Aggregates after 4 and 8 weeks in vivo. Black arrows denote the boarder of the Aggregates, “c” indicated the newly formed cortex and “m” indicates the ongoing restoration of the medullary canal (van der Stok et al., 2014).47

Figure 2.18: Microcomputed tomography angiography demonstrating the level of vessel formation within the implanted collagen-GAG scaffolds after 4 weeks subcutaneous implantation. ECs denote endothelial cells alone and co-culture denotes the addition of both endothelial cells and MSCs (McFadden et al., 2013).50

Figure 2.19: Confocal images of CD31 labelling of MSC/HUVEC spheroids at day 1, 3 and 7. DAPI stains nuclei of cells blue and CD31 stains endothelial cells green (Saleh et al., 2011).51

Figure 3.1: Schematic of the experimental groups harvested samples at each time-point.60

Figure 3.2: The DNA content of the BALB/c groups at days 0, 10, 21, 28, 35 and 49 ($n = 6$ samples per group per time-point). Error bars denote standard deviation.65

Figure 3.3: (A) The sGAG content of the BALB/c groups at days 0, 10, 14, 21, 28, 35 and 49 ($n = 6$ samples per group per time-point). * $p < 0.05$ vs. Chondro group; $\wedge p < 0.05$ vs. Osteo group (see text). Error bars denote standard deviation. (B) Alcian Blue staining of each of the groups at day 49. Each of the images was taken at a magnification of 10 X.67

Figure 3.4: (A) The ALP/DNA content of the of the BALB/c groups at days 0, 10, 14, 21, 28, 35 and 49 ($n = 6$ samples per group per time point). (B) The ALP/DNA content

of the BALB/c groups and compared with the osteogenic group, 1 week and 2 weeks after changing the medium. * $p<0.05$ vs. Chondro group; $^{\wedge}p<0.05$ vs. Osteo group; $^{a}p<0.05$ vs. 10-day priming group; $^{b}p<0.05$ vs. 14-day priming group; $^{c}p<0.05$ vs. 21-day priming group; $^{d}p<0.05$ vs. 28-day priming group (see text). Error bars denote standard deviation.70

Figure 3.5: (A) Calcium content of the BALB/c groups at days 0, 10, 14, 21, 28, 35 and 49 ($n = 6$ samples per group per time-point). (B) Calcium content of the BALB/c groups compared with the osteogenic group 1 week and 2 weeks after changing the media. * $p<0.05$ versus Chondro group; $^{\wedge}p<0.05$ vs. Osteo group; $^{a}p<0.05$ vs. 10-day priming group; $^{b}p<0.05$ vs. 14-day priming group; $^{c}p<0.05$ vs. 21-day priming group; $^{d}p<0.05$ vs. 28-day priming group (see text). Error bars denote standard deviation. 73

Figure 3.6: Alizarin Red and immunohistochemical staining (collagen types I, II and X) of the BALB/c chondrogenically primed groups and osteogenic group at 2 weeks after switching media. Each of the images was taken at a magnification of 10 X.....75

Figure 3.7: (A) The ALP/DNA content of the human mesenchymal stem cell (MSC) priming groups, and compared with the osteogenic group 1 week and 2 weeks post-media change. (B) Calcium content of the human MSCs priming groups compared to the osteogenic group, 1 week and 2 week after switching media. * $p<0.05$ vs. Chondro group; $^{\wedge}p<0.05$ vs. Osteo group; $^{a}p<0.05$ vs. 10-day priming group; $^{b}p<0.05$ vs. 14-day priming group; $^{c}p<0.05$ vs. 21-day priming group; $^{d}p<0.05$ vs. 28-day priming group (see text). Error bars denote standard deviation ($n = 6$).77

Figure 4.1: Schematic of experimental groups.90

Figure 4.2: Characterisation of the multipotency of isolated human MSCs. Calcium deposition is confirmed by positive alizarin red staining by MSCs cultured in

osteogenic differentiation medium (**A**), whereas no staining was observed in the negative control (expansion medium) (**B**). Fat globules are confirmed by Oil Red O staining for MSCs cultured in adipogenic medium (**C**), but not in the negative control (expansion medium) (**D**). Staining with Alcian Blue for chondrocyte-associated glycosaminoglycan was positive within the aggregate cultured in chondrogenic medium (**E**), but was not seen in the negative control (expansion medium) (**F**)..... 96

Figure 4.3: DNA content of the co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) compared with 21 days priming group alone (CP21-HUVECs) pre and 1, 2 and 3 weeks post the addition of HUVECs/MSCs ($n = 6$ samples per group per time point). $^{\wedge}p < 0.05$ vs. CP21 +HUVECs group and $*p < 0.05$ vs. CP21 -HUVECs. Error bars denote standard deviation. 98

Figure 4.4: Alcian Blue staining of all co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) and 21 days priming group alone (CP21-HUVECs) over the course of the experiment. Each of the images was taken at a magnification of 20 X.

..... 99

Figure 4.5: ALP Activity the co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) compared with 21 days priming group alone (CP21-HUVECs) pre and 1, 2 and 3 weeks post the addition of HUVECs/MSCs ($n = 6$ samples per group per time point). Error bars denote standard deviation..... 100

Figure 4.6: Calcium content for the co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) compared with 21 days priming group alone (CP21-HUVECs) pre and 1, 2 and 3 weeks post the addition of HUVECs/MSCs ($n = 6$ samples per group per time point). $^{\wedge}p < 0.05$ vs. CP21 + HUVECs group; $*p < 0.05$ vs. CP21 -HUVECs. Error bars denote standard deviation. 101

- Figure 4.7:** Alizarin Red staining of co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) and 21 days priming group alone (CP21-HUVECs) over the course of the experiment. Arrows denote mineralisation nodules present in the cellular aggregates. Each of the images was taken at a magnification of 20 X.102
- Figure 4.8:** VEGF content the co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) compared with 21 days priming group alone (CP21-HUVECs) pre and 1, 2 and 3 weeks post the addition of HUVECs/MSCs ($n = 6$ samples per group per time point). $^{\wedge}p < 0.05$ vs. CP21 +HUVECs group; $*p < 0.05$ vs. CP21-HUVECs. Error bars denote standard deviation.104
- Figure 4.9:** CD31 staining (green) of co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) and 21 days priming group alone (CP21-HUVECs) over the course of the experiment. Arrows denote positive CD31+ staining present in the cellular aggregates. Each of the images was taken at a magnification of 40 X. Nuclear counterstain: propidium iodide (red).....105
- Figure 4.10:** Representative images of CD31+ (green) stained sections showing the presence of rudimentary vessels (A-D). Each section is $8\mu\text{m}$ thick and each of the images was taken at a magnification of 40X. Nuclear counterstain: propidium iodide (red). Quantitative analysis of Cross-Sectional Area (E) and Vessel Diameter (F) of rudimentary vessels found. Error bars denote standard deviation ($n = 16$).106
- Figure 4.11:** Collagen Type X staining (green) of co-culture groups (CP21+HUVECs (B, E, H), CP21+HUVECs:MSCs (C, F, I)) and 21 days priming group alone (CP21-HUVECs (A, D, G)) over the course of the experiment, compared to the positive control (neo-natal mouse long-bone) (K) and negative control (Blocking solution) (J). Arrows denote positive Collagen Type X staining present in the cellular aggregates.

Each of the images was taken at a magnification of 40 X. Nuclear counterstain: propidium iodide (red) 108

Figure 5.1: DNA content/sample of all three experimental conditions cultured without osteogenic supplements (-OM) compared to the results from Chapter 4 of the same groups cultured in the presence of osteogenic supplements (+OM). $^{\wedge}p < 0.05$ vs. CP21 +HUVECs group, $^{\text{a}}p < 0.05$ vs. CP21 -HUVECs. $^{*}p < 0.05$, and $^{****}p < 0.0001$. ($n = 6$ samples per group per time point). Error bars denote standard deviation. 127

Figure 5.2: Alcian Blue staining of all three groups cultured with (+OM) and without osteogenic supplements (-OM) over the course of the experiment. Each of the images were imaged at a magnification of 20X (aggregates cultured with osteogenic supplements (+OM)) or at 10X (aggregates cultured without osteogenic supplements (-OM)). 129

Figure 5.3: (A) ALP Activity of all of the experimental groups cultured without osteogenic supplements compared with osteogenic group alone 1, 2 and 3 weeks post the addition of HUVECs/MSCs. $^{\wedge}p < 0.05$ vs. CP21 +HUVECs group, $^{\text{a}}p < 0.05$ vs. CP21 -HUVECs, and $^{\text{b}}p < 0.05$ vs. Osteo alone. (B) ALP Activity of all three experimental groups cultured without osteogenic supplements compared to the same groups cultured in the presence of osteogenic supplements and the osteogenic group alone. $*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. ($n = 6$ samples per group per time point). Error bars denote standard deviation. 132

Figure 5.4: (A) Calcium content of all of the experimental groups cultured without osteogenic supplements (-OM) compared with osteogenic group alone 1, 2 and 3 weeks post the addition of HUVECs/MSCs. $^{\wedge}p < 0.05$ vs. CP21 +HUVECs group and $^{\text{a}}p < 0.05$ vs. CP21 -HUVECs. (B) Calcium content of all three experimental groups

cultured without osteogenic supplements compared to the same groups cultured in the presence of osteogenic supplements (+OM) and the osteogenic group alone. **p < 0.01, n = 6 samples per group per time point. Error bars denote standard deviation.

.....134

Figure 5.5: Alizarin Red staining of the aggregates cultured without osteogenic supplements prior to addition of cells and all three groups cultured with (+OM) and without osteogenic supplements (-OM) at 1, 2 and 3 weeks post the addition of cells. Each of the images were imaged at a magnification of 20X (aggregates cultured with osteogenic supplements (+OM)) or at 10X (aggregates cultured without osteogenic supplements (-OM))......135

Figure 5.6: VEGF expression of all three experimental groups cultured without osteogenic supplements (-OM) compared to the same groups cultured in the presence of osteogenic supplements (+OM). [^]p < 0.05 vs. CP21 +HUVECs group, ^ap < 0.05 vs. CP21 -HUVECs, **p < 0.01 and ****p < 0.0001, n = 6 samples per group per time point. Error bars denote standard deviation.137

Figure 5.7: CD 31 staining (green) of all three experimental groups cultured with (+OM) and without (-OM) osteogenic supplements over the course of the experiment. Each of the images of the aggregates cultured without (-OM) osteogenic supplements were imaged at a magnification of 10X with a nuclei counterstain DAPI (blue). The aggregates cultured with osteogenic supplements (+OM) were imaged at a magnification of 40X, with a nuclei counterstain propidium iodide (red).....139

Figure 5.8: (A) Representative images of CD31+ (green) stained sections showing the presence of rudimentary vessels. Each section is 8 μ m thick and each of the images was taken at a magnification of 20X and 60X. Nuclear counterstain: DAPI (blue). (B)

Quantitative analysis of cross-sectional area of the rudimentary vessels present in the aggregates. *** $p < 0.001$ and error bars denote standard deviation ($n = 9$)..... 141

Figure 6.1: Schematic of the Cellular Groups, Hydrogel Methodology, and Experimental Setup..... 157

Figure 6.2: Masson's Trichrome of samples from each of the groups after 4 weeks implantation. A denotes nanofiber mesh, B denotes islands of alginate, C denotes sGAG rich encapsulation, D denotes area of degradation and arrows denote vessels complete with red blood cells. Images were taken at 5X and 20X..... 163

Figure 6.3: Total Mineral Volume. Error bars denote standard error ($n = 8$)..... 164

Figure 6.4: Alizarin Red staining of samples from each of the groups after 4 weeks implantation. Red Staining denotes mineralisation nodules present, A denotes aggregates present within the alginate, and arrows denote vessels present. Images were taken at 4X and 40X. 165

Figure 6.5: (A) Representative BLI heat-maps for representative rat over the time course of the study (B) Total BLI count of all the groups over the course of the study. 167

Figure 6.6: (A) Total Vessel volume, and (B) Average Vessel Diameter demonstrating the level of vessel formation within the implanted constructs after 4 weeks ($n = 8$)..... 169

Figure 6.7: (A) Microcomputed tomography angiography representative images of vessel diameter and (B) Histograms of Vessel Diameter for each group demonstrating location of the varying vessel thickness of the vessels present within each of the implanted constructs after 4 weeks. 170

Figure 6.8: Immunohistochemical staining of the groups after 4 weeks implantation. CD31 stained in green, Nucleus stained in blue, smooth actin stained in red. Boxes denote area of magnification. Arrows denote presence of CD31 (green) within vessel formation. Images were taken at 10X and 60X.....	172
Figure 6.9: Immunohistochemical staining of the groups after 4 weeks implantation. CD146 stained in green, Nucleus stained in blue, smooth actin stained in red. Boxes denote area of magnification. Arrows denote presence of CD146 within vessel formation. Images were taken at 10X and 60X.....	173
Figure 7.1: Graphical representation of the work conducted as part of this PhD Thesis in context with previous studies.....	188
Figure 7.2: Graphical representation of cellular responses due to the application of chondrogenic and vascular priming in vitro as determined in Chapter 3, 4, 5 and 6.	
.....	191
Figure 7.3: (A) Photograph of bioreactor setup. (B) sGAG/DNA content and (C) ALP Activity of aggregates chondrogenically primed for 14 days with (+HP) and without hydrostatic pressure (-HP) applied at both Pre-Medium Switch, 1 and 2 weeks Post Media Switch. Error bars denote standard deviation.	197

Table of Tables

Table 2.1: Clinical Studies using Human MSC seeded tissue engineering constructs	40
---	----

Chapter 1: Introduction

1.1 Clinical Need for Bone Tissue Engineering for Large Bone Defects

Bone is an active material that constantly adapts itself to accommodate the loading conditions imposed on the skeletal system during every day physical activities. This adaptive nature is crucial to the ability of the skeleton to protect and support the organs of the body throughout life. However, during disease and injury fractures can occur, and if these fractures are sufficiently large, non-unions can result, which lack potential to heal without further intervention. The cause of these non-union defects falls into three categories; (1) severe traumatic injuries, (2) non-union due to the age of the patient and (3) osteosarcoma.

In 2011 more than 30,000 people died on the roads of the European Union, however, for every death there was also 8 serious or disabling injuries leading to non-union defects (European Commission, 2011). There has also been nearly 6,800 military fatalities and more than 51,000 injuries (US Department of Defence, 2014) as a result of the wars in Iraq and Afghanistan and the use of advanced explosives in these conflict zones has also lead to a substantial increase in severe blast traumas. Currently, explosive injuries have accounted for approximately three-quarters of all combat-related injuries (Belmont et al., 2012), leading to an increasing number of patients both within the USA and Europe with non-union defects.

Moreover, with an increasing aging population, the average person's risk of hip, vertebral and wrist fractures has increased exponentially. In the USA alone there are approximately 280,000 hip, 700,000 vertebral and 250,000 wrist fractures (Hollinger et al., 2000), with delayed healing or non-union healing occurring in 5% of all fractures, and 20% of high impact fractures (Dickson et al., 2007; Brydone et al., 2010).

Finally, each year over 800,000 new cases of osteosarcoma are diagnosed in the United States (AmericanCancerSociety, 2014). With over 50% of these tumours being relatively resistant to radiation therapy, the standard treatment is tumour resection, leading to non-union bone defects. The management of these defects is a challenging and costly exercise, which has a significant socioeconomic cost (Chapekar, 2000; Rose and Oreffo, 2002), estimated to be €14.7 billion in Europe (Enterprises, 2001) and \$45 million in the USA in the last 18 years (Hollinger et al., 2000).

1.1.1 Current Treatments for Large Bone Defects

The treatment approaches for non-union defects include autografts, allografts and vascularised bone grafts (Perry, 1999; Rose and Oreffo, 2002; Cancedda et al., 2007; Dimitriou et al., 2011; St John et al., 2003; Finkemeier, 2002). Autografts are transplants of tissue from one site to another within an individual and are considered the ideal bone graft, as they incorporate both osteogenic cells and an osteoconductive mineral. An example of this involves autologous tissue transplantation from the patient's iliac crest, ribs or calvarium. However, these procedures have major limitations such as donor site morbidity, pain, bleeding and infections (Coventry and Tapper, 1972; Reid, 1968; Younger and Chapman, 1989; Brydone et al., 2010;

Dimitriou et al., 2011; St John et al., 2003; Finkemeier, 2002; Ahlmann et al., 2002). Moreover, as the tissue is coming from the patient, this method is limited with regards to the amount of bone that can be transplanted and therefore is not a suitable source for the treatment of large bone defects or multiple defects (Brydone et al., 2010). Allografts are transplanted from cadavers or other living donors. To prevent host immune response, the grafts are usually processed to remove all cellular components prior to transplantation (Brydone et al., 2010). Although allografts can provide greater amounts of graft tissue than autografts, there is a risk of disease transmission and infection, with 18% of donated femoral heads having a bacterial or fungal contamination (Boyce et al., 1999; Dick and Strauch, 1994; Chapman and Villar, 1992; Brydone et al., 2010). Vascularised bone grafts are autografts that have been extracted with the original blood supply of the donor, which are then transplanted to the affect area. They have been shown to have some success in the treatment of non-union fractures as there are fewer complications associated with the procedure (Rizzo and Moran, 2008), and as the grafts are already vascularised, they are integrated quickly. However, such grafts are expensive and non-unions are still common, especially in large shaft reconstructions (Perry, 1999; Rose and Oreffo, 2002; Cancedda et al., 2007; Weiland et al., 1983; Sowa and Weiland, 1987; Taylor, 1983; Enneking et al., 1980). Therefore, there is a distinct clinical need for new therapies that provide an effective clinical treatment for large bone defects.

1.1.2 Bone Tissue Engineering Applications for Large Bone Defects

Tissue engineering is a multidisciplinary field, which is focused on the fabrication of tissues for the human body, in order to preserve the physiological function of damaged or diseased tissues. The three key elements of a tissue

engineering construct are morphogenic signalling, responding stem cells and extracellular matrix scaffolding (Reddi, 1998; Reddi, 2007). Among all of the tissues within the body bone has great potential for regeneration and bone tissue engineering has been shown to have some promise for treating bone diseases and reconstructing bone defects (Cancedda et al., 2007; Dawson and Oreffo, 2008; Rose and Oreffo, 2002; Reddi, 2007). These approaches involve the use of responding stem cells, which are often grown on biomaterial scaffolds in an in vitro environment in the presence of osteogenic growth factors and cell culture nutrients. However, many of these strategies are limited due to the occurrence of degradation of the tissue engineered constructs when implanted in vivo (Phelps and Garcia, 2009; Ko et al., 2007; O'Brien, 2011; Amini et al., 2012), which will be discussed in further detail in Chapter 2. Clinical trials of tissue engineered bone products within large bone defects have been undertaken but results are limited to case reports, and have shown less bone healing than was previously seen during animal trials (Quarto et al., 2001; Marcacci et al., 2007; Krecic-Stres et al., 2007; Meijer et al., 2008; Hibi et al., 2006; Meijer et al., 2007; Lee et al., 2010; Zamiri et al., 2013; d'Aquino et al., 2009; Shayesteh et al., 2008), as is discussed in detail in Chapter 2. To date in vitro tissue regeneration for clinical treatment of bone pathologies has not reached its full potential (Ma et al., 2014; Frohlich et al., 2008).

A possible solution to overcome the current limitations of in vitro bone regeneration is to recreate a bone development process that occurs during early fetal development, known as endochondral ossification. This process begins when chondrocytes condense, proliferate and produce a cartilage template, which is subsequently invaded by blood vessels, bone marrow Mesenchymal Stem Cells (MSCs) and osteoblasts, after which bone tissue is deposited upon this template

(Mackie et al., 2008; Kronenberg, 2003; McNamara, 2011). In vitro tissue regeneration approaches, which mimic the formation of the cartilage template through the use of chondrogenic growth factors, have been investigated, and such approaches have been shown to enhance osteogenic differentiation of MSCs and mineral production both in vivo and in vitro (Farrell et al., 2011; Farrell et al., 2009; Jukes et al., 2008; Harada et al., 2014; Scotti et al., 2013; Scotti et al., 2010; van der Stok et al., 2014; Gawlitta et al., 2015; Visser et al., 2015). The duration of chondrogenic priming has been shown to affect the construct's survivability in vivo, however optimum timing has not yet been investigated. To address this need, the first research hypothesis to be investigated is if “There is an optimum duration for chondrogenic priming of MSCs that will enhance osteogenic differentiation of MSCs in vitro”.

Other studies have investigated whether pre-vascularising constructs prior to implantation would allow for faster host infiltration post-implantation. The results from these studies found that pre-vascularisation networks can be formed within aggregates and biomaterial scaffolds both in vivo (Correia et al., 2011; Ghanaati et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Duffy et al., 2011; Scherberich et al., 2007) and in vitro (Fuchs et al., 2007; Gawlitta et al., 2012; Saleh et al., 2011; Verseijden et al., 2010). During endochondral ossification in vivo both the formation of the cartilage template and the vascularisation of this template must occur prior to bone formation. Although researchers have demonstrated the separate effectiveness of chondrogenic priming and prevascularisation, to date no tissue engineering strategy has sought to incorporate both of these crucial events. Therefore, the second hypothesis of this Thesis is that “A tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage aggregates, through the co-culture of Human Umbilical

Vein Endothelial Cells (HUVECs) in vitro, will serve as an effective in vitro bone regeneration approach.”

The standard procedure to induce osteogenic differentiation of MSCs in vitro is through the treatment of a cocktail of dexamethasone (dex), ascorbic acid (asc) and β -glycerolphosphate (β -glyc). However, in vivo bone progenitors and other cells produce various factors that can induce osteogenic differentiation without the use of any of these supplements. In vitro co-culture studies of MSCs with HUVECs (Villars et al., 2000; Villars et al., 2002; Guillotin et al., 2008; Zhao et al., 2012) or chondrocytes (Nakagawa et al., 2000) have found that the direct co-culture can promote the expression of early osteogenic markers (Alkaline Phosphatase (ALP) activity), even without the presence of osteogenic supplements. During endochondral ossification MSCs, endothelial stem cells, osteoblasts and chondrocytes all reside in close proximity within the cartilage template, but the influence of this cellular niche for osteogenic differentiation of MSCs has not been established. Therefore, the third hypothesis of this Thesis is that “An in vitro bone regeneration strategy that mimics the cellular niche of the endochondral template will provide an alternative strategy for in vitro mineralisation of MSCs, and thereby obviate the need for external osteogenic growth factors.”

The fate of tissue engineered bone constructs once implanted in vivo is immensely important, in particular with respect to cell viability, vessel infiltration and mineral formation. Therefore, the final hypothesis of this PhD Thesis is that “A tissue regeneration approach that incorporates both chondrogenic priming of MSC aggregates, to first form a cartilage template, and subsequent pre-vascularisation of

the cartilage aggregates, through the co-culture of HUVECs in vitro, will enhance bone formation in vivo.”

1.2 Objectives and Hypothesis

The global objective of this Thesis is to investigate the effect of endochondral priming of stem cells in vitro on the mineralisation capacity of MSC aggregates both in vitro and ultimately once implanted in vivo. The primary specific objective of this Thesis is to investigate the optimum duration of chondrogenic priming (10-28 days) to enhance osteogenic differentiation of MSC aggregates in vitro. The second specific objective of this Thesis is to compare the in vitro regenerative potential of (a) chondrogenic priming of MSCs in aggregate culture and (b) addition of HUVECs to chondrogenic MSC aggregates, to (c) a novel methodology involving both chondrogenic priming and the co-culture of HUVECs and MSCs. The third specific objective of this Thesis to compare the mineralisation and vessel formation potential of (a) chondrogenic priming of MSCs, (b) addition of HUVECs alone to chondrogenic MSC aggregates, and (c) addition of HUVECs and MSCs to chondrogenic MSCs aggregates all cultured without the use of any osteogenic supplements and compare them to (d) the same experimental groups, which were cultured in the presence of osteogenic supplements and to (e) non co-culture group cultured in the presence of osteogenic supplements alone. The final specific objective is to investigate the in vivo vascularisation and mineralisation potentials of endochondrally primed cellular aggregates in a rat subcutaneous delivery model. To address these objectives, four hypotheses have been defined, each of which will be underpin the research of Chapters 3-6 of this Thesis.

Hypothesis 1: There is an optimum duration for chondrogenic priming of MSCs that will enhance osteogenic differentiation of MSCs in vitro.

Hypothesis 2: A tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage aggregates, through the co-culture of HUVECs in vitro, will serve as an effective in vitro bone regeneration approach.

Hypothesis 3: An in vitro bone regeneration strategy that mimics the cellular niche of the endochondral template will provide an alternative strategy for in vitro mineralisation of MSCs, and thereby obviate the need for external osteogenic growth factors.

Hypothesis 4: A tissue regeneration approach that incorporates both chondrogenic priming of MSC aggregates and subsequent pre-vascularisation of the cartilage aggregates, will improve cell viability, vessel infiltration and thus mineral formation once implanted in vivo.

By testing each of these hypotheses, the research objectives outlined above can be answered and the proposed research will deliver significant advances in the understanding of the conditions under which a bone tissue engineered construct can be generated in vitro, and can survive and integrate once implanted in vivo.

1.3 Thesis Structure

This Thesis comprises the work completed for the duration of the candidate's PhD studies. Chapter 2 presents a thorough review of the literature, detailing bone

function and composition, bone's adaptive nature, bone cells, bone development and current approaches in bone tissue engineering, in particular bone tissue engineering strategies incorporating certain aspects of the endochondral ossification process. Chapter 3, investigates whether there is an optimum time for the formation of the cartilage template that will enhance the mineralisation potential of MSCs *in vitro*, testing Hypothesis 1 of the Thesis. In Chapter 4, a cartilage template is formed for the optimum time, as deemed by the results of Chapter 3, and is subsequently co-cultured with either HUVECs or HUVECs and hMSCs to investigate the vascularisation and mineralisation potential, and as such tests Hypothesis 2 of this research. Chapter 5 investigates the vascularisation and mineralisation potential of the co-culture strategy developed in Chapter 4 without the presence of osteogenic growth factors, thus addressing Hypothesis 3. Finally in Chapter 6, the *in vivo* potential of these endochondrally primed aggregates developed through the studies of Chapters 3-5, is determined in a subcutaneous model, addressing the final Hypothesis of this Thesis. A summary of the main findings of the Thesis is presented in Chapter 7, placing them in the context of current bone tissue engineering strategies, along with recommendations for future research in these fields.

Chapter 2: Literature Review

2.1 Introduction

2.1.1 Bone Function and Anatomy

The skeletal system supports and protects our internal organs, contributes to our shape and allows us to move by providing a bony framework that muscles pull on as they work (Clarke, 2008). The adult skeletal system has in total 206 bones, excluding the sesamoid bones, which are found within the appendicular skeleton (126 bones), the axial skeleton (74 bones), and then finally the auditory ossicles (6 bones) (Gray and Standring, 2008). Each bone is an active material that constantly undergoes modelling and remodelling to accommodate the biomechanical forces imposed by everyday activities. There are two categories of bone, namely flat bones, such as the scapula, ilium and cranium, which function for protection or as surfaces for muscular attachment, and the load-bearing long bones, such as the femur, tibia and fibula, which are essential for skeletal mobility (Clarke, 2008).

2.1.2 Bone Tissue Composition

Bones are lightweight to allow for efficient movement, whilst also providing strength, support, and rigidity to the skeleton. This mechanical behaviour can be attributed to the fact that bone is a composite material with a highly complex hierarchical structure. It is composed of organic proteins and mineral crystals, which are intricately organised to allow bone to be both strong and lightweight and to be able to

serve these functions under the variety of loading conditions experienced during everyday activities. Bone is a porous composite material that is composed of 65% mineral, 35% organic matrix, cells and water. Hydroxyapatite is the principle component of the mineral phase of bone. This is present in the form of nanoscale crystals of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) and other minerals including magnesium, potassium, manganese, silica, fluoride, iron, zinc and citrate (Currey and Brear, 1990; Fratzl et al., 2004). The organic phase is composed of approximately 90% collagen type I, 5% non-collagenous proteins and 2% lipids by weight (Boskey, 2013). The proteins present in the organic matrix can be split into two categories: (1) structural proteins, such as collagen and fibronectin, and (2) proteins with specialised functions. Such specialised functions include those that: (a) regulate collagen fibril diameter, (b) serve as signalling molecules, (c) serve as growth factors, (d) serve as enzymes and (e) have other functions (Boskey, 2013). Although the collagen present in bone is primarily collagen type I there are also trace quantities of collagen type III, IV and VI present (Miller, 1969; Miller, 1984). The non-collagenous proteins, such as osteocalcin, osteonectin, osteopontin, and bone sialoprotein, provide the necessary bonds between collagen fibrils and thus facilitate mineralisation by providing a binding site for osteoclasts (Roach, 1994; Thurner et al., 2009).

2.1.3 Bone Structure and Organisation

Each of the constitutive phases (stiff mineral phase and softer organic phase) are hierarchically organised to provide a tissue that is highly optimised to function under the variety of loading conditions experienced, whilst also being lightweight for efficient movement (Vaughan et al., 2012), as shown in Figure 2.1.

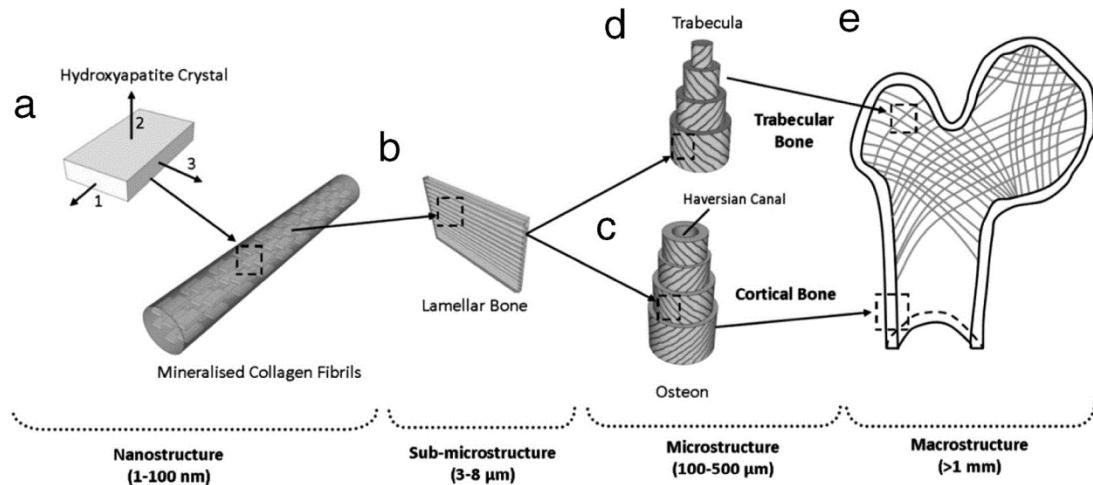


Figure 2.1: Hierarchical Structure of bone demonstrating the composite structure from the nanoscale to the macroscale (a-e) (Vaughan et al., 2012).

Bone tissue is organised in two different types of tissue; cortical bone (also known as compact bone) and trabecular bone (also known as cancellous or spongy bone). Different bones and skeletal sites within bones have different ratios of cortical to trabecular bone. Long bones are composed of a hollow diaphysis, with cone-shaped metaphyses below both growth plates, and a round epiphysis above the growth plates see Figure 2.2.

The diaphysis is comprised mostly of cortical bone, which is a dense solid that surrounds the marrow and is comprised of functional units known as osteons. Each osteon contains a central blood vessel, located within the Haversian canal, which is encircled by rings containing mechanical sensitive bone cells, known as osteocytes (Eriksson et al., 1994). Cortical bone has an outer periosteal surface and an inner endosteal surface. The periosteum consists of two layers, an outer fibrous layer and an inner cambium layer. The fibrous layer contains fibroblasts, collagen and elastin fibrils, while the cambium layer, positioned directly on top of the bone surface contains progenitor cells, which develop into osteoblasts are essential for appositional

growth and fracture repair (Allen et al., 2004). Typically during bone growth, bone formation exceeds bone resorption on the periosteum, whereas bone resorption typically exceeds bone formation on the endosteum (Clarke, 2008).

Both the epiphysis and metaphysis are made predominately of trabecular bone, with a thin shell of cortical bone. Trabecular bone is composed of a series of multidirectional rod- and plate-like struts known as trabeculae. The orientation of the trabeculae has been proposed to be positioned along the lines of regular mechanical stress (Frost, 1990; Meyer von, 1867) and thereby provides mechanical support by dissipating the internal stresses out to the stronger cortical bone shell (Lemaire et al., 2004), whilst also allowing bone to be lightweight (Wolff, 1986).

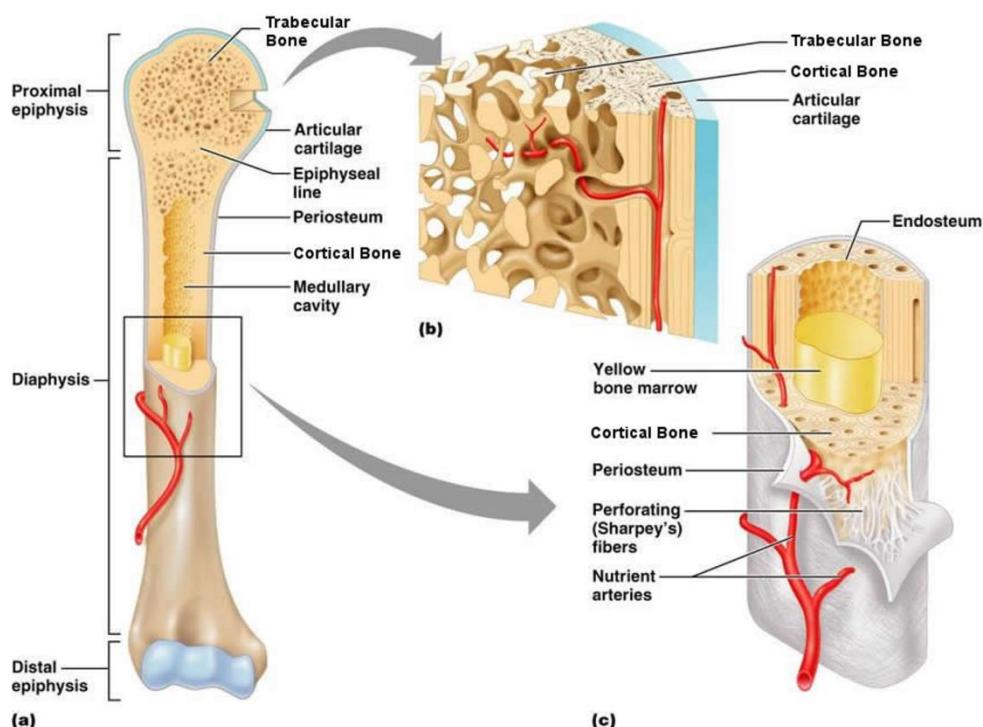


Figure 2.2: (a) Macrostructure of the Long bone showing (b) micro structure of the Epiphysis and (c) macrostructure of the Diaphysis (Marieb and Hoen, 2006).

2.2 Bone Cells

Bone tissue is home to many different cell types including osteoblasts, osteoclasts and osteocytes. These cells derive from one of two lineages: the osteogenic lineage (osteoblasts and osteocytes) or the monocyte-macrophage lineage (osteoclasts), as shown in Figure 2.3. Osteoprogenitor cells, present within the bone marrow, differentiate into osteoblasts and ultimately osteocytes, whereas osteoclasts are formed from multinucleated cells found within the bone marrow and vasculature of bone (Udagawa et al., 1990; Okazaki et al., 2002).

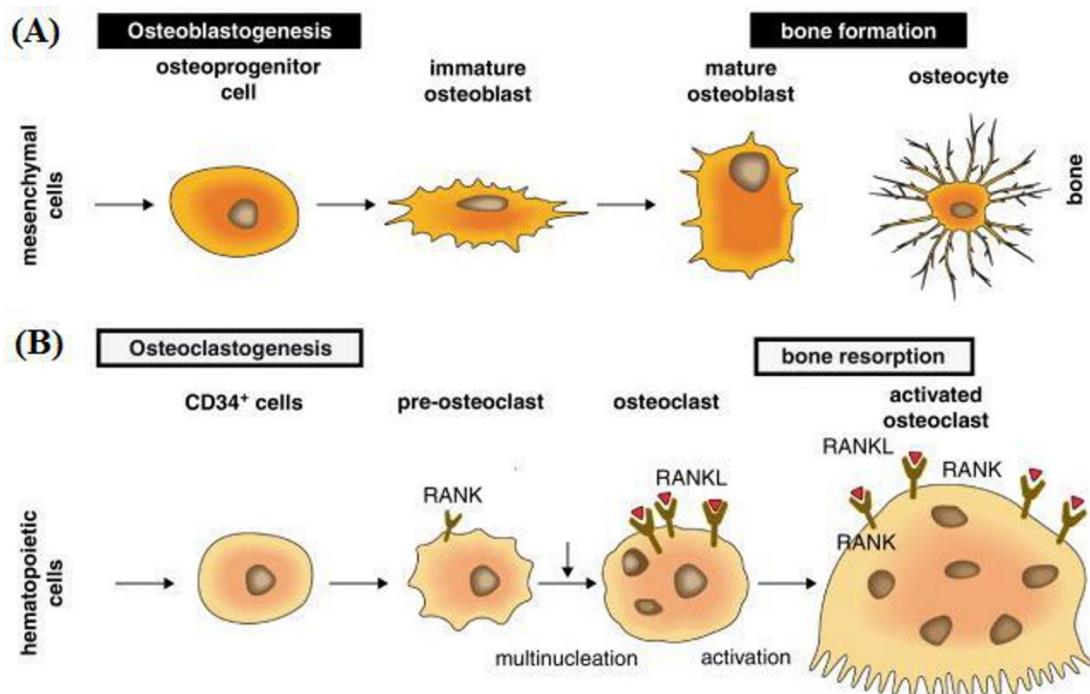


Figure 2.3: Differentiation of cell types along the two lineages: (A) the osteogenic lineage and (B) the osteoclastic lineage (Wang et al., 2012b).

2.2.1 Bone Lining Cells and Osteoblasts

Bone lining cells are quiescent osteoblasts that form the endosteum on trabecular and endosteal surfaces, and underlie the periosteum on the mineralised surface

(Clarke, 2008). They have fewer organelles than the active osteoblasts (Cameron, 1968), which indicates that they are largely inactive cells (Aubin et al., 1993). They are hypothesised to function as a cellular membrane that regulates the fluxes of calcium and phosphate in and out of bone and to control the growth of mineral crystals by maintaining a suitable microenvironment (Miller and Jee, 1987; Miller et al., 1980). They also produce signals that initiate bone resorption and remodelling, as well as playing an important role in the marrow stromal system (Miller et al., 1989; Miller et al., 1980).

Osteoblasts are responsible for synthesising bone matrix proteins and mineral during early fetal development, whilst also controlling bone formation and mineralisation throughout life. They are formed by osteoprogenitor cells found in the bone marrow, which mature and become cuboidal cells that have a large Golgi apparatus, a rough endoplasmic reticulum and a central nucleus (Jee, 2001; McNamara, 2011). Their morphology can be seen in Figure 2.4.

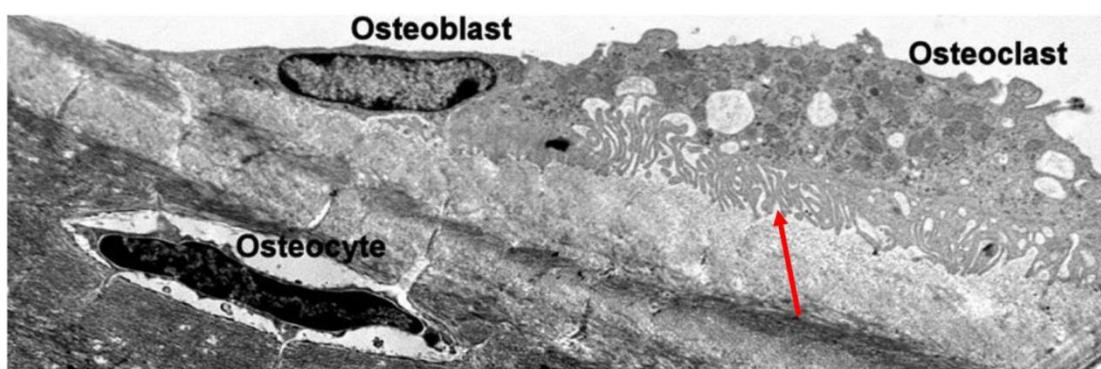


Figure 2.4: TEM image of an osteocyte, osteoblast and osteoclast, with ruffled border of the osteoclast indicated (red arrow). Reproduced with permission of Dr. L.M. McNamara.

During early fetal development, osteoblasts secrete collagen and noncollagenous proteins that act as a template for bone, which will be described further in Section 2.4. Osteoblasts also function throughout life to produce new bone mineral that is required to replace aged or damaged bone (will be described Section 2.5.1). Osteoblasts secrete osteoid and a range of hormones, lipids and enzymes such as ALP, prostaglandins, and Parathyroid hormone (PTH) (Aubin, 2001; Aubin et al., 1993).

The ALP enzyme is produced by both bone lining and osteoblast cells but is absent from the osteocytes (Doty and Schofield, 1976; Aubin et al., 1993), and it is generally accepted that ALP has a role in the regulation of osteoblast differentiation (Aubin et al., 1993), but also regulates local phosphate concentration and promotes mineralisation (Robison, 1923) by removing inhibitor proteins (Clarke, 2008), and providing an initial attachment site for mineral nucleation along the collagen fibrils (McNamara, 2011; Jee, 2001). ALP production is often followed by an up-regulation of the mineral regulating proteins: osteocalcin (Aubin et al., 1993; Bronckers et al., 1987; Aronow et al., 1990; Owen et al., 1990), osteopontin and bone sialoprotein (Aubin, 2001).

Prostaglandins are low molecular weight lipids and are known to have a vital part to play in the biological effects in a variety of cellular systems including bone (Curtis-Prior, 1988). Prostaglandins have been shown to influence expression of many aspects of the osteoblast phenotype (Harvey, 1988), and it has been hypothesised that the ability to respond to prostaglandins (specifically PGE₂) is a marker of a less differentiated osteoblastic cell (Aubin et al., 1993). PTH is a major regulator of mineral ion metabolism (Abou-Samra et al., 1992), which inhibits collagen synthesis (Dietrich et al., 1976) and ALP production (Luben et al., 1976; Felix and Fleisch,

1979), and stimulates calcium influx (Dziak and Brand, 1974; Dziak and Stern, 1975; Rodan and Martin, 1981).

Osteoblasts also produce a range of growth factors and cytokines (interleukin 1), specifically, insulin-like growth factors (IGFs), transforming growth factor β (TGF- β), and the bone morphogenic proteins (BMPs). IGFs have been shown to stimulate osteoblast proliferation and differentiation (Canalis et al., 1993; Mohan et al., 1995; Mundy, 1993), and both TGF (Linkhart et al., 1996b; D'Souza et al., 1993) and BMPs (Finkelman et al., 1992) are expressed at high levels in mature osteoblasts on bone surfaces during bone development and growth (Linkhart et al., 1996a).

Osteoblasts are also responsible for producing bone matrix proteins including collagen type I, osteocalcin, osteonectin, bone sialoprotein and osteopontin. Osteocalcin plays a role in osteoclast recruitment and bone resorption (Mundy and Poser, 1983; Lian et al., 1984; Glowacki et al., 1991) and is secreted after mineralisation has begun (Groot et al., 1986; Bronckers et al., 1987; Vermeulen et al., 1989). Osteonectin is produced in both embryonic and adult bone tissue that is undergoing remodelling and/or morphogenesis (Howe et al., 1988; Nomura et al., 1988; Sage et al., 1989). Osteopontin, also known as bone sialoprotein I, and bone sialoprotein are present in the bone matrix, osteocytes, and stromal cells in the marrow as well as osteoblasts, and their expression/production is highest in new bone and 21-day old fetal bone and then decline thereafter (Aubin et al., 1993).

2.2.2 Osteocytes

Osteocytes are terminally differentiated osteoblasts (as seen in Figure 2.3), and are the most abundant cell in mature bone, representing more than 90% of the total

number of bone cells (Kato et al., 1997; Bonewald, 2011). They reside within lacunae of the mineralised bone matrix, embedded within their secreted osteoid (Clarke, 2008). Differentiation of osteoblasts into osteocytes occurs during the deposition of new bone matrix. During this process osteocytes lose much of the organelle from their predecessors and acquire elongated dendritic processes, which are extensions of their plasma membrane (Jee, 2001; McNamara, 2011). Osteocytes express osteocalcin, osteopontin and a cell adhesion receptor for hyaluronate (Clarke, 2008; Thompson et al., 2011; Noonan et al., 1996; Noble, 2008), as well as other bone matrix proteins including Dentin Matrix Protein 1 (DMP1), cell surface glycoproteins (CD-44, E11, and β -3 integrin), phosphate regulating genes (Sost) (Hall, 1941), and other signalling molecules including Nitric Oxide (NO). DMP1 and E11 are expressed by osteoblasts that are developing into osteocytes. Sclerostin is the secreted gene product of the Sost gene, and it has been shown to inhibit osteoblasts and is only produced by osteocytes in late stage bone formation, as it is not expressed in osteocytes associated with recently deposited osteoid (Hall, 1941). Sclerostin is only expressed in the osteocyte once cell processes have formed (Winkler et al., 2003) and has been shown to regulate bone formation by interrupting the Wnt signalling pathways (Poole et al., 2005; Li et al., 2005). In bone, NO has been shown to mediate bone formation by protecting osteocytes against apoptosis (Tan et al., 2006) and high levels of NO rapidly reduce osteoclast activity (Tan et al., 2007; MacIntyre et al., 1991).

Osteocytes maintain connections with each other, bone lining cells and bone surface via their multiple filipodial cellular processes that reside in canaliculi or channels within the bone matrix (Jee, 2001; Clarke, 2008). Osteocyte cell processes are connected to one and another and to other cells via gap junctions (Li et al., 2000; Xia et al., 2010), which are composed primarily of connexin proteins (Clarke, 2008).

Connexin 43 is found on the membrane of the cell body as well as on the cell processes (Jones et al., 1993). Gap junctions are also required for osteocyte maturation, activity, and survival (Clarke, 2008). A thin layer of pericellular fluid is present within the lacunar-canalicular system, which separates the osteocyte body and processes from the surrounding bone and its matrix, as shown in Figure 2.5. This pericellular fluid also supplies nutrients and allows for the dispose of waste through the lacunar-canalicular system (Jee, 2001).

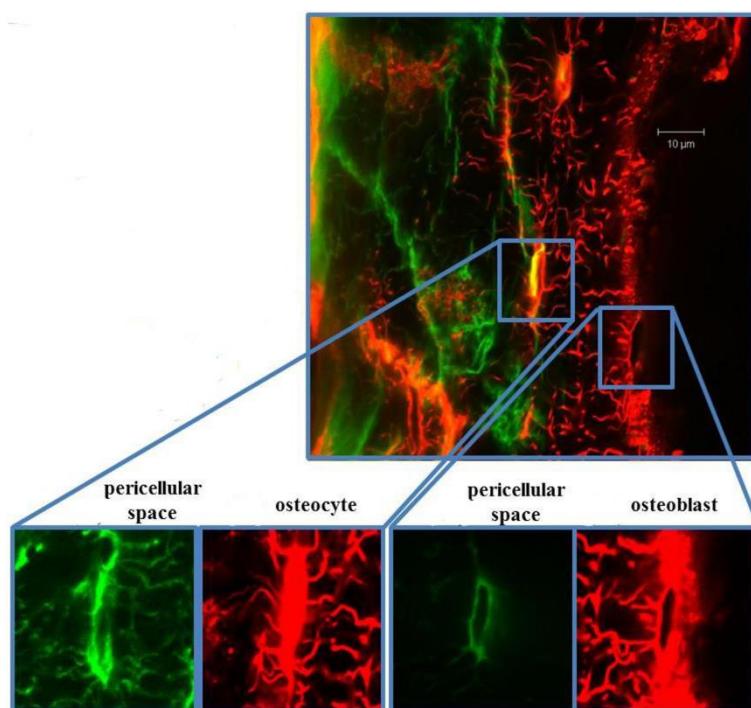


Figure 2.5: Confocal scans performed from the surface of a cut rat femur allowing for visualisation of the pericellular space, osteocytes and osteoblasts (Verbruggen et al., 2015).

It is widely considered that the primary function of osteocytes is to respond to mechanical stimuli, whereby the cells transduce mechanical signals, created from bending or stretching of bone, into biological signals to govern cellular activity. The mechanical signals are created through the flow of the pericellular fluid within the

lacunar-canalicular system or through direct strain via the integrin attachments (McNamara et al., 2009; Wang et al., 2007), which then leads to external forces on osteocytes. These external forces have been shown to include fluid shear (Imai et al., 2009; Taylor et al., 2007; Hoey et al., 2011) and hydrostatic pressure (Takai et al., 2004; Liu et al., 2010), and these forces have been shown to lead to a variety of responses within the osteocyte (Clarke, 2008). Responses include the production or reduction of signal molecules such as BMPs, Sclerostin (Robling et al., 2006), PGE₂, and NO (Tan et al., 2006; Tan et al., 2007), which as stated previously can modulate recruitment, differentiation, and activity of osteoblasts and osteoclasts (Klein-Nulend et al., 2012). Osteocytes have also been shown to monitor the mechanical environment within bone tissue (Adachi et al., 2009; Cowin et al., 1995; Bonewald, 2002; Cowin et al., 1991; Lanyon, 1993; Klein-Nulend et al., 1995; Burger and Klein-Nulend, 1999; Wang et al., 2007), to regulate bone resorption to micro-damage (Zhao et al., 2002; Schaffler et al., 2014; Kennedy et al., 2012; Schaffler and Kennedy, 2012), and finally have been shown to be more influential than osteoblasts in stimulating the differentiation of nearby MSCs (Birmingham et al., 2012).

2.2.3 Osteoclasts

Osteoclasts are derived from mononuclear precursor cells in the hematopoietic vascular channels in bone (Udagawa et al., 1990; Boyle et al., 2003). They are large multi-nucleated cells that can range in diameter from 20-100µm (Roodman, 1996), with the main purpose of resorbing damaged or aged bone matrix. They are scarcely found in bone, as they temporarily reside in resorption cavities or Howship's lacunae on the bone surface when undergoing bone resorption (Figure 2.6) (Watanabe et al., 1995; Clarke, 2008) and only cover less than 1% of the bone surface at any given time

during adolescence. These highly motile cells can possess up to 50 nuclei and contain multiple cellular adhesions, cytoskeleton proteins, granules, vacuoles and an abundance of mitochondria (Horne, 1995; Watanabe et al., 1995).

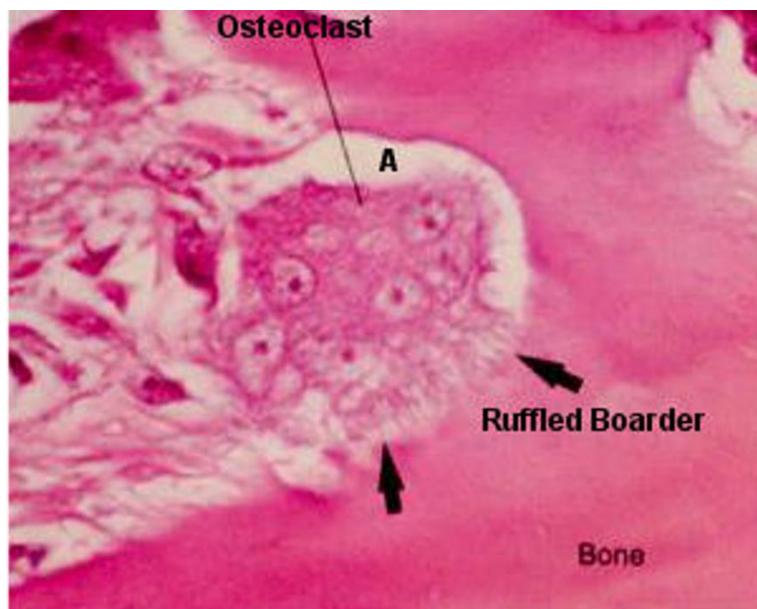


Figure 2.6: Multinucleated Osteoclast in its resorption cavity or Howship's Lacunae denoted by the letter (A) (Cormack, 2001).

Osteoclastogenesis is governed by stromal cells and osteoblasts present in the bone marrow, which produce RANKL and macrophage cytokines CSF (M-CSF), in both membrane-bound and soluble forms, which are crucial for osteoclast formation. RANKL is critical for osteoclast formation, whereas M-CSF is required for the proliferation, survival and differentiation of osteoclast precursors, as well as osteoclast survival and the cytoskeletal rearrangement required for bone resorption (Clarke, 2008; Teitelbaum and Ross, 2003). The life cycle of an osteoclast has been shown to be up to six weeks, after which it migrates to the marrow space and undergoes apoptosis (Marks Jr and Seifert, 1985).

2.2.4 Mesenchymal Stem Cells

MSCs were first discovered in the bone marrow by Friedenstein et al., and were described as clonal and fibroblastic stromal cells that form adherent colonies in culture and had osteogenic potential (Friedenstein et al., 1968; Friedenstein et al., 1970; Friedenstein et al., 1987). The MSC has a basic cell morphology consisting of a small cell body, a large round nucleus with a prominent nucleolus that is surrounded by chromatin particles, a Golgi apparatus, rough endoplasmic reticulum, mitochondria, and polyribosomes. Within the cytoplasm there is a 3D network of proteins known as the cytoskeleton. The cytoskeleton dictates the morphology and shape of MSCs and is able to extend their cytoplasm into the surrounding matrix in which they reside by means of cell processes (McNamara, 2011).

MSCs are usually isolated from an aspirate of bone marrow harvested from the iliac crest of the pelvis in humans (Pittenger et al., 1999; Barry and Murphy, 2004; Digirolamo et al., 1999), or in rodents it is generally isolated from the mid-diaphysis of the tibia or the femur (Barry and Murphy, 2004). The International Society for Cellular Therapy (ISCT) has established the three main criteria for defining MSCs, which include (1) adherence to plastic in standard culture conditions, (2) specific surface antigen expression, and (3) differential potential to give rise to specific mesenchymal tissues including bone, cartilage, muscle, bone marrow stoma, fat, dermis and other connective tissues, see Figure 2.7 (Kuhn and Tuan, 2010; Caplan, 1990; Caplan, 1991; Caplan, 2005; Barry and Murphy, 2004; Dominici et al., 2006). Recently, the ISCT has further modified these criteria to include surface markers. By flow cytometry >95% of the MSC population must express the MSC cell markers

CD73, CD90 and CD105, and no more than 2% of the cells must express CD34 (Kuhn and Tuan, 2010; Dominici et al., 2006).

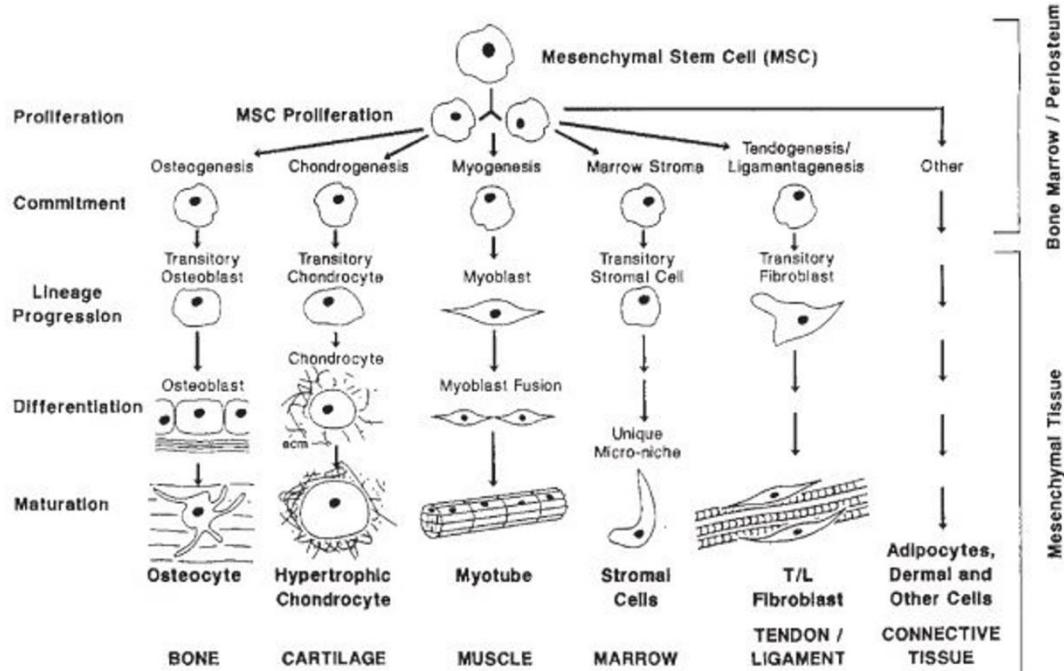


Figure 2.7: Mesenchymal Cell Lineages - MSCs have the capacity to differentiate into bone, cartilage, muscle, marrow stroma, tendon/ligament, fat and other connective tissues (Caplan, 2005).

The therapeutic use of MSCs has been used to address a wide variety of indications, including cardiovascular repair (Orlic et al., 2001; Deb et al., 2003; Saito et al., 2002; Toma et al., 2002), treatment of lung fibrosis (Ortiz et al., 2003), and spinal cord injury (Teng et al., 2002; Barry and Murphy, 2004). In the area of orthopaedic medicine local delivery of MSCs has been investigated in numerous applications including, spinal fusion (Muschler et al., 2003), craniotomy (Krebsbach et al., 1998) and repair of segmental defects which will be discussed further in Section 2.7 (Barry and Murphy, 2004).

2.3 Bone Marrow as a Stem Cell Niche

Stem cells within bone marrow are not randomly distributed. They reside in a complex microenvironment that is otherwise known as a stem cell niche. Scholfield first described a stem cell niche as a specialized microenvironment housing haematopoietic stem cells (HSC) that ensure their self-maintenance (Schofield, 1978; Kuhn and Tuan, 2010). This microenvironment contains the support cells and thus the necessary growth factors to maintain and regulate a particular stem cell or progenitor, including both HSCs and MSCs (Kuhn and Tuan, 2010; Morrison and Scadden, 2014; Morrison and Spradling, 2008). The niche is thought to have three functions: (1) maintaining quiescence, (2) promoting cell number and (3) directing differentiation of the two distinct developmental systems: the haematopoietic system and the stromal system (Fuchs et al., 2004). The bone marrow niche can be conceptually divided into two parts: an osteoblastic niche and a vascular niche. The osteoblastic niche is located near the trabecular bone and is a hypoxic environment and generally hosts quiescent haemopoietic stem cells, whereas the vascular niche is located near blood vessels and is an oxygenated niche, where stem cells and progenitor cells actively proliferate (Xaymardan et al., 2010). It is this direct contact between the two systems that provides the appropriate growth factors and cell-to-cell and cell-to-extracellular matrix contact needed for the development of bone tissue (Mauney et al., 2005) see Figure 2.8.

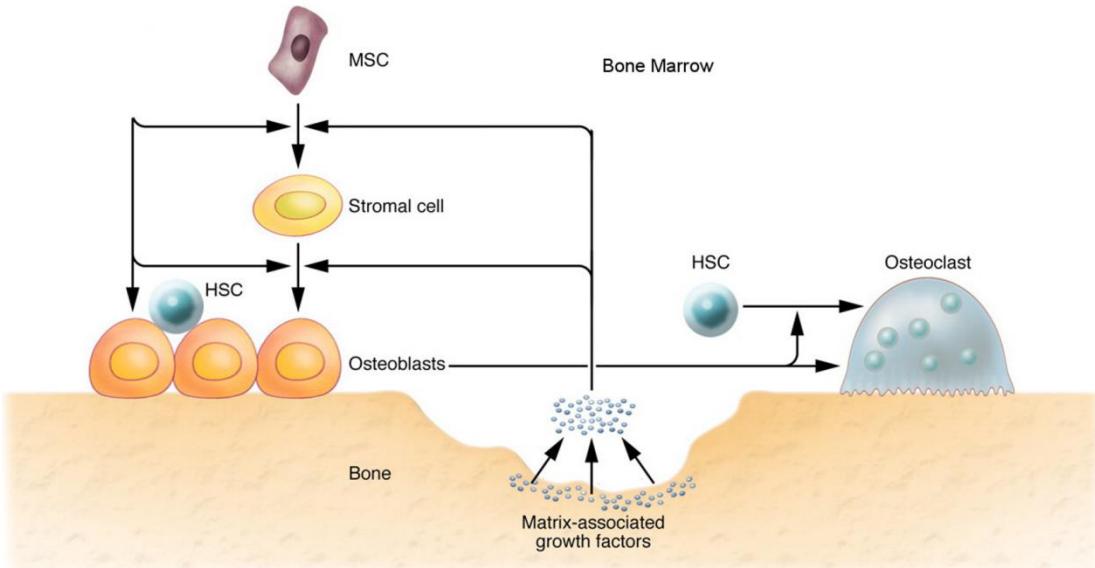


Figure 2.8: Schematic of the co-localisation of the osteoblastic niche and vascular niche present within bone marrow (Ross and Christiano, 2006).

There have also been many studies that have looked at this link between bone marrow formation/vascularisation and bone development. Specifically studies have investigated whether pre-vascularising 3D tissue engineered scaffolds in vitro, through the co-culture of MSCs and HUVECs, would allow for faster host integration post-implantation (Correia et al., 2011; Ghanaati et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Scherberich et al., 2007; Duffy et al., 2011) and these studies will be further discussed in Section 2.8.2.

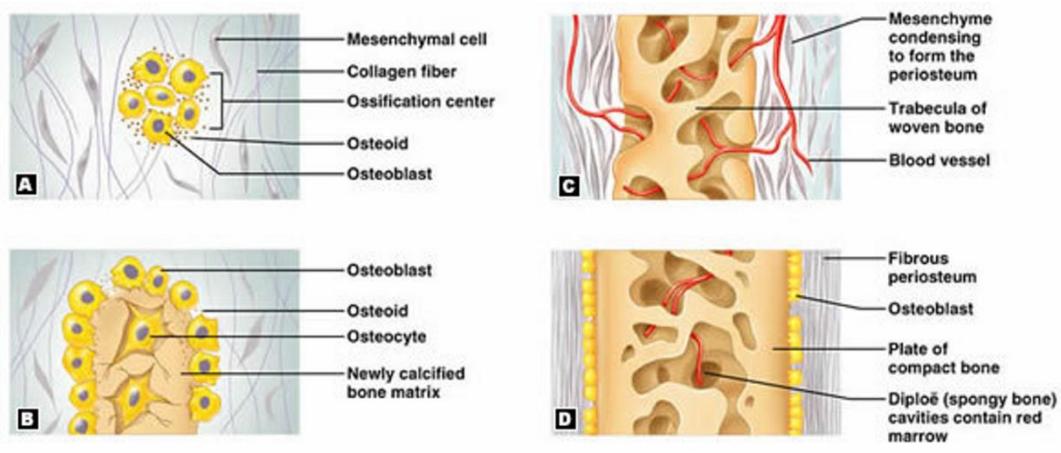
2.4 Bone Development

Bone is formed during early fetal development via two specific mechanisms: intramembranous ossification and endochondral ossification. Bone formation persists throughout life to accommodate for its mechanical environment. Both mechanisms begin with a two-step process whereby an organic matrix (osteoid/cartilage template)

is initially laid down by osteoblasts and then mineral crystals are produced and grow slowly over time to produce bone tissue (McNamara, 2011).

2.4.1 Intramembranous Ossification

All flat bones such as the skull and the clavicle are developed by a process known as intramembranous ossification. Mesenchymal progenitor cells first condense and form a cellular aggregate, which is then surrounded by a membrane, after which the cells within the membrane begin to differentiate to form osteoprogenitor cells and then osteoblasts, see Figure 2.9. Osteoblasts surround the cellular aggregate and begin to excrete Extracellular Matrix (ECM) containing type I collagen. Other osteoblasts become embedded in the newly formed matrix and differentiate and begin to form interconnecting processes and eventually osteocytes. The cells on the periphery of the aggregate form a periosteum and further growth occurs at the surface of the trabeculae. By this time the aggregate is mineralised and begins to form rudimentary bone tissue populated by osteocytes and lined by osteoblasts (McNamara, 2011; Kanczler and Oreffo, 2008; Cowin et al., 1995).



Copyright © 2006 Pearson Education, Inc., publishing as Benjamin Cummings.

Figure 2.9: The Intramembranous Ossification process: **(A)** MSCs condense form ossification centre, **(B)** the deposited osteoid entombs osteoblasts, which then become osteocytes, **(C)** periosteum and trabeculae form and vessels invade, **(D)** compact bone develops superficial to the trabecular bone (Marieb and Hoen, 2006).

2.4.2 Endochondral Ossification

Most of the bones within the body are created via a process known as endochondral ossification. This process is most commonly seen during early fetal development. Bone formation begins when pre-chondrogenic cells condense to become clusters of cells that adhere through the expression of adhesion molecules (Koyama et al., 2008). These progenitor cells begin to differentiate to become chondroblast cells and form a membrane around a cartilage template known as the perichondrium. These chondrocytes are known to have a characteristic shape, and to secrete a matrix rich in collagen type II and the proteoglycan aggrecan. As time passes the chondroblasts begin to differentiate and become chondrocytes. This leads to a cartilage aggregate growing through chondrocyte proliferation and matrix production (Mackie et al., 2008; McNamara, 2011; Kronenberg, 2003). No matter what the

location or stage of development, chondrocytes present in the cartilage template are arranged in morphologically distinct zones (see Figure 2.10), which reflect changes in the functional state of cells. The zone furthest from the ossification front is known as the zone of resting chondrocytes, also known as the germinal zone. Adjacent to this is the zone of proliferation, which consists of round proliferating chondrocytes that become flattened as they are packed into multicellular clusters. Chondrocytes pass through a transition stage (zone of maturation) in which the cells are known as “pre-hypertrophic” chondrocytes. In the hypertrophic zone chondrocytes stop proliferating, enlarge, and change their genetic program to synthesize type X collagen. The hypertrophic chondrocyte, simply through its size, is the principle engine of bone growth and is also known as a regulatory cell. Hypertrophic chondrocytes are known to direct mineralization of their surrounding matrix as well as attracting blood vessels by producing vascular endothelial growth factor (VEGF). Hypertrophic chondrocytes directly adjacent to the perichondrial cells become osteoblasts, which form an extracellular matrix containing alkaline phosphatase (Mackie et al., 2008). This matrix forms an outer membrane known as the periosteum, which consists mainly of irregular connective tissue.

ALP is an enzyme that acts as a nucleator for the deposition of minerals upon the cartilage template. It is also known to secrete the growth factors responsible for the promotion of blood vessel invasion into the perichondrium and is produced by osteoblasts and hypertrophic chondrocytes (Fell and Robison, 1934; Fell and Robison, 1929). During endochondral ossification the cartilage template undergoes a series of changes involving chondrocyte proliferation, hypertrophy and matrix calcification (Leboy et al., 1989). As the chondrocytes within the cartilage template begin to undergo hypertrophy they release ALP on the cell surface and in matrix vesicles

(extracellular membrane-invested particles), which are usually produced at the sites of initial mineralisation (Anderson, 2003; Leboy et al., 1989). During early mineralisation ALP is up regulated, however as the mineralisation process progresses and other genes (osteocalcin) are upregulated, ALP expression begins to decline (Golub et al., 1992).

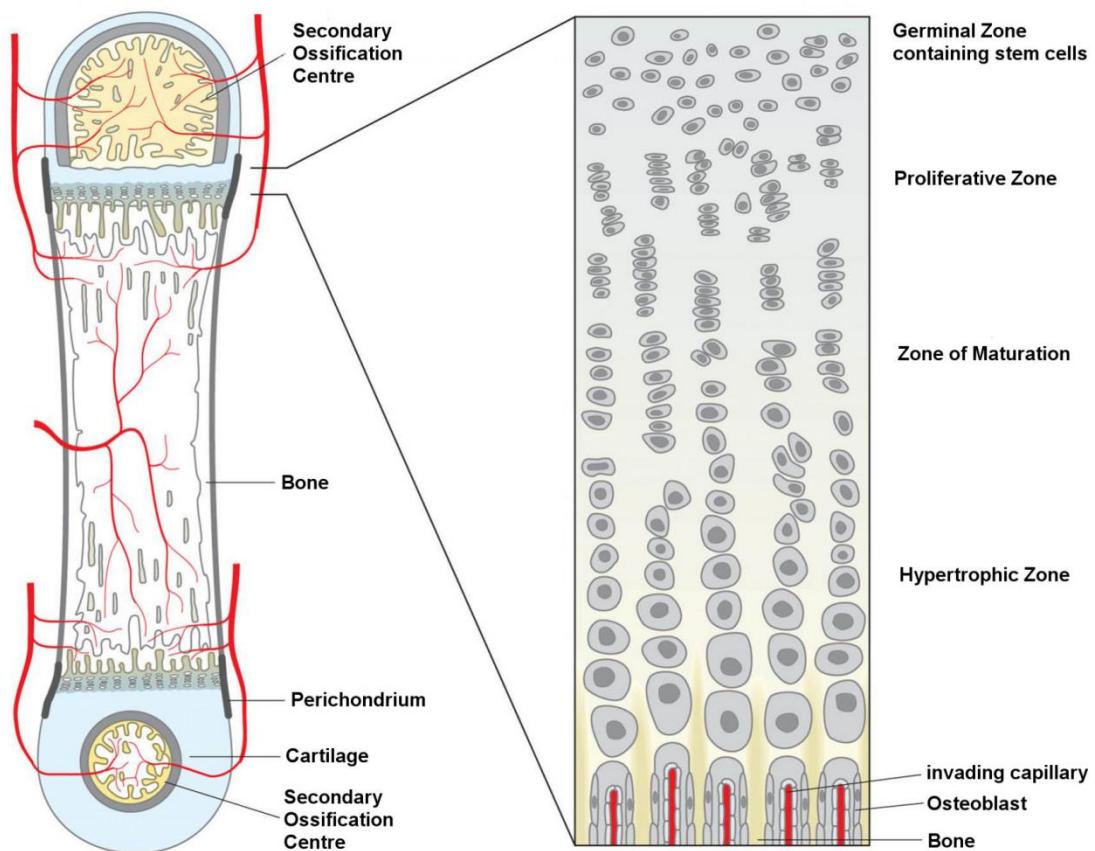


Figure 2.10: Schematic of the different zones present in the cartilage template (Zuniga, 2015).

The periosteum is an important source for undifferentiated osteoprogenitor cells. It is divided into an outer layer, which is a source for fibroblasts, and an inner osteogenic layer, which is a source for osteoprogenitor cells that develop into osteoblasts. The periosteum also provides sites for attachment for ligaments, tendons and muscles (McNamara, 2011). While hypertrophic chondrocytes perform these

multiple tasks in the centre of the cartilage template, the template enlarges further through continuous proliferation of chondrocytes. As the bone enlarges, haematopoietic stem cells interact with the bone marrow stroma to establish the main site for haematopoiesis. It is here, in the presence of VEGF, that vascularisation of the template occurs. This process is vital as blood vessels are a source of endothelial cells, which produce essential growth factors that control recruitment, proliferation, and differentiation of osteoblasts; haemopoietic cells, that form the bone marrow; and osteoprogenitor cells, which differentiate to become osteoblast cells and secrete bone proteins and minerals (Kronenberg, 2003; McNamara, 2011). Cells in the perichondrium are signalled to become osteoblasts and to secrete collagen type I-rich matrix resulting in the formation of the bone collar. Hypertrophic chondrocytes undergo apoptosis and are replaced by osteoblasts that form the bone matrix. Secondary ossification centres develop at the ends of the cartilage model, where again, chondrocytes stop proliferating, become hypertrophic and signal the influx of blood vessels and osteoblasts (Mirjam Fröhlich, 2008), see Figure 2.11.

Any further bone growth occurs within the secondary ossification centre. This is regulated by the epiphyseal or growth plate, which continues to produce new cartilage that is replaced by bone, and thereby facilitates lengthening of bone. After puberty, the lengthening of bones stops and the growth plate fuses and is replaced by bone, known as the epiphyseal line (McNamara, 2011).

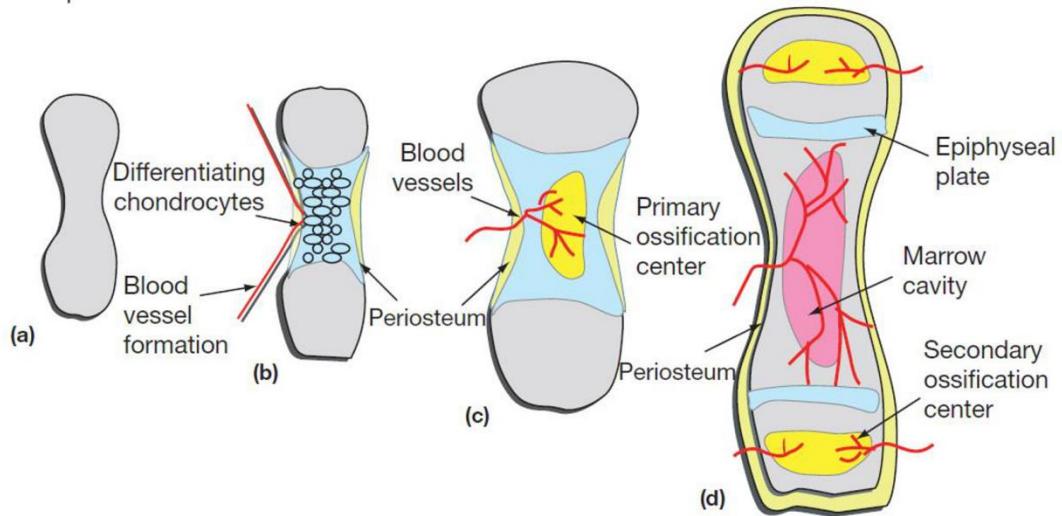


Figure 2.11: Endochondral Ossification: **(A)** Formation of the cartilage template, **(B)** Chondrocytes become hypertrophic and vessels begin to invade, **(C)** osteoid and mineral is formed upon the cartilage template and the primary ossification centre is formed, **(D)** Marrow, secondary ossification centre and epiphyseal plate are formed (McNamara, 2011).

2.5 Bone as an Adaptive material

2.5.1 Bone Modelling and Remodelling

Bone is a dynamic material that constantly adapts itself, by altering its shape and microstructural architecture, through processes known as modelling and remodelling, to accommodate the loading conditions imposed during everyday activities. This adaptive nature is crucial to bone's ability to protect and support the body through time.

Modelling is the process by which bones change their overall shape during growth or in response to physiological conditions or mechanical loads due to everyday

activities. Bones may widen or change axis by bone modelling. This occurs when bone resorption performed by osteoclasts, usually on the endosteum, is simultaneous to bone formation by osteoblasts, usually on the periosteum, such that the dimensions are altered (Frost, 1990; Clarke, 2008). The bone remodelling is a cell-controlled process by which bone is replaced to maintain bone strength and mineral homeostasis. This process is divided into four successive phases; Activation Phase, Resorption Phase, Reverse Phase, and Formation Phase (see Figure 2.12).

Activation Phase. Bone remodelling process begins after different inputs, such as an alteration to mechanical loading is sensed by the osteocytes or certain factors (insulin growth factor (IGF), tumour necrosis factor- α (TNF- α), PTH) and interleukin-6 (IL-6)) are released in the bone microenvironment, then activation of the bone lining cells incurs. As a consequence, the bone lining cells begin to express the surface marker Receptor Activator of Nuclear Ligand (RANKL). RANKL in turn interacts with its receptor RANK which is expressed by pre-osteoclasts. This RANKL/RANK interaction leads to the differentiation of pre-osteoclasts into multinucleated osteoclasts.

Resorption Phase. The differentiated osteoclast polarises and attaches to the underlying bone matrix (Rucci, 2008; Favus and Christakos, 1996). Osteoclast resorption depends on osteoclast secretion of hydrogen ions and the cathepsin K enzyme. The hydrogen ions acidify the area beneath the osteoclast to dissolve the mineral component of the bone matrix, whereas the cathepsin K digests the protein matrix, which is mostly composed of collagen type I (Clarke, 2008; Boyle et al., 2003; Rucci, 2008). Once the resorption process has been completed and the osteoclasts have accomplished their function, the osteoclasts undergo apoptosis (Rucci, 2008).

Reverse Phase. The reverse cells whose role is still yet unknown, perform this phase. It has been hypothesised that they are macrophage-like cells, whose function is the removal of debris produced during the resorption phase.

Formation Phase. Bone resorption leads to a release of several growth factors including BMPs, fibroblast growth factors (FGFs) and TGF- β , which are responsible for the recruitment of osteoblasts to the reabsorbed area. The recruited osteoblasts produce new bone matrix, which is initially not calcified and also known as osteoid, and they promote mineralisation, thus completing the bone remodelling process.

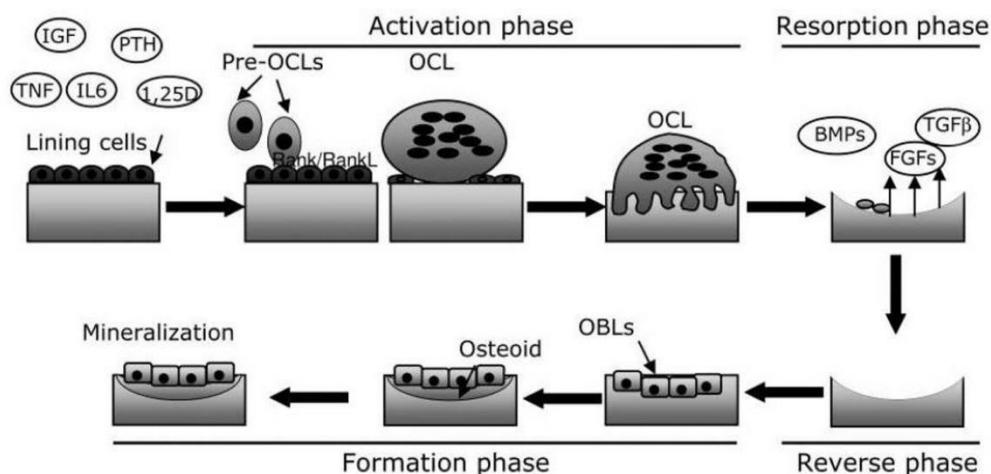
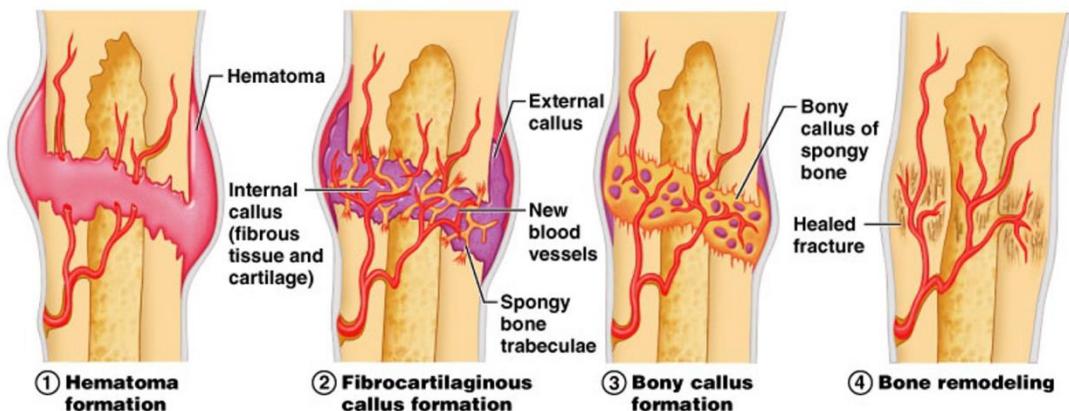


Figure 2.12: Diagram showing the phases of the bone remodelling cycle: Activation Phase, Resorption Phase, Reverse Phase, and Formation Phase. OCL denote osteoclasts and OBLs denote osteoblasts.

2.5.2 Fracture Healing

Although bone is constantly renewing itself throughout its lifetime, traumatic injuries or pathological conditions can occur, which can lead to fracture of the bone. Bone fracture can be repaired through synchronised activity of osteoprogenitor,

chondroblasts, and osteoblasts by a process known as fracture healing, as shown in Figure 2.13 (McKibbin, 1978; Einhorn, 1998).



Copyright © 2004 Pearson Education, Inc., publishing as Benjamin Cummings.

Figure 2.13: The healing process of bone fracture (1) Hematoma formation, (2) Fibrocartilaginous callus formation, (3) Bony callus formation, and (4) Bone Remodelling (Marieb and Hoen, 2006).

This process can be divided into four stages: hematoma formation, fibrocartilaginous callus formation, bony callus formation and bone remodelling. When a bone breaks, blood vessels present in the periosteum and the surrounding tissues are torn and haemorrhage. As a result, a hematoma or mass of clotted blood forms around the fracture site. Cells around the break are deprived of nutrients and die, and the tissue surrounding it becomes swollen and inflamed. Within a few days, new capillaries grow into the hematoma and phagocytes invade the area and begin to clean up the site. Meanwhile, fibroblasts and osteoblasts invade the site from the nearby periosteum or endosteum and begin to reconstruct new bone. Within a week, new bone trabeculae begin to appear in the callus and this continues until a firm union is formed after approximately two months. Over time the bone callus is remodelled and the excess material on the diaphysis exterior is removed and compact bone is laid down in the shaft walls (Marieb and Hoen, 2006; Tsiridis et al., 2007). This

remodelling process is largely dependent on the mechanical forces applied to the bone (McNamara, 2011; McKibbin, 1978; Crockett et al., 2011). The precise mechanical stimulus that is sensed by bone cells during the bone remodelling process and the signal produced as a result has yet to be elucidated. Despite this, studies have found that the presence of microcracks is currently considered a crucial driver for the remodelling process as it initiates the activation of osteocytes to begin the resorption process (Crockett et al., 2011; Cardoso et al., 2009).

2.6 Bone Pathology

Large bone defects can occur due to traumatic injuries or pathological conditions. Healthy bones do not develop spontaneous fractures as a result of the mechanical loads exerted due to everyday activities. However, large bone fracture can occur during trauma. These types of fractures usually occur due to sporting activities, high impact loading such as road accidents, and explosion and blast injuries (Samuel et al., 2009).

Pathologic fractures are usually caused by the weakening of the bone due to metabolic bone diseases including osteoporosis or osteomalacia, or due to the presence of bone forming tumours. In general, osteoporosis is known as a metabolic disease, in which progressive degradation of both bone mass and bone microarchitecture occurs, with corresponding deterioration in bone mechanical strength. It is classified as either primary or secondary osteoporosis. Primary osteoporosis is more common and is associated with decreased levels of oestrogen and typically occurs in postmenopausal women and elderly patients. Secondary osteoporosis is a consequence of aging and is usually caused by inadequate calcium and vitamin D intake (Wensel et al., 2011; Hoch

et al., 2009). Osteomalacia is a disorder characterised by inadequate mineralisation of newly formed bone matrix, and is also known as rickets in children (Hoch et al., 2009). It often develops because of a Vitamin D deficiency. Both diseases lead to greater fragility of bones and as such can lead to a greater risk of large bone fractures occurring.

Bone-forming tumours represent a broad spectrum of neoplasms (abnormal growths) and vary in both morphology and biology. A bone-forming tumour is defined as a neoplasm, which arises within or on the surface of bone tissue and synthesises and secretes organic matrix that may or may not mineralise. Tumour cells on the surface of the bone tend have a similar phenotype to osteoblasts, and in tumours where the cells are creating matrix, the cells they have a large, polyhedral morphology. On the other hand, tumour cells found within the bone are entrapped within the matrix and tend to be smaller than those neoplastic cells on the surface (Nielsen and Rosenberg, 2010). The growth of bone tumours and the architecture of neoplastic bone tend to differ based on the nature of the bone tumour. For example Bone Island (enostosis), osteoma, and osteoblastoma are all benign bone tumours and are usually well confined, and the bone matrix is typically deposited upon the trabeculae of lamellar bone. Conversely, osteosarcoma is a malignant tumour where the neoplastic cells grow with an infiltrative pattern and the bone is woven in architecture and deposited in a coarse, lace like pattern (Nielsen and Rosenberg, 2010). The tumours are also clinically problematic to treat as over 50% are resistant to radiation therapy, therefore the standard therapy is tumour resection leading to large non-union bone defects.

As stated in Section 1.1.1 current therapies for these defects include autografting and allografting cancellous bone, applying vascularised grafts and other bone transport

methodologies (Perry, 1999; Rose and Oreffo, 2002; Cancedda et al., 2007). However, these treatments have their own limitations, the procedure is typically expensive and donor site morbidity due to infection and haemotoma. Moreover, with an increasing aging population there is a significant increase in fractures due to osteoporosis, with 30-50% of the replacement operations requiring revision surgeries. Therefore, there is clearly a clinical need for new therapies to treat these large bone defects.

2.7 Bone Tissue Engineering

Bone tissue engineering has been heralded as the alternative strategy to regenerate bone tissue in a large defect. In essence, bone tissue engineering requires three aspects: (1) a biomaterial scaffold conductive to cell attachment and cell viability, (2) osteoprogenitor cells and (3) osteoconductive/inductive growth factors, see Figure 2.14.

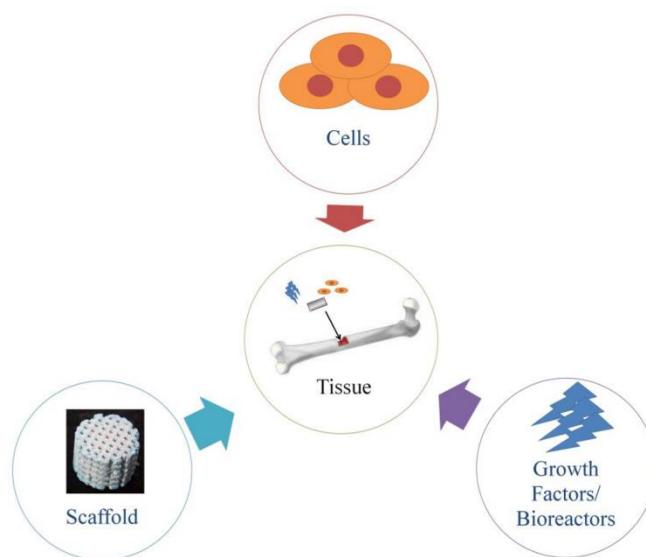


Figure 2.14: Schematic of the three stages of the bone tissue engineering triad: **(1) Cells, (2) Scaffold and (3) Growth Factors.**

Most bone tissue engineering strategies have incorporated one or all of these aspects to develop an approach to heal a segmental defect. As described in Section 2.2.4, bone marrow MSCs have a vast capacity for tissue regeneration. Ectopic bone formation can occur when untreated MSCs are subcutaneously implanted on biomaterial scaffolds (Goshima et al., 1991c; Goshima et al., 1991b; Goshima et al., 1991a; Haynesworth et al., 1992; Ohgushi et al., 1993) and MSCs seeded on scaffolds have also been shown to heal critical-sized segmental defects in both large (canine) and small animals (rats) (Bruder et al., 1998a; Bruder et al., 1998b; Arinzech et al., 2003; Kadiyala et al., 1997a; Kadiyala et al., 1997b).

The main criteria for bone tissue engineering scaffolds are to provide the cells an appropriate platform for cell attachment and proliferation, whilst also having the mechanical stability needed to deal with the physiological and biological challenges in the *in vivo* environment. In general, a scaffold should be made out of a material that is biocompatible and not elicit an immunological response, due to the body recognising the scaffold as a foreign body when implanted *in vivo* (Chapekar, 2000; Logeart-Avramoglou et al., 2005; Kneser et al., 2006; Hutmacher, 2000). The material properties of scaffolds used in current tissue engineering strategies fall into two categories: (1) ceramic biomaterials and (2) natural and synthetic polymers. The advantages of ceramic biomaterials, including hydroxyapatite and calcium phosphate based ceramics, are that they stimulate cell differentiation, are osteoconductive and have an ability to integrate with the host bone (Heise et al., 1990; Sartoris et al., 1991; Marcacci et al., 1999; Elsinger and Leal, 1996; Ge et al., 2004; Mastrogiacomo et al., 2006; Kruyt et al., 2004; Rezwan et al., 2006; Ramay and Zhang, 2004). They also have a strong structural integrity that is needed from a bone tissue engineering scaffold to meet the loading demands of everyday activity. However, the ceramic scaffolds are

resorbed by the body, but in a relatively slow manner. On the other hand, natural and synthetic polymers, such as collagen, silk, polylactic acid (PLA) and polycaprolactone (PCL), can be chemically modified and their degradation rate controlled (Rose and Oreffo, 2002; Burg et al., 2000; Liu and Ma, 2004; Athanasiou et al., 1996; Prestwich and Matthew, 2002; Wang et al., 2006; Meinel et al., 2004a; Li et al., 2006b; Shin et al., 2004; Williams et al., 2005; Hollister, 2005; Yoshimoto et al., 2003; Meinel et al., 2005; Meinel et al., 2004b; Farrell et al., 2006; Farrell et al., 2007; Hofmann et al., 2007; Martin et al., 1998; Lyons et al., 2010). These biodegradable scaffolds provide the initial structure that is needed for cells to attach and proliferate but then degrade as the tissue forms, providing room for matrix deposition and tissue growth. They can also be processed easily into 3D structures with the morphological characteristics needed to fit into the defect site. However, despite these advantages these scaffolds do not have the mechanical integrity needed to be placed in a load bearing location (Rose and Oreffo, 2002; Burg et al., 2000; Liu and Ma, 2004; Athanasiou et al., 1996; Prestwich and Matthew, 2002; Wang et al., 2006; Meinel et al., 2004a; Hollister, 2005).

Moreover, scaffolds can not only be a vector for cell delivery but also for the delivery of growth factors. Growth factors that are commonly used in bone tissue engineering are the BMPs. The BMPs have been shown to have a promising effect on bone regeneration through the recruitment, commitment and differentiation of bone progenitor cells (Reddi, 1992; Kawakami et al., 1996; Hollinger et al., 1998; Rose and Oreffo, 2002). One of the main limitations to the use of BMPs in bone tissue regeneration is the failure to find a suitable carrier to deliver the growth factor with the necessary dosage to maintain the biological activity. One way of overcoming this is the incorporation of the growth factor within the natural and synthetic polymer

scaffolds. Studies have looked at the incorporation of BMP and Vascular Endothelial Growth Factor (VEGF) within the scaffolds (Boden, 1999; Winn et al., 1999; Schmitt et al., 1999; Kanczler et al., 2010; Yang et al., 2004; Kanczler et al., 2008; Kolambkar et al., 2011; Oest et al., 2007; Boerckel et al., 2011) in order to aid in the repair of large bone defects.

When designing a scaffold for the delivery of growth factors the choice of scaffold material is crucial. When ceramic biomaterials are used as carriers for osteoinductive factors, there have been problems associated with biodegradability, inflammatory and immunologic reactions when implanted in vivo (Oreffo and Triffitt, 1999; Horisaka et al., 1991; Doll et al., 1990). Synthetic and natural polymers have been used to make biodegradable composite scaffolds through the addition of osteoconductive factors such as hydroxyapatite and tricalcium phosphate (Middleton and Tipton, 2000; Agrawal and Ray, 2001; Hutmacher, 2000; Rose and Offrefo, 2002; Rezwan et al., 2006; Wei and Ma, 2004; Zhang and Ma, 1999; Li et al., 2006a; Ma et al., 2001; Lyons et al., 2014; Murphy et al., 2014; Gleeson et al., 2010) and have been able to overcome some limitations of ceramic biomaterials. The field of bone tissue engineering is rapidly growing and bone tissue engineering products are beginning to hit clinical trials, which are summarised in Table 2.1.

Table 2.1: Clinical Studies using Human MSC seeded tissue engineering constructs

Patient No.	Scaffold	Results
<u>Long Bone Defects</u>		
(Quarto et al., 2001)	3 Hydroxyapatite Scaffold	Callus Formation along the implants and good integration at the interface with host bones 2 months post-surgery.

(Marcacci et al., 2007)	4	Hydroxyapatite Scaffold	Complete fusion between the implant and the host bone was seen 5 to 7 months post-surgery and was maintained 6 to 7 years later.
(Krecic-Stres et al., 2007)	1	Calcium-Triphosphate Scaffold	Results unpublished.
Aveolar Cleft Defect			
(Hibi et al., 2006)	1	Titanium Plate	Regenerated bone bridge in the defect 6 months post-surgery.
(Meijer et al., 2007)	10	Hydroxyapatite Scaffold	Only one patient had new bone formation.
Mandibular Defect			
(Lee et al., 2010)	1	Fibrin Glue and Titanium Plate	Successful regeneration of large bone defect in the jaw.
(Meijer et al., 2008)	6	Hydroxyapatite Particles	4 months post-surgery 3 patients had bone formation, however in only 1 of these patients was the bone formation induced due to the implant.
(Zamiri et al., 2013)	3	Allogenic Mandible	Vascularisation and bone healing was observed in 2 out of 3 patients.
(d'Aquino et al., 2009)	17	Collagen Sponge	Complete bone regeneration was evident one year post-surgery.
Maxillary Defect			
(Shayesteh et al., 2008)	6	Hydroxyapatite/beta-tricalcium scaffold	Mean bone regeneration within the defect was reported to be 41.34% and all implanted were considered clinically integrated 4 months post-surgery.

In general, the results from these studies demonstrated that it is safe to use human MSCs for bone regeneration, however, the results thus far have been substantially less promising than those of animal studies (Ma et al., 2014; Frohlich et al., 2008). To date, no current bone tissue engineering strategy has successfully created a bone tissue engineered construct that can be placed in a load-bearing location. The reason for that is that engineering bone tissue is not only highly dependent on cellular and molecular developmental biology but also very strongly guided by bioengineering and bio-mechanics. The majority of limitations seen by current bone tissue engineering studies is due to the issue of core degradation, which occurs due to lack of vascularisation (Phelps and Garcia, 2009; Ko et al., 2007; O'Brien, 2011; Amini et al., 2012). Another limitation seen in current tissue engineering strategies is fibrous tissue encapsulation of the construct due to the host's reaction to a foreign body (Lyons et al., 2010; Amini et al., 2012; O'Brien, 2011), as seen in Figure 2.15.

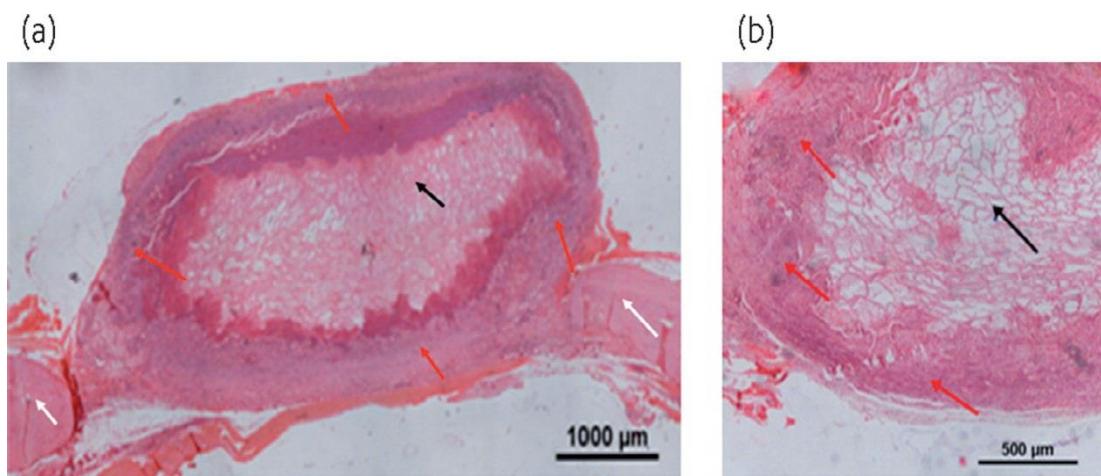


Figure 2.15: Example of Core degradation in a rat calvarial defect treated with tissue engineered collagen-calcium phosphate scaffold for 4 weeks post implantation. **(a)** Shows full defect site and **(b)** shows higher magnification of the same image. Red arrows show the fibrous tissue capsule and black arrow shows the acellular core (O'Brien, 2011).

Despite the great advances that have been made in the field of material science, the design of biomaterials for bone tissue engineering is constantly changing. In vivo the ECM of bone is a structurally complex environment composed of a variety of structural elements, in which growth factors, integrins, and functional peptides are incorporated, as previously discussed in Section 2.1. A relatively new strategy is ECM-based tissue engineering, in which the scaffolding material contains natural ECM sources. The main advantage to this is that it supports and encourages specific tissue formation due to the presence of the bioactive molecules present within the ECM driving tissue homeostasis and regeneration (Benders et al., 2013). To date most of the ECM-based tissue engineering studies have been cartilage based (Benders et al., 2013; Ye et al., 2013; Schwarz et al., 2012a; Schwarz et al., 2012b; Elder et al., 2009), however, some studies have begun investigating the use of decellularised cartilage within the scaffolds to induce bone formation (Visser et al., 2015; Gawlitta et al., 2015). However, the underlying mechanisms of this strategy are still not fully understood and the functional outcome of ECM-derived scaffolds depends on a number of variable factors, including retention of growth factors, surface topology, immune response, and the microenvironmental cues exerted on the cells (Benders et al., 2013). There have clearly been major strides in bone tissue engineering in the past few years but it is clear that further investigation within the field is needed.

2.8 Endochondral Ossification as a Bone Tissue Engineering approach

To date the promising bone tissue engineering strategies have either tried to produce a construct that mimics the function and mechanical properties of the bone

tissue (Heise et al., 1990; Sartoris et al., 1991; Marcacci et al., 1999; Elsinger and Leal, 1996; Ge et al., 2004; Mastrogiacomo et al., 2006; Kruyt et al., 2004; Rezwan et al., 2006; Ramay and Zhang, 2004; Quarto et al., 2001; Marcacci et al., 2007; Hibi et al., 2006; Meijer et al., 2007; Lee et al., 2010; Meijer et al., 2008; Shayesteh et al., 2008) or produce a construct that has the necessary factors to induce bone formation but not the mechanical integrity (Rose and Oreffo, 2002; Burg et al., 2000; Liu and Ma, 2004; Athanasiou et al., 1996; Prestwich and Matthew, 2002; Wang et al., 2006; Meinel et al., 2004a; Li et al., 2006b; Shin et al., 2004; Williams et al., 2005; Hollister, 2005; Yoshimoto et al., 2003; Meinel et al., 2005; Meinel et al., 2004b; Farrell et al., 2006; Farrell et al., 2007; Hofmann et al., 2007; Martin et al., 1998; Lyons et al., 2010; Middleton and Tipton, 2000; Agrawal and Ray, 2001; Hutmacher, 2000; Rezwan et al., 2006; Wei and Ma, 2004; Zhang and Ma, 1999; Li et al., 2006a; Ma et al., 2001; Lyons et al., 2014; Murphy et al., 2014; Gleeson et al., 2010; d'Aquino et al., 2009). However, although this strategy has produced extensive amounts of research, recently it has been proposed that perhaps an ideal construct does not have to replicate the properties of the fully functional tissue but to replicate the bone formation process from the beginning, a process known as “developmental engineering” (Martin, 2014; Farrell et al., 2011; Miot et al., 2012; Scotti et al., 2010; Farrell et al., 2009; Scotti et al., 2013; Jukes et al., 2008; Harada et al., 2014; Gawlitta et al., 2015; Visser et al., 2015; Lenas et al., 2009). The principle of this strategy is to develop *in vitro* approaches that mimic and take advantage of the natural mechanisms by which ossification occurs in the body. In this vein, recent tissue engineering strategies have sought to replicate features that occur during embryogenesis (Martin, 2014; Farrell et al., 2011; Miot et al., 2012; Scotti et al., 2010; Farrell et al., 2009; Scotti et al., 2013; Jukes et al., 2008; Harada et al., 2014; Gawlitta et al., 2015; Visser et al., 2015; Lenas

et al., 2009), in particular to mimic the endochondral ossification process responsible for the formation of all long bones during embryogenesis. The endochondral ossification process relies on the production of a cartilage template, and the invasion of a vascular network into this template (McNamara, 2011; Mackie et al., 2008), and each of these events has been investigated separately as a precursor strategy to entice bone formation, as is outlined further below.

2.8.1 Formation of the Cartilage Template

Endochondral Ossification is a tightly regulated process, which is highly controlled by the presence and expression of biochemical signals. Researchers have sought to investigate if the application of certain biochemical cues *in vitro* could manipulate the cells to undergo the endochondral ossification process both *in vitro* and following implantation *in vivo*. One methodology is to chondrogenically prime MSCs *in vitro*, by exposing cells to chondrogenic differentiation supplements, prior to implantation *in vivo*. The idea is that this strategy would allow the cells to form a cartilage template prior to mineralisation, similar to what occurs naturally during early fetal development. Previous subcutaneous mouse/rat studies have found that chondrogenic priming of human MSCs seeded on transwell inserts (Scotti et al., 2010) and collagen glycosaminoglycan (GAG) scaffolds (Scotti et al., 2013; Farrell et al., 2011; Farrell et al., 2009) or of embryonic stem cells on calcium phosphate ceramics (Jukes et al., 2008) can overcome issues with poor oxygen and nutrient supply in tissue engineered constructs. Moreover such constructs have been shown to mineralise when implanted subcutaneously for up to 8 weeks, see Figure 2.16.

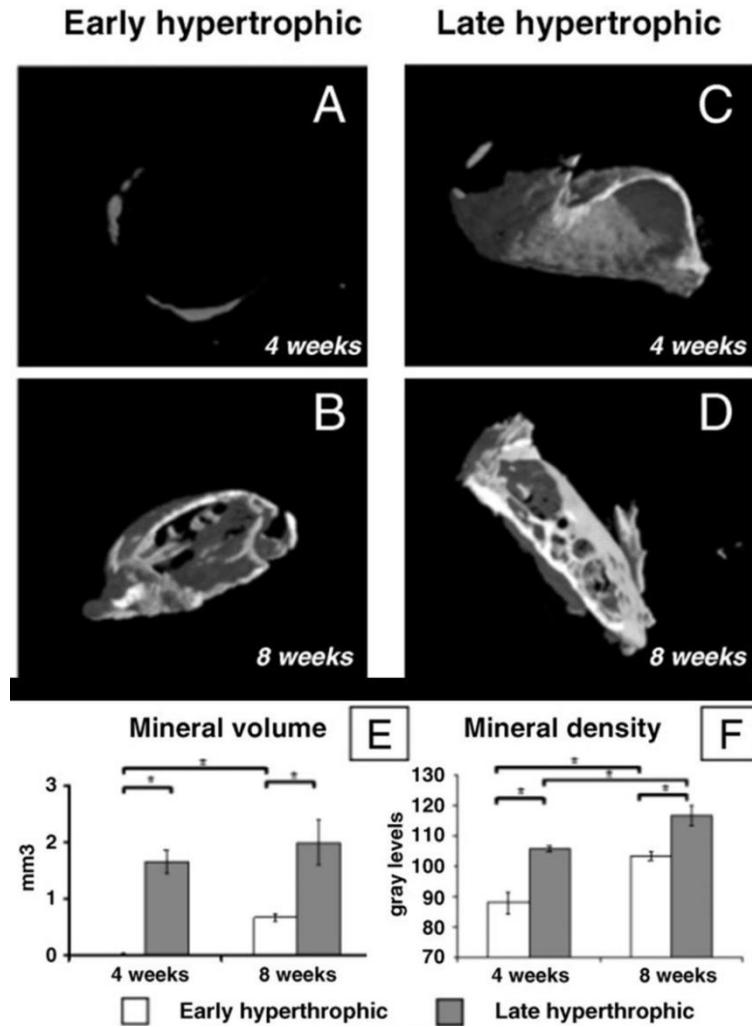


Figure 2.16: Three-dimensional μCT reconstructions (A-D) and quantitative histomorphometric data ($n = 4$) of mineral volume (E-F) and density of the constructs chondrogenically primed for 14 days (early hypertrophic) versus constructs that were chondrogenically primed for 21 days followed by 2 weeks in hypertrophic medium (late hypertrophic) (* indicates significant differences; $p < 0.01$) (Scotti et al., 2010).

Other studies have investigated the implantation of chondrogenically primed MSCs in both aggregate and scaffold form to heal a large segmental defect (Harada et al., 2014; van der Stok et al., 2014). Chondrogenically primed rat MSCs were seeded on a poly(lactic-co-glycolic acid) (PLGA) scaffold in an effort to repair both a critical sized (5mm) and a full thickness femur defect (15mm) (Harada et al., 2014). The

results showed that bone union occurred in all of the constructs examined radiographically at 8 or 16 weeks post-surgery. Chondrogenically priming human MSC cellular aggregates were also investigated for the purpose of regenerating a 6mm femoral defect (van der Stok et al., 2014). Micro-CT scanning was implemented to show that after 4 and 8 weeks there was significantly more bone formation in the defect treated with chondrogenically primed MSCs compared to undifferentiated MSCs, as seen in Figure 2.17.

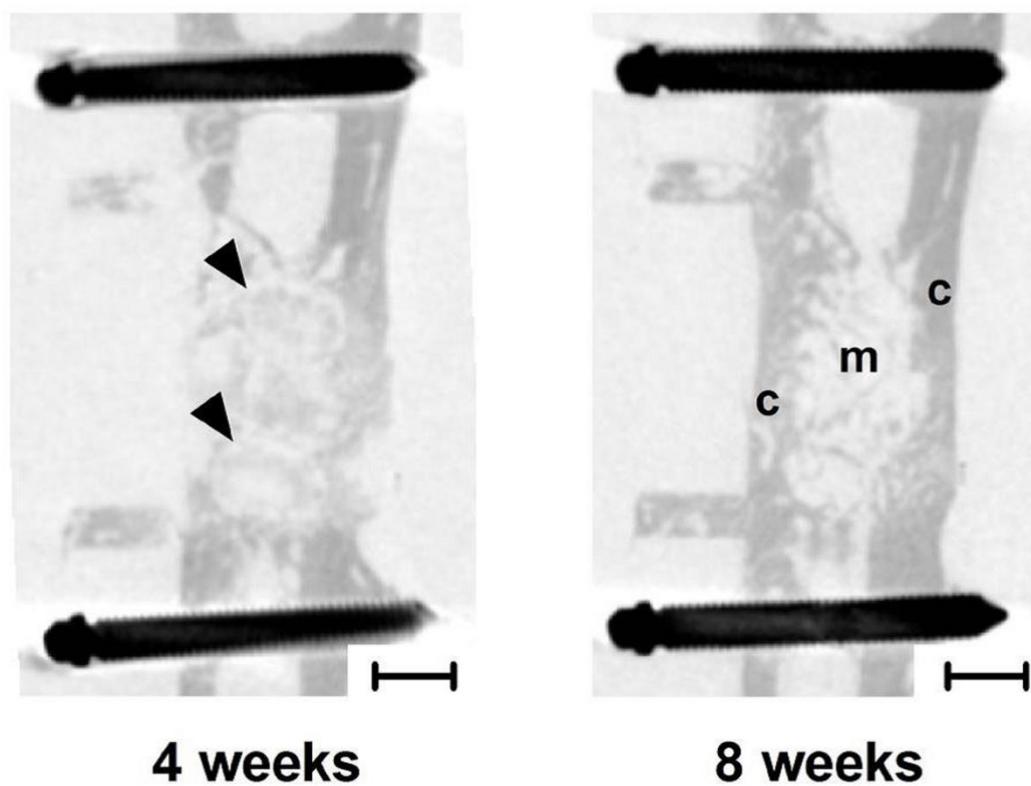


Figure 2.17: Micro-CT images of the mineralised Aggregates after 4 and 8 weeks in vivo. Black arrows denote the boarder of the Aggregates, “c” indicated the newly formed cortex and “m” indicates the ongoing restoration of the medullary canal (van der Stok et al., 2014).

These studies have established that chondrogenic priming of MSCs can entice bone formation in both an ectopic and critical defect model. However, some of these

studies observed core degradation and an uneven distribution of bone mineral throughout the construct (Farrell et al., 2009; Scotti et al., 2010; Farrell et al., 2011), and only a few studies (Scotti et al., 2013; Scotti et al., 2010; Farrell et al., 2009; Farrell et al., 2011) examined human MSCs rather than animal MSCs. It is clear that chondrogenic priming may have potential as an in vitro bone tissue regeneration strategy but approaches must be further optimised to overcome these limitations.

In vivo, the endochondral ossification process relies on the establishment of a cartilaginous template prior to vessel invasion and bone formation. Therefore the timing of cartilage template formation is important for bone formation by endochondral ossification. Embryologists have estimated that cartilaginous limb buds are first seen in humans by the 26th day (Merz and Bahlmann, 2004; Webster, 2012; Smith, 1968), whereas in vivo studies have shown that bone formation begins in chick bones when the embryo is 9-18 days old (Roach, 1992; Roach, 1997; Nowlan et al., 2007; Hunziker and Schenk, 1989; Hall, 1987). One study has shown that the timing of media supplementation is important for mineralisation of bone tissue constructs in vivo (Scotti et al., 2010). Human MSCs were chondrogenically primed on transwell inserts for either 1 or 2 weeks before subcutaneous mice implantation. The constructs that were chondrogenically primed for 1 week were unable to be retrieved 4 weeks post implantation, whereas constructs primed for 2 weeks developed an extracellular matrix rich in GAG and contained cells embedded in large lacunae. By 8 weeks this group had reduced levels of GAG however, the centre region of the construct was rich in collagen type X, indicating hypertrophy, and the beginning of core degradation. This study shows that the timing for chondrogenic priming does have an effect on the in vivo survivability of the constructs. However, to date, no study has investigated

what the optimum time for chondrogenic priming is and what effect it would have on the mineralisation capacity of MSCs in vitro. This deficit of knowledge will be explored further in Chapter 3 of this Thesis.

2.8.2 Vascularisation of the Cartilage Template

In vivo, vessel formation is an integral part of the bone formation process that occurs during the endochondral ossification process. As described in Section 2.4.2, vessel invasion occurs after the cartilage template has formed by a process known as quiescent angiogenesis, which involves endothelial cells invading through the cartilage canals already present in the developing bone tissue (Mackie et al., 2008; McNamara, 2011; Gerber and Ferrara, 2000; Kronenberg, 2003), and this process typically occurs between 14 and 18 days of embryogenesis of mice (Gerber and Ferrara, 2000; Carlevaro et al., 2000). It is believed that bone vasculature is necessary to provide appropriate growth factors, hormones, chemokines and nutrients, and without a vascular supply, cells within tissue engineered constructs do not get the necessary requirements to regenerate the damaged bone tissue and ultimately die when implanted in vivo (Rivron et al., 2008; Kanczler and Oreffo, 2008).

In vitro, co-culture studies have investigated whether pre-vascularising 3D tissue engineered scaffolds, such as trabecular bone (Correia et al., 2011), PCL (Ghanaati et al., 2011), poly(LLA-co-DXO) (Pedersen et al., 2013), collagen GAG (McFadden et al., 2013; Duffy et al., 2011), and hydroxyapatite (Scherberich et al., 2007) scaffolds in vitro, through the co-culture of MSCs and HUVECs, would allow faster host integration post-implantation (Correia et al., 2011; Ghanaati et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Scherberich et al., 2007; Duffy et al.,

2011). It has been shown that pre-vascular networks can be formed in a subcutaneous animal model *in vivo* when human MSCs (Correia et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Scherberich et al., 2007) are first co-cultured with HUVECs *in vitro* see Figure 2.18.

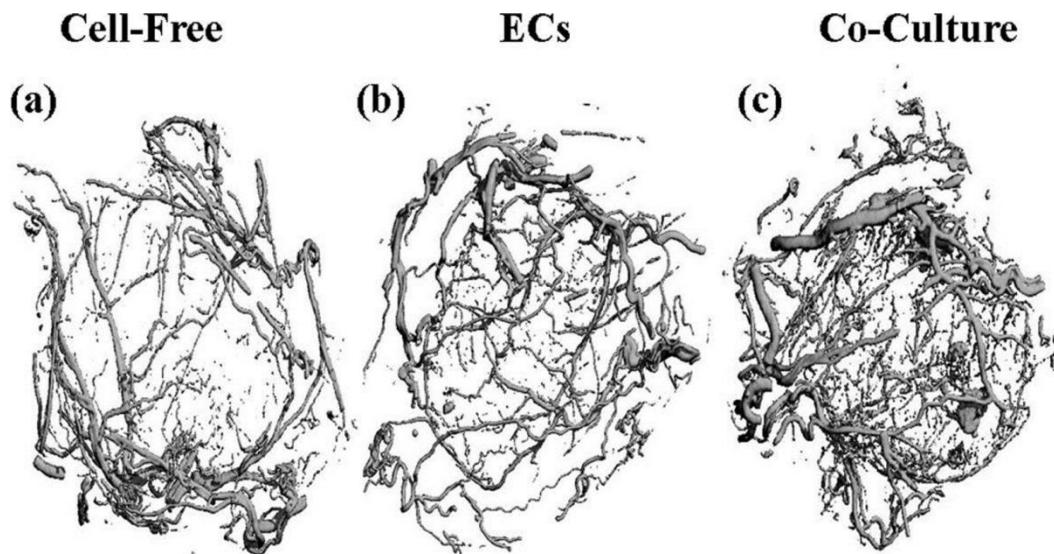


Figure 2.18: Microcomputed tomography angiography demonstrating the level of vessel formation within the implanted collagen-GAG scaffolds after 4 weeks subcutaneous implantation. ECs denote endothelial cells alone and co-culture denotes the addition of both endothelial cells and MSCs (McFadden et al., 2013).

Studies have also investigated direct cell-cell contact co-culture approaches through the formation of cellular aggregates (Gawlitta et al., 2012; Fuchs et al., 2007; Saleh et al., 2011; Rouwkema et al., 2006; Verseijden et al., 2010), and pre-vascular networks have been observed in cellular aggregates co-cultured *in vitro* (Gawlitta et al., 2012; Fuchs et al., 2007; Saleh et al., 2011; Verseijden et al., 2010). An example of these cellular networks can be seen in Figure 2.19. The presence of both MSCs and HUVECs within the cellular aggregate has not only been shown to promote the

formation of vascular networks, but has also led to an upregulation in early osteogenic markers like ALP activity (Fuchs et al., 2007; Saleh et al., 2011).

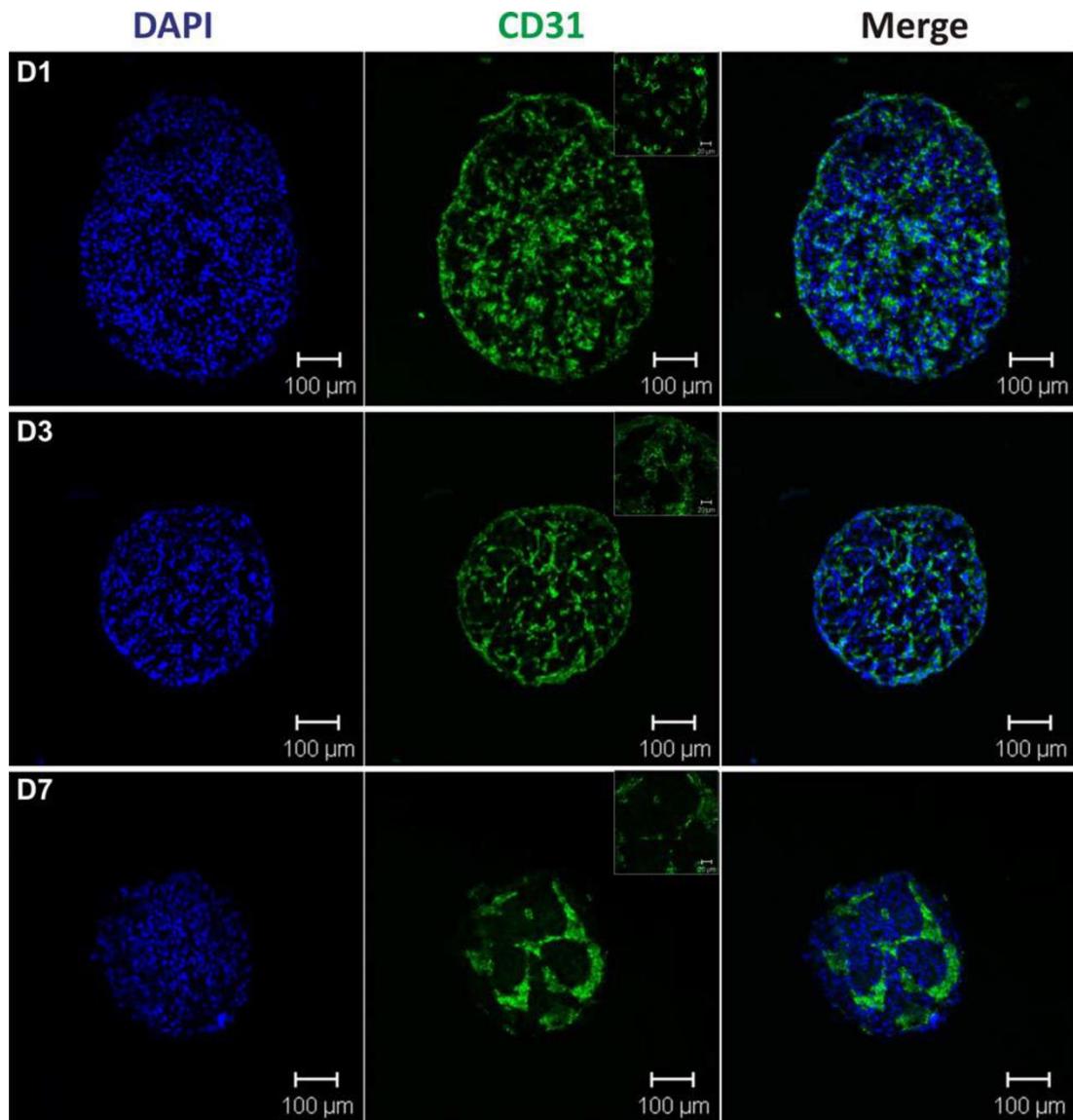


Figure 2.19: Confocal images of CD31 labelling of MSC/HUVEC spheroids at day 1, 3 and 7. DAPI stains nuclei of cells blue and CD31 stains endothelial cells green (Saleh et al., 2011).

While in vitro bone regeneration strategies have sought to incorporate either the production of the cartilage template or the vascularisation of the construct, no strategy has sought to incorporate both events simultaneously, albeit that both are crucial

precursors for bone formation *in vivo* during endochondral ossification. Chapter 4 and Chapter 5 seek to explore this deficit of knowledge.

2.9 Summary

This Chapter has presented a detailed overview of bone function, composition and structure with a particular focus on the cellular constituents of bone. In summary, bone is a highly adaptive tissue that is constantly repaired and renewed to accommodate for everyday activities throughout life, through the coupled activities of osteocytes and osteoblasts. However, when the fracture is too large, and the bone cannot repair itself, other means are needed. The current clinical treatment for non-union defects involves autologous tissue transplantation from the patient's own iliac crest, ribs or calvarium. This procedure is painful to the patient and major complications can occur including donor site morbidity due to infection and haemotoma. Therefore, there is a distinct clinical need for new therapies that provide an effective clinical treatment for large bone defects. The most promising strategy is the use of bone tissue engineering with bone marrow derived MSCs for treating bone diseases and reconstructing bone defects. However, current bone tissue engineering strategies have their own challenges including core degradation and fibrous capsule encapsulation. One way of overcoming these issues is the concept of "developmental engineering", which relies on replicating features that occur during bone development, in particular the endochondral ossification process. By manipulating cells to undertake the endochondral ossification process prior to the point of mineralisation, a bone tissue engineered construct can be designed that once implanted *in vivo* would lead to enhance vascularisation and construct survivability. Although strategies that mimic

certain aspects of this process have been shown to have potential, to date no approach has sought to recreate multiple aspects of the endochondral ossification process such as the presence of multiple cell populations, or their spatiotemporal differentiation.

To address this deficit in knowledge, Chapter 3 of this PhD Thesis investigates whether there is an optimum time for chondrogenic priming of MSCs in vitro that will enhance osteogenic differentiation. Chapter 4 examines the hypothesis that a tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage aggregates, through the co-culture of endothelial cells in vitro, will serve as an effective in vitro bone regeneration approach. In Chapter 5, the effectiveness of the application of chondrogenic priming and the co-culture of MSCs and HUVECs for inducing mineralisation, even without the addition of osteogenic supplements, is investigated. Finally, using the pre-vascularised aggregates developed through Chapters 3-5, the fate these aggregates once implanted subcutaneously, in particular with respect to cell viability, vessel infiltration and mineral formation, is investigated in Chapter 6.

Chapter 3: Is there an optimum time for chondrogenic priming of MSCs in vitro that will enhance mineralisation in vitro?

3.1 Introduction

Clinical treatments for bone defects and extensive fractures involve autologous tissue transplantation or implantation of vascularized bone grafts ((Rose and Oreffo, 2002; Cancedda et al., 2007; Dawson and Oreffo, 2008; Zomorodian and Baghaban Eslaminejad, 2012). However, such approaches are limited in their ability to ensure union of large bone defects and are also associated with high cost and risk of infection (Rose and Oreffo, 2002; Cancedda et al., 2007; Dawson and Oreffo, 2008; Zomorodian and Baghaban Eslaminejad, 2012). Bone tissue engineering has been heralded as a promising strategy for treating bone diseases and reconstructing bone defects (Rose and Oreffo, 2002; Cancedda et al., 2007; Dawson and Oreffo, 2008). As stated in Section 2.7, MSCs have shown promise for producing a bone-like matrix when subcutaneously implanted (Goshima et al., 1991c; Goshima et al., 1991b; Goshima et al., 1991a; Haynesworth et al., 1992; Ohgushi et al., 1993). However, existing approaches are limited as they are unable to produce functional bone tissue for clinical use in load-bearing locations (Meijer et al., 2008) and have also been shown to act as a barrier to healing in rodent cranial defects (Lyons et al., 2010; Amini et al., 2012; O'Brien, 2011).

A possible solution to overcome such limitations and harness the osteogenic potential of MSCs is to mimic bone development during early fetal development and recreate it in vitro. Endochondral ossification is a tightly regulated process, which is highly controlled by the presence and expression of biochemical signalling (as described in Section 2.4.2). Recently bone tissue regeneration studies have been conducted to investigate whether the application of certain biochemical cues in vitro could manipulate the cells to undergo the endochondral ossification process when implanted in vivo. In particular, chondrogenic priming of MSCs has been investigated in vitro, prior to implantation in vivo (Farrell et al., 2011; Farrell et al., 2009; Jukes et al., 2008; Scotti et al., 2013; Scotti et al., 2010; Harada et al., 2014; van der Stok et al., 2014; Visser et al., 2015; Gawlitta et al., 2015). This methodology has been shown to overcome issues with poor oxygen and nutrient supply in cellular aggregates and biomaterial scaffolds, and has been shown to induce mineralisation within the chondrogenic MSCs when implanted subcutaneously (Farrell et al., 2011; Farrell et al., 2009; Jukes et al., 2008; Scotti et al., 2013; Scotti et al., 2010; Gawlitta et al., 2015; Visser et al., 2015) and in large segmental defects (Harada et al., 2014; van der Stok et al., 2014). Specifically, the implantation of MSCs which were pre-differentiated towards the chondrogenic lineage, led to bone regeneration in vivo whereas undifferentiated MSCs were not capable of eliciting bone formation. However, a number of these studies observed core degradation and an uneven distribution of bone mineral throughout the construct (Farrell et al., 2009; Scotti et al., 2010; Farrell et al., 2011) and only one study investigated different time periods for chondrogenic priming in vitro prior to subcutaneous implantation (Scotti et al., 2010). The results from this study found that if the construct was primed for 1 week, the construct was irretrievable

4 weeks post subcutaneous implantation, and if primed for 2 weeks, core degradation was observed within the constructs 8 weeks post implantation.

The timing of cartilage template formation is important for bone formation by endochondral ossification. Embryologists have estimated that cartilaginous limb buds are first seen in humans by the 26th day (Merz and Bahlmann, 2004; Webster, 2012; Smith, 1968), and in vivo animal studies have shown that bone formation begins in chick embryos which are 9-18 days old (Roach, 1992; Roach, 1997; Nowlan et al., 2007; Hunziker and Schenk, 1989; Hall, 1987). It is clear from both in vitro and in vivo studies that the length of time for chondrogenic priming has a significant effect on a construct's survivability in vivo. However, to date, the optimum timing and what the role timing has on the mineralisation capacity of MSCs in vitro has not yet been investigated.

Therefore, the hypothesis that “There is an optimum duration for chondrogenic priming of MSCs that will enhance osteogenic differentiation of MSCs in vitro” was tested in this Chapter. The overall objective of the study was to test a variety of times for chondrogenic priming (10-28 days) and investigate if this would enhance osteogenic differentiation of MSCs in vitro. The specific objectives of this study were to investigate the effects of treatment with chondrogenic and osteogenic medium for various durations on (1) DNA content, (2) sGAG production, (3) collagen content, (4) ALP production and (5) calcium content of MSCs in aggregate culture through biochemical and histological analyses. This Chapter presents an adapted version of work previously published in the Journal of Tissue Engineering and Regenerative medicine (Freeman et al., 2013).

3.2 Methods

3.2.1 Isolation and Characterisation of BALB/c and Human bone marrow derived MSCs

Cultures of Bagg Albino (BALB/c) primary MSCs were obtained and characterized according to protocols of Peister et al. as previously described (Peister et al., 2004; Birmingham et al., 2012). Briefly, 8- 10 week-old female and male mice were killed under ethical approval from the Animal Care Research Ethics (ACREC) committee at the National University of Ireland, Galway. The femurs and tibiae of the mice were removed and the ends of the bones were cut off. Bones were placed in RPMI-1640 medium (Sigma-Aldrich, Dublin, Ireland) supplemented with 9% fetal bovine serum (FBS; EU Thermo Scientific, Loughborough, UK), 9% horse serum (HS), 100 U/mL penicillin (Sigma-Aldrich), 100 g/mL streptomycin (Sigma Aldrich) and 2 M L-glutamine (Sigma-Aldrich) and centrifuged at 400 g for 2 minutes. The cellular aggregates were collected and resuspended in medium in T175 flasks, and were then washed with sterile phosphate buffered saline (PBS) solutions. After approximately 4 days, large colonies had formed; these were re-plated and cultured for a further 10 days.

Human bone marrow-derived MSCs were extracted from bone marrow aspirates. The bone marrow aspirates were obtained from the iliac crest of normal human donors under ethical approval, from the Research Ethics Committee of the National University of Ireland Galway and the Clinical Research Ethical Committee at University College Hospital, Galway, and following informed consent. Bone marrow aspirates were obtained from four donors aged 45, 48, 56 and 59 years. The

MSCs were isolated on the basis of plastic adherence from bone marrow aspirates as previously described (Farrell et al., 2009).

Once suitable colonies had formed in both BALB/c and human MSCs the chondrogenic, osteogenic and adipogenic potential of these MSCs was confirmed as previously described (Birmingham et al., 2012).

BALB/c MSCs for experiments were maintained in expansion medium containing Iscove's minimum essential medium (MEM; Sigma Aldrich) supplemented with 10% FBS, 10% HS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin until confluent. Human bone marrow MSCs for experiments were maintained in expansion medium containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (EU Thermo Scientific), 100 U/mL penicillin, 100 g/mL streptomycin and 2 M L-glutamine (all from Sigma-Aldrich).

3.2.2 Aggregate Formation

Once the cells reached a confluence of ~90% the cells were trypsinised, counted, and centrifuged at 650 g, at 22°C for 5 minutes. The cells were then resuspended in expansion medium so that there was 1×10^6 cells/mL. This cell suspension was divided into 1.5mL tubes so that there were 500,000 cells (for BALB/c) and 250,000 cells (for human MSCs) in each tube, and then centrifuged for 5 minutes at 400 g to create cellular aggregates. Carefully avoiding the newly formed aggregate, the medium was removed from each of the aggregates and 0.5 mL of either chondrogenic medium or osteogenic medium was added depending on experimental conditions (described in detail below). Chondrogenic medium consisted of a chemically defined medium which contained high-glucose DMEM GlutaMAX (Gibco, Life Sciences, Dublin,

Ireland), 10 ng/mL TGF- β 3 (ProSpec-Tany TechnoGene Ltd, Ness-Ziona, Israel), 50 μ g/mL ascorbic acid (Sigma-Aldrich), 4.7 μ g/mL linoleic acid (Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich) and 1 \times insulin–transferrin–selenium (ITS; BD Biosciences, Bedford, MA, USA). Osteogenic medium consisted of the expansion medium supplemented with 100 nM of dexamethasone, 50 μ g/mL ascorbic acid and 10 mM β -glycerol phosphate (Sigma-Aldrich). For all experiments, aggregate cultures were fed twice per week by performing a 50% medium exchange. During each feed the aggregates were agitated to prevent them from adhering to the microtube. This was achieved through aspirating the medium beneath the aggregate with a micropipette.

The BALB/c aggregates were cultured under the following experimental conditions: osteogenic medium for 49 days (Osteo group); chondrogenic medium for 49 days (Chondro group); 10 days chondrogenic priming and osteogenic medium for 39 days; 14 days chondrogenic priming and osteogenic medium for 35 days; 21 days chondrogenic priming and osteogenic medium for 28 days; 28 days chondrogenic priming and osteogenic medium for 21 days (Figure 3.1).

The human MSC aggregates were cultured under the following experimental conditions: osteogenic medium for 14 days (Osteo group); 14 days chondrogenic priming and osteogenic medium for 14 days; 21 days chondrogenic priming and osteogenic medium for 14 days.

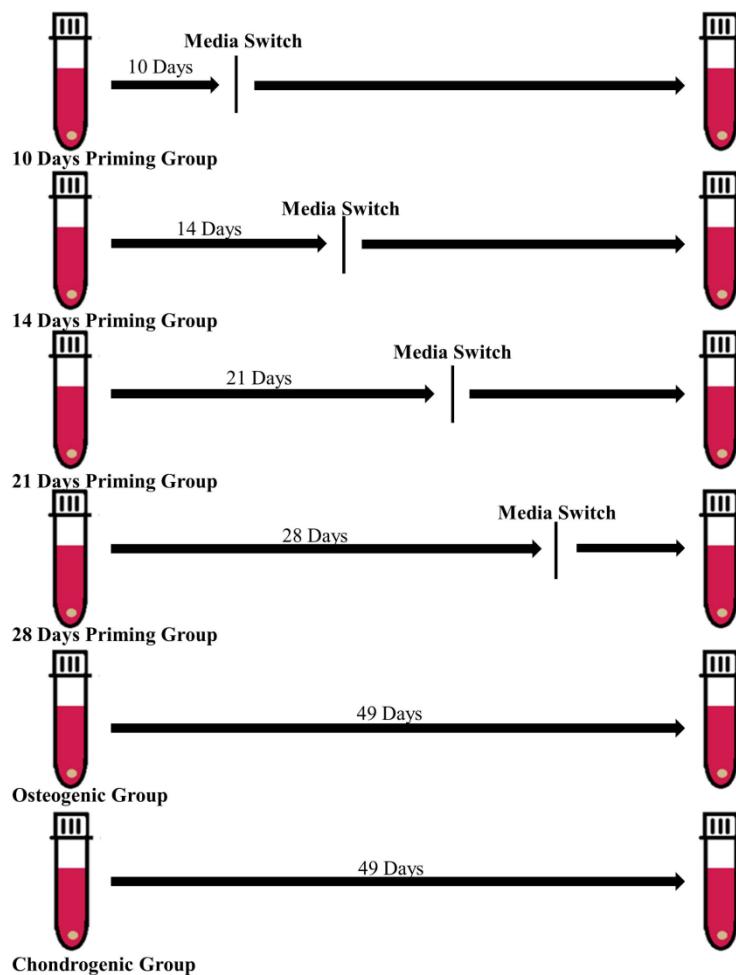


Figure 3.1: Schematic of the experimental groups harvested samples at each time-point for BALB/c MSCs.

3.2.3 Aggregate Harvesting

Aggregates from BALB/c MSCs were examined at 0, 10, 14, 21, 28, 35 and 49 days and were prepared for either histochemical analysis or biochemical analysis. Aggregates from human MSCs were examined at day 0, 1 week post media switch, and 2 weeks post media switch and prepared for biochemical analysis. At each of the time points, the culture medium from the aggregates was collected, frozen and stored at -80°C until biochemical assays could be performed. The remaining aggregates

were washed with PBS and then treated in one of two ways: (1) frozen and stored at –80 °C for biochemical analysis or (2) fixed overnight in paraformaldehyde before being placed in PBS and refrigerated for histochemical analysis. For the above study, two independent experiments were carried out with at least two repeats in each experiment ($n = 2$ for histological analysis and $n = 3$ for biochemical analysis).

3.2.4 Quantitative Biochemical Analysis

3.2.4.1 DNA Content

The DNA content was analysed in the BALB/c and human derived MSC aggregates. Firstly, 500 µl of papain digest [100 mM sodium phosphate buffer containing 10mM L-cysteine, 125 µg/mL papain and 5 mM Na₂ethylenediaminetetraacetic acid (EDTA) (all from Sigma-Aldrich) in double-distilled H₂O at pH 6.5] was added to the aggregates and aggregates were placed in an oven at 60 °C overnight, as previously described (Haugh et al., 2011). When the aggregates were digested the biochemical assays were performed immediately or the solutions were restored at –80 °C until the assays could be performed. Determination of DNA content was performed using Hoechst 33258 DNA assay with calf thymus DNA (Sigma-Aldrich) as a standard, following a previously published protocol (Haugh et al., 2011; Kim et al., 1988). Briefly, in minimal light 40 µl of papain digest of the sample/standard was added to a 96-well plate in triplicate. To this 200 µl of working solution [assay buffer and 1 mg/mL Hoechst dye solution (Sigma-Aldrich)] was added. The plate was incubated away from light for 10 minutes and then read on a microplate reader (Wallac Victor3 1420 Multilabel Counter, PerkinElmer, Ireland), excitation at 350nm and emission at 450 nm, as previously described (Haugh et al., 2011; Birmingham et al., 2012).

3.2.4.2 sGAG Content

The sGAG content was measured in the BALB/c-derived MSC aggregates using the papain-digested samples (prepared for the Hoechst assay) and 1,9 dimethylmethylene blue (DMB) dye [26.25 µg/mL DMB, 0.625% ethanol and 2.5 µg/mL sodium formate (all Sigma Aldrich) in double distilled H₂O at pH 3] with 10 mg/mL bovine-derived chondroitin-4-sulphate (Sigma-Aldrich) as a standard, according to a previously published protocol (Estes et al., 2010). Then 40 µl of each sample and standard in triplicate was added to a 96-well plate and 125 µl of the DMB dye was added. The plate was read on the microplate reader at an optical density of 595 nm.

3.2.4.3 ALP Production

ALP production was determined in the BALB/c and human-derived MSC aggregates using a colorimetric assay of enzyme activity (SIGMAFAST p-NPP Kit; Sigma-Aldrich) which uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate with ALP (Sigma-Aldrich) as a standard. A total of 40 µl of the medium was added to a 96-well plate in triplicate with 50 µl of pNPP solution, which contained both pNPP and assay buffer. The samples were shielded from direct light at room temperature for 1 hour. After this, 20 µl of stop solution (3 N NaOH) was added to the wells and the plate was read at 405 nm in a microplate reader, as described elsewhere (Birmingham et al., 2012).

3.2.4.4 Calcium Content

Calcium deposition within the aggregates was measured in the BALB/c and human-derived MSC aggregates using the Calcium Liquicolour kit (Stanbio Laboratories, Texas, USA) according to the manufacturer's protocol. Briefly,

aggregates were digested by adding 1 mL of 0.5 M HCL and rotating the solution overnight in a cold room; 10 µl of each of the digested samples and assay standard was then added to a 96-well plate and 200 µl of the working solution was added. The plate was read on a microplate reader at an absorbance of 550 nm, as described previously (Curtin et al., 2012).

3.2.5 Histology

Aggregates from BALB/c MSCs were examined by histological methods at days 0, 10, 14, 21, 28, 35 and 49. After each sample had been fixed overnight in paraformaldehyde, samples were dehydrated and embedded in paraffin using an automatic tissue processor (Leica ASP300, Leica). All samples were sectioned with a thickness of 8 µm using a rotary microtome (Leica microtome, Leica). Sections were stained with 1% Alcian Blue 8GX solution (pH of 2.5) for sGAG, as previously described (Haugh et al., 2011), and finally 2% Alizarin Red solution for mineralisation (all Sigma Aldrich).

3.2.6 Immunohistochemical Analysis

Immunohistochemical analysis was used to test the samples from BALB/c derived MSCs for Collagen type I, II and X as previously described (Vinardell et al., 2009). Briefly, sections were treated with chondroitinase ABC (Sigma Aldrich), rinsed with PBS, quenched of peroxidase activity, and blocked with goat serum (Sigma Aldrich) for 1 hour. Sections were then incubated at room temperature with either rabbit polyclonal anti-collagen type I (1:400), rabbit polyclonal anti-collagen type II (1:100), or rabbit polyclonal anti-collagen type X (1:200) (All Abcam, UK) for 1 hour. After washing in PBS again the sections were incubated with secondary antibody for

type I, type II and type X collagen (Anti-Rabbit IgG Biotin antibody produced in goat (1:200)) (Abcam UK) for 1 hour. Colour was developed using the Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, UK) and exposure to Peroxidase DAB substrate kit (Vector laboratories, UK). Positive controls (whole femur sections) for mice were included in the immunohistochemical staining protocol for each batch.

3.2.7 Statistical Analysis

Results are expressed as mean \pm standard deviation. For all the biochemical analysis a two-way analysis of variance (ANOVA) with time and medium type as the independent factors followed by pair-wise multiple comparison procedure (Tukey's HSD test) was used. All analyses were performed with Minitab. For all comparisons, the level of significance was $p \leq 0.05$.

3.3 Results

3.3.1 Chondrogenic priming of BALB/c MSC's

3.3.1.1 DNA Content

Although there were trends towards increased DNA content in the chondrogenically primed groups compared to the control groups, there was no statistical difference in the DNA content between any of the groups at any time point over the course of the 49 days, or within the groups at each time point, see Figure 3.2.

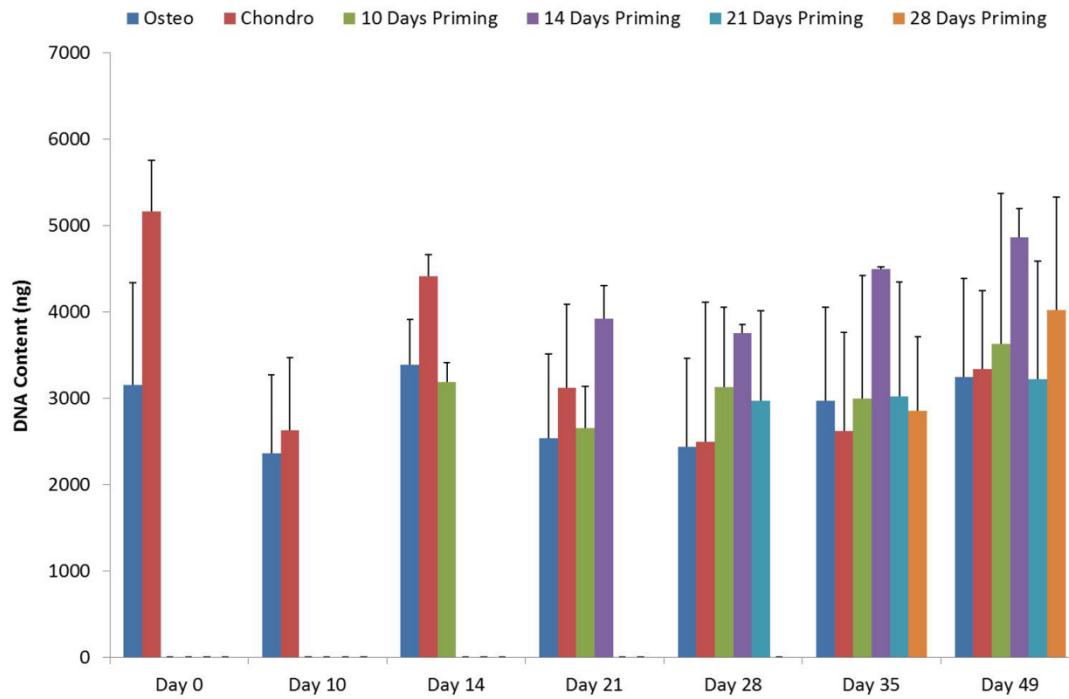


Figure 3.2: The DNA content of the BALB/c groups at days 0, 10, 21, 28, 35 and 49

($n = 6$ samples per group per time-point). Error bars denote standard deviation.

3.3.1.2 Production of the Cartilage Template

3.3.1.2.1 sGAG Content

After the initial surge in sGAG content present in the chondrogenic group at day 14, there was no statistical difference in the accumulative sGAG content between the chondrogenic and osteogenic groups at any of the time points (see Figure 3.3 (a)). Furthermore, there was no significant difference in the accumulative sGAG content between the chondrogenic, osteogenic and the chondrogenic priming groups until day 21. By day 35, there was no statistical difference in sGAG content between the 10, 14 days priming group and the chondrogenic group. In contrast, there was significantly higher sGAG content ($p < 0.01$) in the 14, 21 and 28 days priming groups compared to the chondrogenic control. These differences persisted to day 49, in every group but the 14 days priming group which had a drop in sGAG content. There was a significant

increase in sGAG content from day 35 to 49 in the 21 days priming group, and notably the sGAG content was significantly higher than the chondrogenic group ($p<0.01$) and the osteogenic group ($p<0.01$) at 49 days. On the other hand, the 14 days priming group decreased sGAG content between day 35 and day 49.

3.3.1.2.2 Alcian blue staining

The sGAG results are supported by the alcian blue stained sections of each of the groups at day 49; the 21 days priming group has the highest intensity of staining for sGAG (blue), see Figure 3.3 (b). Comparing each of the groups, the intensity of staining for sGAG throughout the entire aggregate is higher in the 21 and 28 priming groups compared to the osteogenic, chondrogenic, and the 10 and 14 days priming groups.

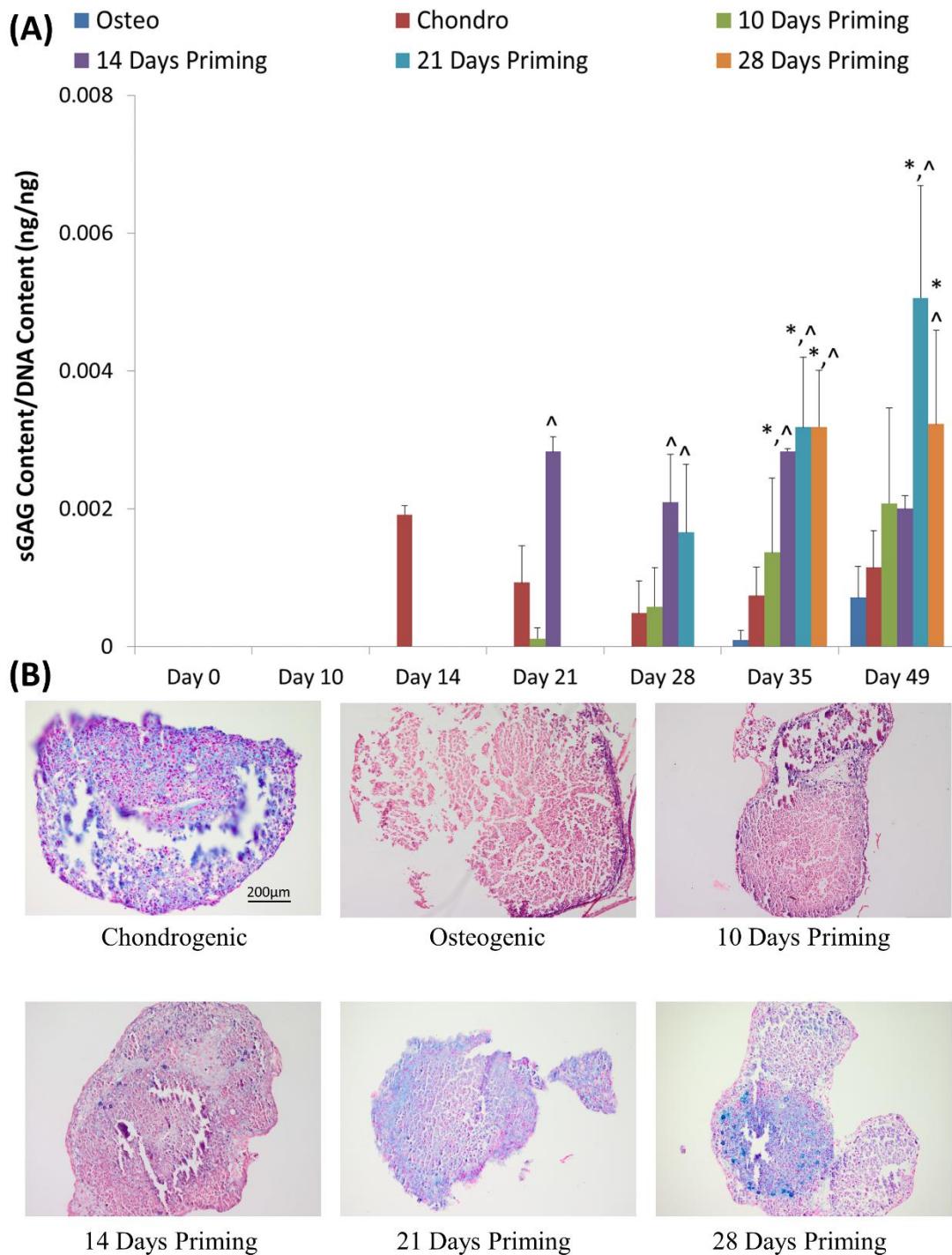


Figure 3.3: (A) The sGAG content of the BALB/c groups at days 0, 10, 14, 21, 28, 35 and 49 ($n = 6$ samples per group per time-point). * $p < 0.05$ vs. Chondro group; ^ $p < 0.05$ vs. Osteo group (see text). Error bars denote standard deviation. (B) Alcian Blue staining of each of the groups at day 49. Each of the images was taken at a magnification of 10 X.

3.3.1.3 Mineralisation of the Cartilage Template

In order to effectively depict the influence of chondrogenic priming on ALP and calcium secretion by BALB/c MSCs the data was analysed in two ways; 1) the amount of ALP/calcium secreted over the entire duration of the experiment and compared with both control groups; Chondrogenic group (chondrogenic medium alone for 49 days) and Osteogenic group (osteogenic medium alone for 49 days) (Figure 3.4 (a), Figure 3.5 (a)); and 2) the amount of ALP/calcium secreted after the switch from chondrogenic to osteogenic medium, which was only compared with the osteogenic control group (Figure 3.4 (b), Figure 3.5 (b)). These results are further analysed below.

3.3.1.3.1 *ALP secreted over the entire duration of the experiment*

ALP production in the osteogenic group increased until day 21, after which it began to plateau, see Figure 3.4 (a). There was higher extracellular ALP production by the osteogenic group than the chondrogenic group at all of the time points, however, these differences were only statistically different ($p<0.05$) at day 21. In contrast, all of the chondrogenic priming groups showed an initial increase in ALP production after changing the medium from chondrogenic to osteogenic. By day 49 most of the chondrogenic priming groups had statistically higher ALP production ($p<0.01$) than both the osteogenic and chondrogenic groups.

3.3.1.3.2 *ALP secreted by MSC's after the switch from chondrogenic to osteogenic medium*

Comparing each of the chondrogenic priming groups after the medium change revealed an initial increase in ALP production both 1 week and 2 weeks after the switch from chondrogenic to osteogenic medium (see Figure 3.4 (b)). The 14 days priming groups showed the smallest increase in ALP production 1 week after the initial

medium change. On the other hand, the 10, 21 and 28 days priming groups had a significant ($p<0.05$) increase in ALP production 1 week after medium change. By two weeks after the medium change all of the chondrogenic priming groups had significantly higher ALP activity than the osteogenic group. The 21 days priming group also had significantly higher ALP activity compared to the 28 days priming group. Examining the chondrogenically primed groups 2 week post-medium change the highest amount of ALP expressed was in the 14 and 21 days priming groups.

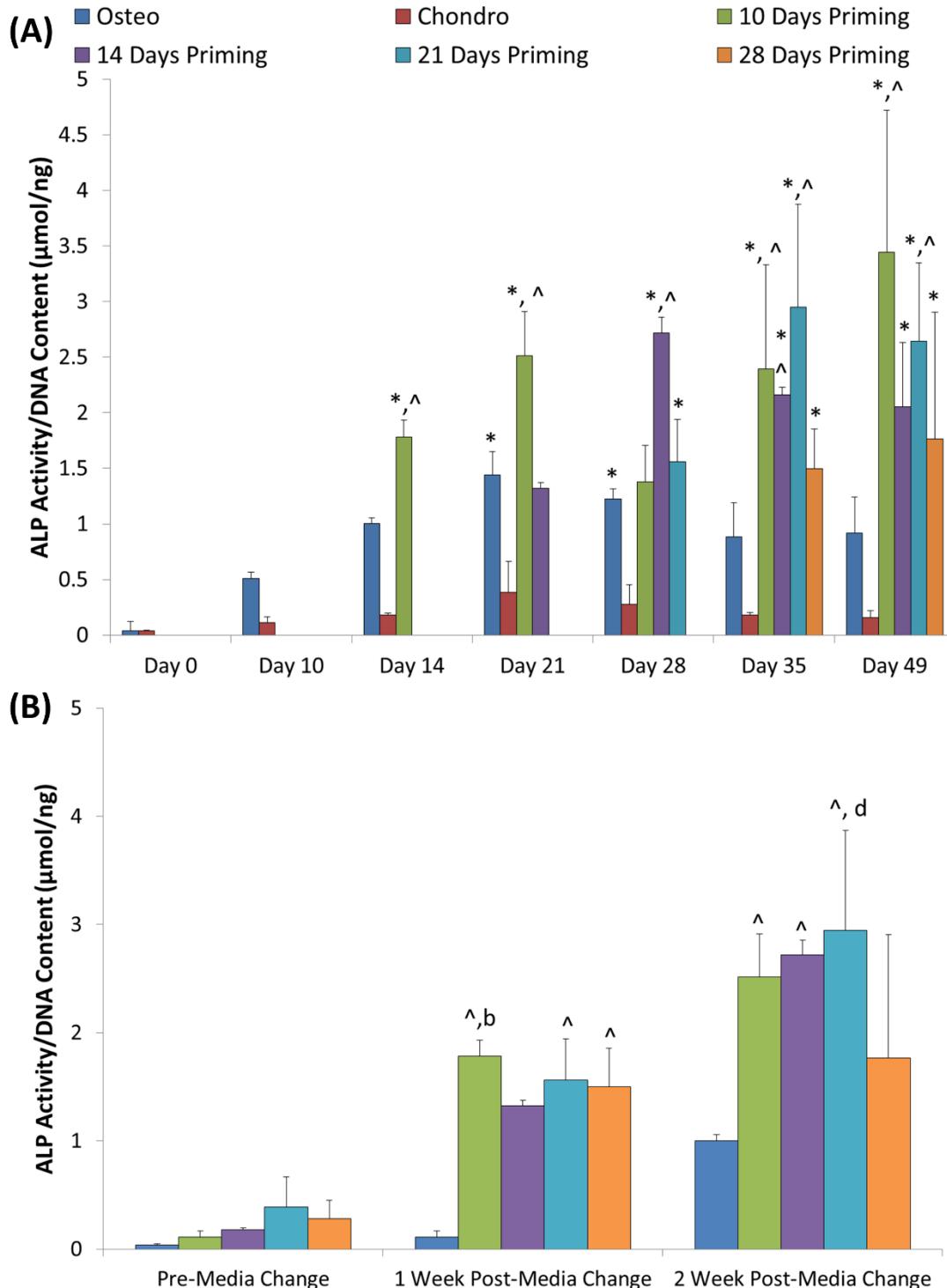


Figure 3.4: (A) The ALP/DNA content of the of the BALB/c groups at days 0, 10, 14, 21, 28, 35 and 49 ($n = 6$ samples per group per time point). (B) The ALP/DNA content of the BALB/c groups and compared with the osteogenic group, 1 week and 2 weeks after changing the medium. * $p < 0.05$ vs. Chondro group; ^ $p < 0.05$ vs. Osteo group;

^ap<0.05 vs. 10-day priming group; ^bp<0.05 vs. 14-day priming group; ^cp<0.05 vs. 21-day priming group; ^dp<0.05 vs. 28-day priming group (see text). Error bars denote standard deviation.

3.3.1.3.3 Calcium production by MSCs over the entire duration of the experiment

The osteogenic group increased calcium content throughout the time course of the experiment (see Figure 3.5 (a)), whereas there was no evidence of calcium production in the chondrogenic group at any time-point. The chondrogenic priming groups' calcium content increased throughout the time course of the experiment. By day 35 both the osteogenic and 10 days priming group had similar calcium content whereas the 14 and 21 days priming groups had significantly (p<0.05) lower calcium content than the osteogenic group. By day 49, both the 10 and 14 days priming groups had higher calcium content than the osteogenic group albeit that the 10 days priming group was the only one with significantly higher calcium content (p<0.05). There was no difference in calcium content between the 21 days priming group and the osteogenic group, whereas the 28 days priming group had significantly (p<0.05) lower calcium content when compared to the osteogenic group.

3.3.1.3.4 Calcium production by MSCs after the switch from chondrogenic to osteogenic medium

Comparing the primed groups with the osteogenic group directly the later chondrogenic priming groups (14, 21, and 28 days priming groups) only had an increase in calcium content two weeks after the media switch (see Figure 3.5 (b)). The 10 days priming groups had a small increase in calcium content one week post media switch. However, two weeks post media switch, all of the chondrogenic priming groups had significantly higher (all p<0.01) calcium content than the osteogenic group

alone, which had no calcium content until 3 weeks in osteogenic medium. The 28 days priming group had significantly higher calcium content than all other chondrogenic priming groups as well as the osteogenic group, and the 10 days priming group had the lowest calcium content when compared to the other chondrogenically primed groups.

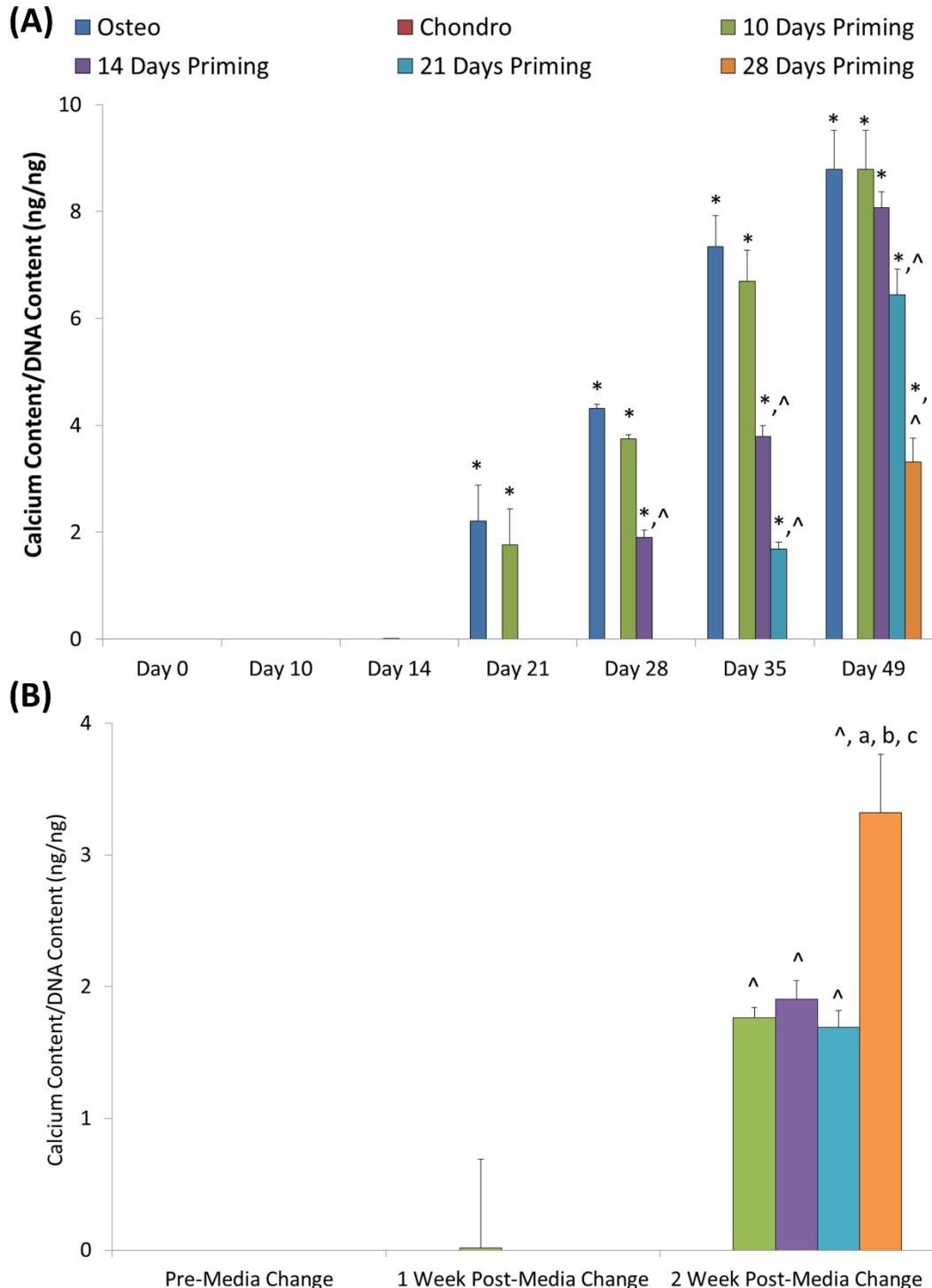


Figure 3.5: (A) Calcium content of the BALB/c groups at days 0, 10, 14, 21, 28, 35 and 49 ($n = 6$ samples per group per time-point). (B) Calcium content of the BALB/c groups compared with the osteogenic group 1 week and 2 weeks after changing the media. * $p < 0.05$ versus Chondro group; $^p < 0.05$ vs. Osteo group; $^a p < 0.05$ vs. 10-day

priming group; ^bp<0.05 vs. 14-day priming group; ^cp<0.05 vs. 21-day priming group;
^dp<0.05 vs. 28-day priming group (see text). Error bars denote standard deviation.

3.3.1.3.5 Alizarin Red Staining

The mineralisation differences observed were further verified through alizarin red staining (see Figure 3.6). Two weeks after the media switch, mineralisation was only seen in the chondrogenic priming groups and not in the osteogenic group. In particular, mineralisation was seen throughout the aggregate in the 14 and 21 days priming groups, but only in the periphery in the other chondrogenic priming groups. This correlates with the results of the calcium assay, where the highest amount of mineralisation occurred in the later chondrogenic priming groups.

3.3.1.3.6 Immunohistochemical Analysis

The results from the calcium content assay were further verified in the immunohistochemical staining of the groups two weeks after the media switch. The highest amount of positive staining for Collagen type I was seen in the 28 days priming group and the lowest amount is seen in the osteogenic group. The highest amount of Collagen type II was seen in the later chondrogenic priming groups (i.e. 14-28 days priming), therefore correlating with the findings of the alcian blue staining and sGAG assay. Finally, Collagen type X staining was positive on the periphery of all of the aggregates two weeks after the media switch with the highest amount of (p<0.05) staining in 28 days priming group (see Figure 3.6).

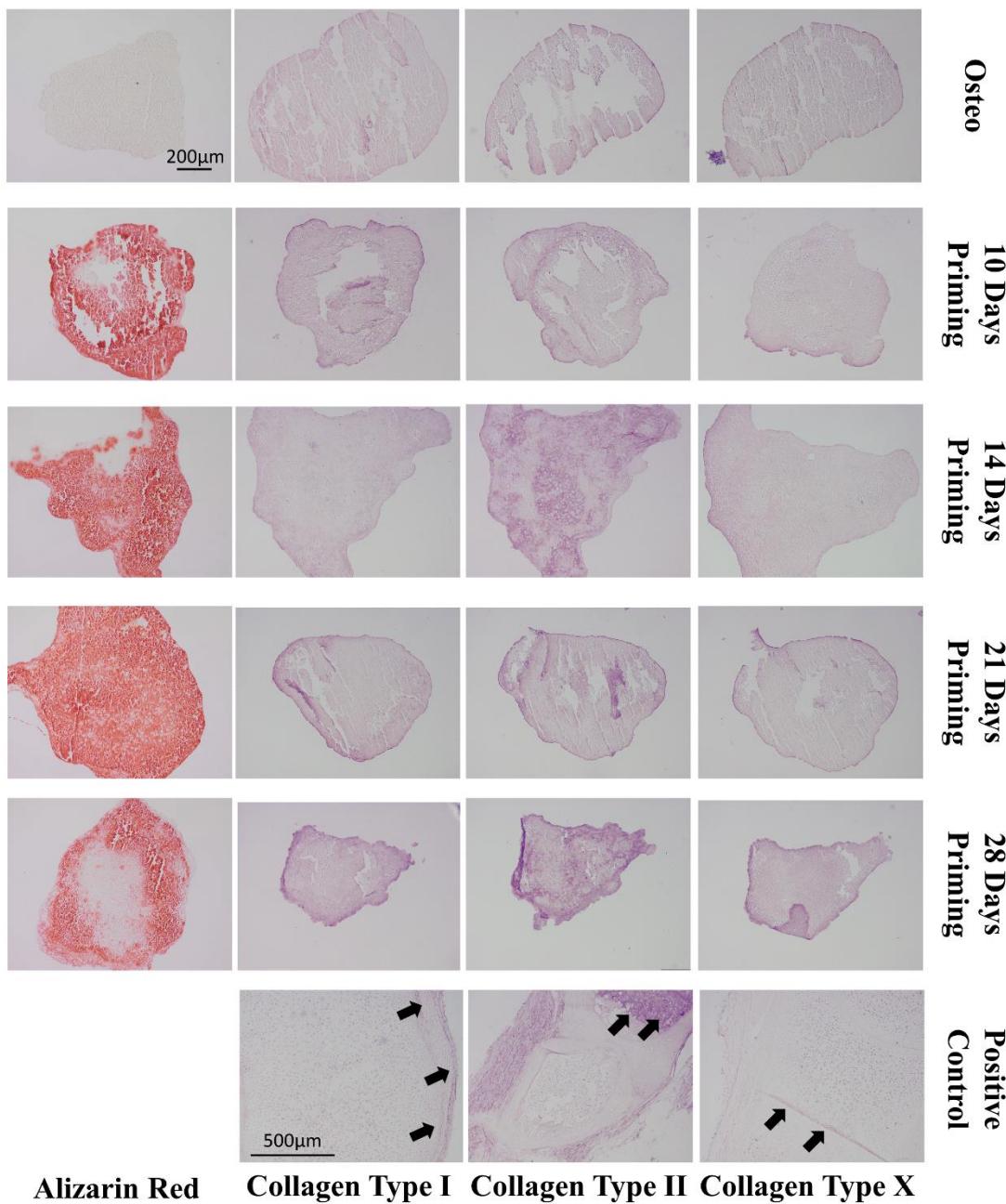


Figure 3.6: Alizarin Red and immunohistochemical staining (collagen types I, II and X) of the BALB/c chondrogenically primed groups and osteogenic group at 2 weeks after switching media. Each of the images was taken at a magnification of 10 X.

3.3.2 Chondrogenic priming of Human MSC's

3.3.2.1 Mineralisation of the Cartilage Template

In order to effectively depict the influence of chondrogenic priming on ALP and calcium secretion by human MSCs the data was analysed by the amount of ALP/calcium secreted after the switch from chondrogenic to osteogenic medium, which was only compared with the osteogenic control group (see Figure 3.7). These results are further analysed below.

3.3.2.1.1 ALP secreted by human MSC's after the switch from chondrogenic to osteogenic medium

Examining the ALP production/DNA content for the human cells both the 14 and 21 days priming groups had significantly higher ALP production than the osteogenic group both at 1 week and 2 weeks post media switch. The 21 days priming group also had significantly higher ALP activity compared to the 14 days priming group 1 week post media change.

3.3.2.1.2 Calcium production by MSCs after the switch from chondrogenic to osteogenic medium

Examining the Calcium content/ DNA content for human cells both the 14 and 21 days priming groups had higher Calcium content than the osteogenic group at all-time points. However, the 21 days priming had significantly higher Calcium content than both the 14 days priming and the osteogenic group at both 1 week and 2 weeks post media switch.

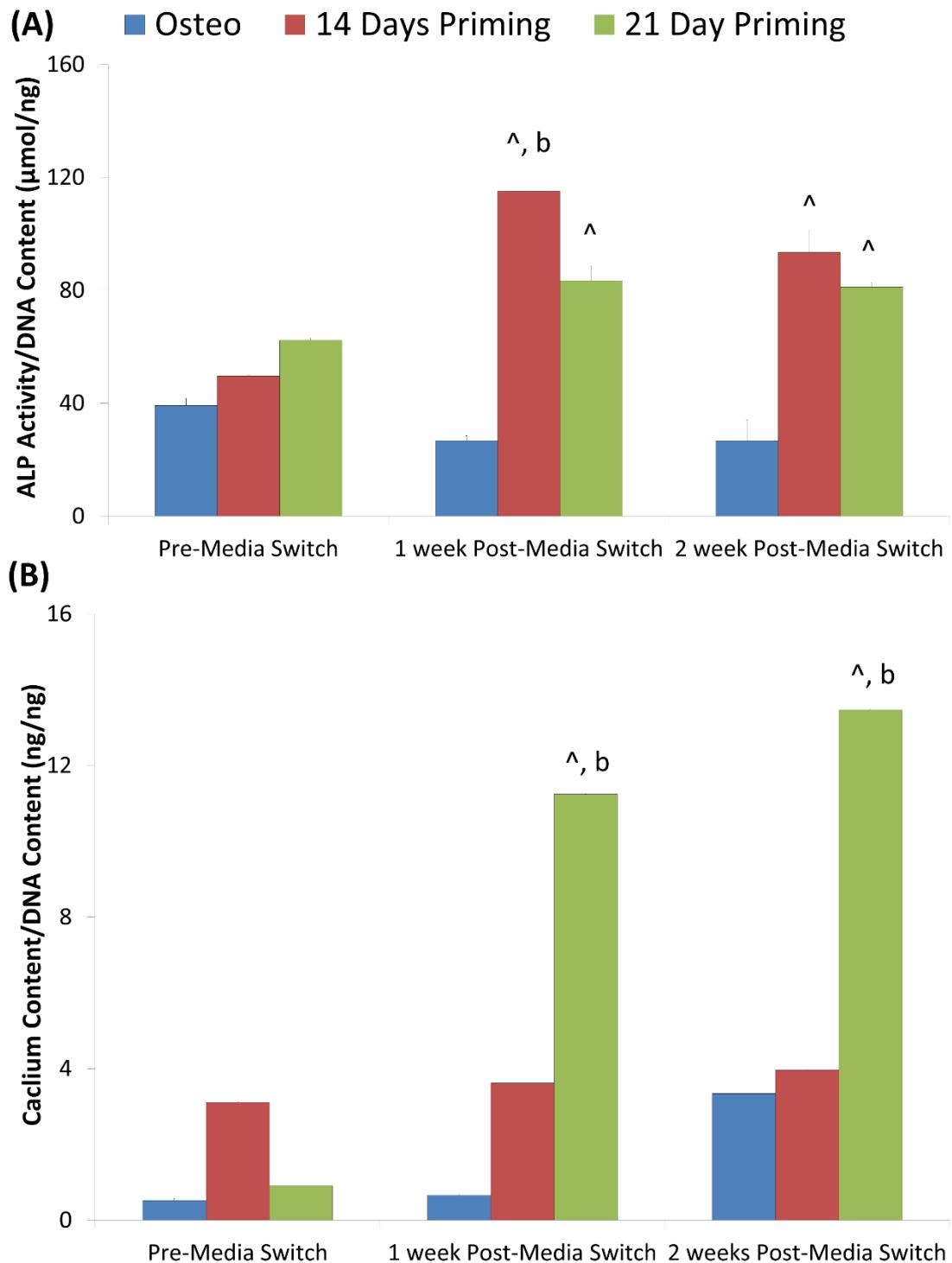


Figure 3.7: (A) The ALP/DNA content of the human MSC priming groups, and compared with the osteogenic group 1 week and 2 weeks post-media change. (B) Calcium content of the human MSCs priming groups compared to the osteogenic group, 1 week and 2 week after switching media. * $p<0.05$ vs. Chondro group; ^ $p<0.05$

vs. Osteo group; ^ap<0.05 vs. 10-day priming group; ^bp<0.05 vs. 14-day priming group; ^cp<0.05 vs. 21-day priming group; ^dp<0.05 vs. 28-day priming group (see text). Error bars denote standard deviation ($n = 6$).

3.4 Discussion

The results of this Chapter show for the first time that chondrogenic priming for specific durations (14, 21 days), prior to being exposed to osteogenic factors, allows BALB/c and human derived MSCs to differentiate and produce a chondrogenic template which can enhance osteogenic differentiation of MSCs (as indicated by ALP, Calcium content and Collagen type I and Alizarin Red staining). Most importantly, chondrogenic priming induced more mineralisation by BALB/c and human MSCs compared to growth in osteogenic medium alone. Furthermore, chondrogenic priming for a period of 21 days enhanced the distribution of mineral through the entire aggregate, whereas other durations led to mineralisation on the periphery alone. Interestingly, chondrogenic differentiation was also enhanced in MSCs that were chondrogenically primed for longer durations, and this was significantly higher than the MSCs cultured in chondrogenic medium for the entire experiment. Taken together, these results indicate for the first time that there is an optimum time, approximately 21 days, for chondrogenic priming of MSCs to enhance both osteogenic and chondrogenic differentiation *in vitro*.

One limitation of this Chapter was that osteogenic and chondrogenic factors (ascorbic acid dexamethasone, β -glycerol and TGF- β 3) were introduced into the culture medium of MSCs to encourage MSC differentiation down specific pathways. It is unlikely that these factors are present in the combinations used here during

endochondral ossification *in vivo*, and these may alter cellular viability in long term *in vitro* studies. However, previous research studies have exposed MSCs to both chondrogenic and osteogenic differentiating agents for long durations (14 days to 5 weeks), and have demonstrated long term viability and matrix production by MSCs (Thorpe et al., 2010; Farrell et al., 2011; Farrell et al., 2009; Vinardell et al., 2012; Haugh et al., 2011; Jaiswal et al., 1997b; Coleman et al., 2013; Cheng et al., 2009; Estes et al., 2010; Mauck et al., 2006; Mauck et al., 2003). Furthermore, the control groups were exposed to the same osteogenic or chondrogenic factors, so the differences observed in osteogenesis due to the duration of priming cannot be explained by differences in cell viability. Another limitation is that human MSCs were only investigated for a subset of conditions, and that BALB/c cells rather than human MSCs were used for histological and immunohistochemical analyses. However, it was not feasible to obtain a large enough stock of human bone marrow MSCs to conduct all of the necessary experiments, involving numerous treatment groups and time points, to address this question. The BALB/c cells provide a suitable model to test the hypothesis (Peister et al., 2004; Tropel et al., 2004; Baddoo et al., 2003) and can overcome limitations of intraspecies variability, due to the ability to obtain relatively large litters of animals from the same parents. They can also provide the large volume of bone marrow required for undertaking large studies without having to expand MSCs beyond passage 3. Furthermore, this study also confirmed the BALB/c ALP and calcium results, in the subset of conditions using human derived MSCs, thus supporting the use of BALB/c cells to test the hypothesis there is an optimum time for chondrogenic priming of MSCs *in vitro* that will enhance osteogenic differentiation. Furthermore, the optimum conditions determined through this study were only optimum for BALBc MSCs/ or human MSCs under the conditions mentioned.

Moreover, the human donors were also chosen for their tri-potentiality potential. Therefore, the results for this study proved that there is an optimum condition for chondrogenesis and osteogenesis of stem cells in vitro however, future studies should investigate if the same is true for other sources of MSCs (e.g. adipose or embryonic derived stem cells).

The first stage of endochondral ossification involves MSCs condensing and aligning to become clusters of cells, and subsequently these MSCs differentiate down the chondrogenic pathway (Mackie et al., 2008; McNamara, 2011; Kronenberg, 2003). In order to investigate the optimum timing for production of a cartilage template, biochemical and histological analyses were performed at various time points. In this work there is an increase in sGAG content from day 21 to day 49. These results are in keeping with those of previous studies, which cultured human MSCs in aggregate form (Angele et al., 2003) or in agarose scaffolds (Haugh et al., 2011). Unlike other studies these results report a significant drop in sGAG content in the chondrogenic group from day 14 to 21. This initial drop in sGAG content and subsequent gradual increase in sGAG content was also seen in all of the chondrogenic priming groups. The histological analyses confirmed the formation of a cartilage-like tissue, which was similar to the histological staining reported in previous cartilage tissue engineering studies using MSCs seeded on agarose hydrogel scaffolds (Mauck et al., 2006; Erickson et al., 2009; Vinardell et al., 2009). However, these results show for the first time that there is an optimum time for chondrogenic priming that enhances chondrogenic differentiation, as is demonstrated by elevated sGAG production in MSCs that were chondrogenically primed for 21 and 28 days.

Previous in vitro studies have also shown that MSCs that were chondrogenically primed in vitro for 21 days can mineralise when subsequently implanted into an animal model in vivo (Scotti et al., 2013; Scotti et al., 2010; Farrell et al., 2011; Farrell et al., 2009; Miot et al., 2012; Harada et al., 2014; van der Stok et al., 2014). Other studies, have also reported that chondrogenic priming of MSCs can enhance chondrogenic and osteogenic differentiation of the cells (Farrell et al., 2011; Farrell et al., 2009), but to date the beneficial effect of chondrogenic priming in vitro for production of bone tissue mineral has not yet been identified. The most interesting observation of this Chapter is that chondrogenic priming of BALB/c MSCs and human bone marrow MSCs for specific time periods (14 and 21 days) can enhance ALP production, calcium content, collagen type I production and mineralisation when compared to the osteogenic group and other chondrogenic priming groups. Moreover, histology results from the BALB/c MSCs show that Collagen type I and Alizarin Red staining were present throughout the aggregate for both groups, whereas the 28 days chondrogenic priming group had the highest amount of calcium but also had the highest amount of Collagen type X which is known to be a marker for the expression of cartilage hypertrophy (Mueller and Tuan, 2008). Alizarin Red staining was also limited to the periphery indicating that the centre of these aggregates may have become hypertrophic. Previous chondrogenic priming studies have been limited by the occurrence of core degradation, arising from lack of nutrient delivery and waste removal when implanted in vivo, and mineralisation occurring around the periphery or in the core alone, but not throughout the aggregate (Farrell et al., 2009; Scotti et al., 2010; Farrell et al., 2011). The results show that mineralisation throughout an aggregate is possible in vitro if chondrogenically primed for a specific amount of time (14, 21 days).

It is clear from these results that the amount of time the cells are chondrogenically primed for can have a positive or negative effect on both the osteogenic and chondrogenic differentiation potential of MSCs *in vitro*. This may be due to the fact that allowing the cells to form a chondrogenic phenotype and then an osteogenic phenotype, thus recreating the *in vivo* endochondral ossification process, can significantly increase the mineralisation potential of MSCs. However, these results also demonstrate that a mature cartilage template needs to be formed to significantly increase the mineralisation potential of the MSCs. However, if the template is allowed to form for too long the core of the aggregate can become hypertrophic, and the periphery of the aggregate will only mineralise and may lead to complications *in vivo* as seen in Section 2.7.2. Chondrogenic priming of MSCs for specific periods of time (14, 21 days) enhances *in vitro* osteogenesis due to the fact that it more closely mimics the endochondral ossification *in vivo*, which begins after cartilage differentiation has occurred. There is a distinct lack of knowledge regarding the exact timing of endochondral ossification during human development. However, as previously mentioned embryologists have estimated through ultrasound techniques that the first sign of limb buds are seen by approximately the 26th day of gestation, but the exact day in which the endochondral ossification process begins is still unknown (Smith, 1968; Webster, 2012; Merz and Bahlmann, 2004). The optimum timing observed in this Chapter will be used in the subsequent Chapters of this Thesis to investigate if mimicking the endochondral ossification process *in vitro* is an effective bone tissue regeneration process.

3.5 Conclusion

The results of this Chapter show that chondrogenic priming of BALB/c derived MSCs and human bone marrow MSCs can significantly increase their potential for osteogenic differentiation and mineralisation, even more so than culturing the cells in osteogenic growth factors alone. Moreover, this Chapter demonstrates for the first time that chondrogenic priming for specific durations (14, 21 days), can enhance osteogenic differentiation by MSCs in vitro and can produce an aggregate that is mineralised throughout the core. Determining the optimum time for chondrogenic priming to enhance osteogenic differentiation in vitro provides vital information that might lead to a novel regenerative treatment for large bone defects, as well as addressing the major limitation of core degradation and construct failure.

Chapter 4: Does Chondrogenic and Vascular priming enhance mineralisation whilst allowing for vessel formation in vitro?

4.1 Introduction

Bone tissue engineering constructs have been shown to act as a barrier to healing in rodent cranial defects (Lyons et al., 2010). Once implanted, the scaffolds become encapsulated and host vasculature is inhibited, which leads to core necrosis and ultimately failure of the implant construct (Lyons et al., 2010; Amini et al., 2012; O'Brien, 2011). Recent studies have suggested a developmental engineering approach, which mimics certain aspects of the endochondral ossification process, might enhance bone tissue regeneration both in vitro and in vivo (Scotti et al., 2013; Jukes et al., 2008; Oliveira et al., 2009a; Oliveira et al., 2009b; Pelttari et al., 2006; Scotti et al., 2010; van der Stok et al., 2014; Sheehy et al., 2013; Harada et al., 2014; Gawlitta et al., 2015; Visser et al., 2015). As discussed in Chapter 3 the formation of the cartilage template through chondrogenic priming in vitro, prior to implantation in vivo, has been shown to induce mineralisation in MSCs following subcutaneous implantation in both tissue engineered constructs (Farrell et al., 2011; Miot et al., 2012; Scotti et al., 2010; Farrell et al., 2009; Scotti et al., 2013; Jukes et al., 2008; Harada et al., 2014; Gawlitta et al., 2015; Visser et al., 2015) and scaffoldless aggregates (van der Stok et al., 2014; Farrell et al., 2011; Farrell et al., 2009). However, a number of these studies observed core

degradation and an uneven distribution of bone mineral throughout the construct (Farrell et al., 2009; Scotti et al., 2010; Farrell et al., 2011). The results of Chapter 3 of this Thesis found that chondrogenic priming of BALB/c mice MSCs and human MSCs in vitro for specific durations (14, 21 days) has a significant influence on their mineralization capacity, allowing mineral to form throughout the aggregates rather than just around the periphery. Without the buildup of mineral around the periphery, nutrients can also reach the core of the aggregates with ease thus reducing the likelihood of core degradation seen in previous studies (Freeman et al., 2013).

In vivo vessel formation is an integral part of the bone formation process that occurs during the endochondral ossification process. As described in Section 2.4.2, vessel invasion occurs once the cartilage template has formed by a process known as quiescent angiogenesis. This involves endothelial cells invading through the cartilage canals already present in the developing bone tissue (Mackie et al., 2008; McNamara, 2011; Kronenberg, 2003; Gerber and Ferrara, 2000), and during early fetal development this process typically occurs between 14 and 18 days of embryogenesis of mice (Gerber and Ferrara, 2000; Carlevaro et al., 2000). For in vitro bone regeneration approaches, it is believed that bone vasculature is necessary to provide appropriate growth factors, hormones, chemokines and nutrients, and without a vascular supply. Cells within tissue engineered constructs do not get the necessary requirements to regenerate the damaged bone tissue and ultimately die when implanted in vivo (Rivron et al., 2008; Kanczler and Oreffo, 2008). Co-culture studies of MSCs with endothelial stem cells, such as HUVECs, have investigated whether pre-vascularising tissue engineering constructs in vitro would allow faster host integration post-implantation (Correia et al., 2011; Ghanaati et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Scherberich et al., 2007; Duffy et al., 2011). Pre-

vascular networks can be formed in vivo as early as 7 days in co-culture of HUVECs (Correia et al., 2011; Ghanaati et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Scherberich et al., 2007) and MSCs (Duffy et al., 2011; McFadden et al., 2013), however, they are only sustained in the presence of the MSCs (Duffy et al., 2011; McFadden et al., 2013). Other studies have looked into direct cell-cell contact co-culture approaches through the formation of cellular aggregates, and have shown that pre-vascular networks can form in vitro in cellular aggregates (Fuchs et al., 2007; Saleh et al., 2011; Rouwkema et al., 2006; Verseijden et al., 2010; Gawlitta et al., 2012). Moreover, the presence of both MSCs and HUVECs promotes the formation of vascular networks and upregulates early osteogenic markers such as ALP activity (Fuchs et al., 2007; Saleh et al., 2011).

While in vitro bone regeneration strategies have sought to incorporate either the production of the cartilage template or the vascularisation of the construct, no strategy has sought to incorporate both events simultaneously, albeit that both are crucial precursors for bone formation in vivo during endochondral ossification. This Chapter tests the hypothesis that “A tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage aggregates, through the co-culture of HUVECs in vitro, will serve as an effective in vitro bone regeneration approach”. The overall objective for this Chapter was to compare the regenerative potential of **(a)** chondrogenic priming of MSCs in aggregate culture and **(b)** addition of HUVECs to chondrogenic MSC aggregates, to **(c)** a novel methodology involving both chondrogenic priming and the co-culture of HUVECs and MSCs. The specific objectives were to compare mineralisation and vessel formation by means of biochemical and histological analysis for DNA content, sGAG production, ALP production, Calcium Content, Vessel

Endothelial Growth Factor (VEGF) production, CD31+ and Collagen Type X production . This Chapter presents an adapted version of work previously published in the Journal of Tissue Engineering Part C (Freeman et al., 2015b).

4.2 Methods

4.2.1 Cell Isolation and Characterisation

4.2.1.1 Human Donor MSC: Isolation and Characterisation

As described in Chapter 3, in section 3.2.1, human bone marrow derived MSCs were extracted from bone marrow aspirates. Once suitable colonies had formed the chondrogenic, osteogenic and adipogenic potential of these MSCs were confirmed as outlined below.

To confirm adipogenic potential of the MSCs used, cells were plated at 2×10^4 cells/cm² and incubated in expansion medium until they reached 80% confluency. Then, adipogenic induction medium was added containing DMEM with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 µM Dexamethasone, 10 µg/mL insulin, 200 µM Indomethacin, 500 µM Isobutyl-1-Methyl-Xanthine (All Sigma Aldrich). After 3 days, maintenance medium was added containing DMEM, 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 g/mL streptomycin and 10 µg/mL insulin (All Sigma Aldrich). This cycle was repeated for a 21 days cell culture period, after which cells were fixed with 10 % formalin and stained with 0.5 % Oil Red O in methanol (All Sigma Aldrich). A negative control group was

also stained for comparison, in which MSCs had been incubated in expansion medium for the same duration.

To confirm osteogenic differentiation cells were plated at 2×10^4 cells/cm² and incubated in expansion medium until they reached 80% confluence. Osteogenic medium was added containing DMEM supplemented with 10 % FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 mM β-glycerol phosphate, 100 nM dexamethasone and 50 µM ascorbic acid (All Sigma Aldrich). Medium was changed every 3 days and cells were cultured for 21 days. After 21 days, cells were fixed in 10% formalin and stained with 10% Alizarin Red (All Sigma Aldrich). A negative control group was also stained for comparison, in which MSCs had been incubated in expansion medium for the same duration.

To confirm the chondrogenic potential of the MSCs, cells were seeded in aggregate culture with 2.5×10^5 cells per aggregate and incubated in 500 µL of complete chondrogenic medium, which consisted of a chemically defined medium containing high glucose DMEM GlutaMAX (Gibco, Life Sciences, Dublin, Ireland), 10 ng/mL TGF-β3 (ProSpec-Tany TechnoGene Ltd., Ness-Ziona, Israel), 50 µg/mL Ascorbic Acid, 4.7 µg/mL Linoleic Acid, 100 nM Dexamethasone (All Sigma Aldrich) and 1x insulin-transferrin-selenium (ITS, BD Biosciences, Bedford, MA). The medium was changed three times a week. After 21 days, aggregates were harvested, embedded in paraffin, sectioned and stained with Alcian Blue (Sigma Aldrich). A negative control group was also stained for comparison, in which MSCs had been incubated in expansion medium for the same duration.

In order to obtain sufficient stocks of MSCs, human MSCs (24 year old donor) were also purchased from Lonza, Braine-l'Alleud, Belgium and cultured in standard

human MSC culture conditions (DMEM plus 10% FBS, 100 U/mL penicillin, 100 U/mL of streptomycin and 2 mM L-glutamine) as described in Chapter 3. Both human MSCs were cultured to passage 4.

4.2.1.2 HUVECs Culture

HUVECs were purchased from PromoCell, Heidelberg, Germany and cultured in Endothelial Growth Medium (EGM) (Promocell). Medium was replaced every 3 days and upon reaching 80-90% confluency, cells were passaged using trypsin-EDTA solution. HUVECs were further cultured to passage 4.

4.2.2 Aggregate Formation

As described in Chapter 3, once the cells reached 80-90% confluency, aggregates of 250,000 cells/aggregate were formed by centrifuging the human MSCs at 400 g at a temperature of 22°C for 5 minutes. For this study all aggregates were formed from individual donors, cells were not pooled. Carefully avoiding the newly formed aggregate, the medium was removed from each of the aggregates and 0.5 mL of either Chondrogenic Medium or Endothelial Growth Medium plus Osteogenic Growth factors was added depending on experimental conditions (described in detail below). The Chondrogenic medium used for the duration of this study was the same as the Chondrogenic medium (high glucose DMEM, 10 ng/mL TGF- β 3, 50 μ g/mL Ascorbic Acid, 4.7 μ g/mL Linoleic Acid, 100 nM Dexamethasone, and 1x insulin-transferrin-selenium) described in Chapter 3 in section 3.2.2. EGM plus Osteogenic Growth factors consisted of EGM Medium (Promocell) supplemented with 8% FBS, 100 nM of Dexamethasone, 50 μ g/mL Ascorbic Acid and 10 mM β -Glycerol Phosphate (Sigma Aldrich). For all experiments aggregate cultures were fed twice per

week by performing a 50% medium exchange. During each feed the aggregates were agitated so as to prevent them from adhering to the micro-tube. This was achieved through aspirating the medium beneath the aggregate with a micro-pipette.

These cells were further cultured under the following conditions: (1) *CP21-HUVECs*: Chondrogenic priming for 21 days (no cells added); (2) *CP21+HUVECs*: Chondrogenic priming for 21 days and addition of HUVECs (250,000 cells); (3) *CP21+HUVECs:MSCs*: Chondrogenic Priming for 21 days and co-culture of HUVECs and MSCs at a ratio of 1:1 (125,000:125,000 cells) as seen in Figure 4.1. A ratio of 1:1 was chosen as previous studies which investigated the direct co-culture of HUVECs and MSCs, found that mineralisation potential was highest using a ratio of 1:1 (Villars et al., 2002; Ma et al., 2011; Kaigler et al., 2005).

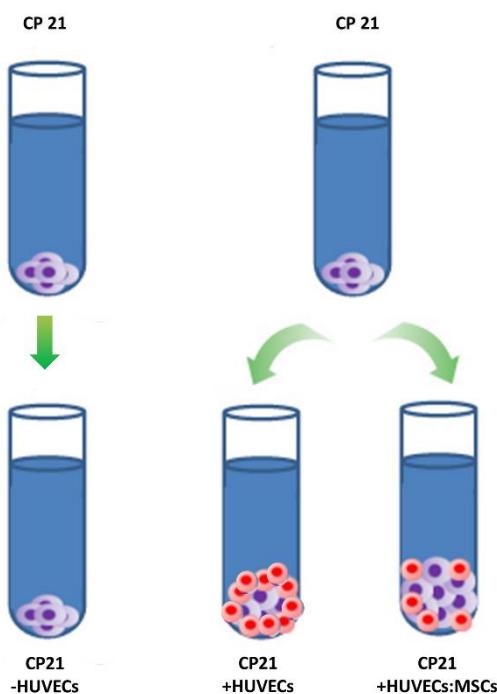


Figure 4.1: Schematic of experimental groups.

For the co-culture groups, confluent layers of HUVECs/MSCs were trypsinised and counted. Cells were suspended depending on experimental conditions so that there

was 0.5×10^6 cells/mL. In the case of the CP21+HUVECs:MSCs, the ratio of cells was 1:1 HUVECs:MSCs. The cells were suspended in EGM medium containing osteogenic growth factors and 20% methocel from a stock solution that was generated by dissolving 6 g of carboxymethylcellulose (Sigma Aldrich) in 500mL of DMEM as previously described (Korff and Augustin, 1998). The medium was removed from the chondrogenically primed group and EGM medium containing osteogenic growth factors was added. In the case of the group that contained HUVECs, the medium added also contained suspended HUVECs alone and in the case of the group that contained both HUVECs and MSCs, the medium added also contained suspended MSCs and HUVECs. After 24 hours the medium that contained methocel was removed and was replaced with EGM medium containing osteogenic growth factors and the aggregates were cultured for a further 21 days.

4.2.3 Histochemical analysis and Biochemical Analysis

Aggregates were examined at day 0, 1 week, 2 weeks, and 3 weeks after the start of culture in their respective conditions and were prepared for either histochemical analysis or biochemical analysis. At each of the time points, the culture medium from the aggregates was collected, frozen and stored at -80°C until biochemical assays could be performed. The remaining aggregates were washed with PBS and then treated in one of the following two ways; (1) frozen and stored at -80°C for biochemical analysis or (2) fixed overnight in paraformaldehyde before being placed in PBS and refrigerated for histochemical analysis. For the above study, two independent experiments were carried out with at least two repeats in each experiment ($n = 4$ for histological analysis and $n = 6$ for biochemical analysis).

4.2.3.1 Quantitative Biochemical Analysis

4.2.3.1.1 DNA Content

To assess DNA content, aggregates were papain digested as previously described in Chapter 3 in section 3.2.4.1. Briefly, 500 µL of Papain digest was added to the aggregates and aggregates were placed in an oven at 60°C overnight. Once the aggregates were digested DNA content was performed using Hoechst 33258 DNA assay with calf thymus DNA as a standard, following a previously published protocol (Freeman et al., 2013; Haugh et al., 2011; Kim et al., 1988; Dolan et al., 2012). In minimal light 40 µL of papain digest of the sample/standard was added to a 96-well plate in triplicate. To this 200 µL of working solution (assay buffer and 1 mg/mL Hoechst Dye solution) was added. The plate was incubated away from light for 10 minutes and then read on a microplate reader (Synergy HT BioTek Multi-Mode Microplate Reader) at an excitation of 350 nm and emission of 450 nm.

4.2.3.1.2 ALP Production

As described in Chapter 3, media samples were used to examine extracellular ALP production using a colorimetric assay of enzyme activity, which uses *p*-nitrophenyl phosphate (*p*NPP) as a phosphatase substrate with ALP enzyme as a standard. Briefly, 40 µL of the medium was added to a 96-well plate in triplicate with a 50 µL of *p*NPP solution, which contains both *p*NPP and assay buffer. The samples were shielded from direct light at room temperature for one hour. After this, 20 µL of Stop Solution (3N NaOH) was added to the wells and the plate was read at 405 nm in a micro-plate reader.

4.2.3.1.3 Calcium Content

Calcium was measured using the Calcium Liquicolour kit (Stanbio Laboratories) according to the manufacturer's protocol, as previously described in Chapter 3. Briefly aggregates were digested by adding 0.5 mL of 0.5 M HCL and rotating the solution overnight in a cold room. Next 10 µL of each of the digested samples and assay standard was added to a 96-well plate and 200 µL of the working solution was added. The plate was analysed on a microplate reader at an absorbance of 550 nm.

4.2.3.1.4 Enzyme-linked Immunosorbent Assay (ELISA) for Vascular Growth Factor

An ELISA (R&D Systems) was used to quantify the levels of VEGF expressed by the aggregates. The cell culture medium was collected and analysed at the specific time points. Assays were carried out according to the manufacturer's protocol (R&D Systems) and analysed on a microplate reader at a wavelength of 450 nm.

4.2.3.2 Histology

Aggregates were obtained at day 0, 1 week, 2 weeks and 3 weeks post media switch for histological analyses. After each sample had been fixed overnight in 4% paraformaldehyde, samples were dehydrated and embedded in paraffin using an automatic tissue processor (Leica ASP300, Leica). All samples were sectioned with a thickness of 8 µm using a rotary microtome (Leica microtome, Leica). Sections were stained with 1% Alcian Blue 8GX solution for sGAG and 2% Alizarin Red solution for mineralisation (all Sigma Aldrich), as previously described in Chapter 3.

4.2.3.2.1 Quantitative Analysis of Alizarin Red Staining

ImageJ software was used to quantify the amount of mineralisation deposited (positive alizarin red staining) between each of the groups. Four samples from each group and nine images from each sample were analysed. The value of positive staining

was determined using the colour threshold plugin on Image J. A threshold of 180 was chosen as it was high enough that strong staining passed but not weak staining. The percentage of the positive staining was determined per total area of aggregate imaged.

4.2.3.3 CD31 and Collagen Type X Immunohistochemical Analysis

Immunohistochemical analysis was used to detect CD31 and Collagen Type X. Sections were deparaffinised overnight before a series of rehydration steps. The samples were then treated with 40 µg/mL of proteinase K for 20 minutes at 37 °C (Sigma Aldrich), rinsed with PBS-Tween and blocked with PBS with 1% w/v Bovine Serum Albumin (BSA) and 3% w/v Normal Goat serum (NGS) (Sigma Aldrich) for 60 minutes. Sections were then incubated overnight at 4°C with either rabbit polyclonal anti-CD31 (ab28364 Abcam, 1:50) or rabbit polyclonal anti-collagen type X (ab58632 Abcam, 1:200). After three washing steps with PBS with 1% w/v BSA the sections were incubated with Dylight 488 goat anti-Rabbit secondary antibody (Jackson Immunoresearch, 115-485-209, 1/200), for one hour at room temperature in the dark. The samples were washed three times in PBS with 1% w/v BSA, and the slides were mounted using Propidium Iodide (PI) mounting medium (Sigma Aldrich). Serum containing blocking solution (PBS with 1% w/v BSA and 3% w/v NGS) was used to replace primary antibodies as a negative control for both CD31 and Collagen Type X staining. HUVECs provided a positive control for CD31 staining. Chondrocytes within the hypertrophic zone of neonatal mice provided a positive control for Collagen Type X staining.

4.2.3.3.1 Quantitative Analysis of Vessel Diameter

ImageJ software was used to quantify vessel diameter and cross-sectional area of the rudimentary vessels (positive CD31 staining) in the CP21+HUVECs:MSCs

group. Four aggregates were analysed which led to a total of sixteen rudimentary vessels analysed. The average cross-sectional area was measured using the free-hand selector tool on Image J software. As the vessels were not completely circular vessels the diameter was measured across four different areas and the average was used as the diameter of the vessels.

4.2.4 Statistical Analysis

Results are expressed as mean \pm standard deviation. For all the biochemical analysis a two-way analysis of variance (ANOVA) with time and medium type as the independent factors followed by a pair-wise multiple comparison procedure (Tukey's HSD test) was used to test for significance. All analyses were performed with Minitab. For all comparisons, the level of significance was $p \leq 0.05$.

4.3 Results

4.3.1 Characterisation of Human Donor MSCs

The differentiation assays confirmed the multipotency of the isolated MSCs which displayed positive markers of (1) osteogenic differentiation; as seen by the positive alizarin red staining by MSCs cultured in osteogenic differentiation medium (Figure 4.2 (A)) and lack of staining in the negative control group (expansion medium) (Figure 4.2 (B)), (2) adipogenic differentiation; as seen by the positive Oil Red O stained lipid nodules with MSCs cultured in adipogenic induction medium (Figure 4.2 (C)) and lack of lipid staining in the negative control group (expansion medium) (Figure 4.2 (D)), and (3) chondrogenic differentiation, as seen by the positive sGAG

staining (Figure 4.2 (E)) compared to a lack of sGAG staining in the negative control group (expansion medium) (Figure 4.2 (F)).

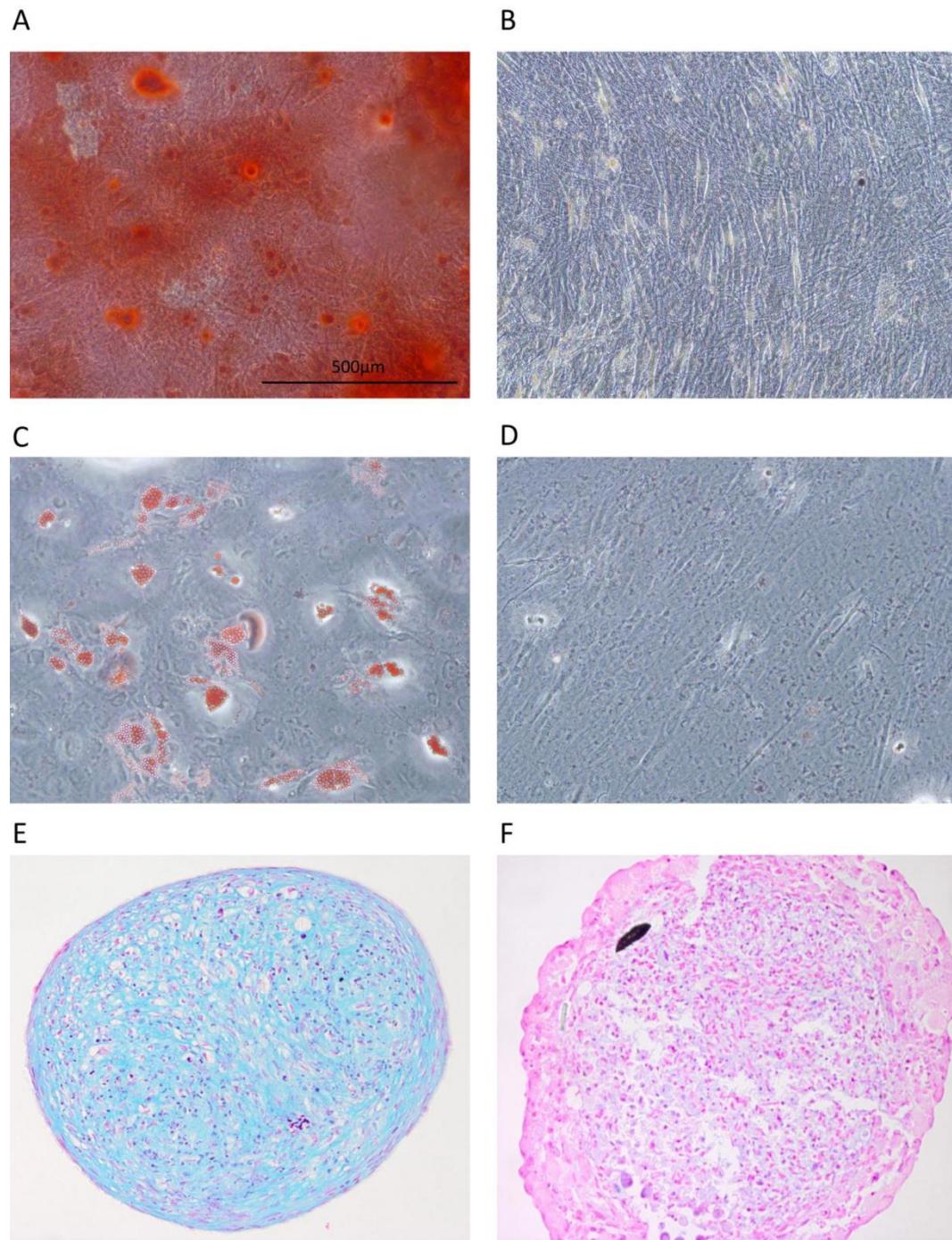


Figure 4.2: Characterisation of the multipotency of isolated human MSCs. Calcium deposition is confirmed by positive alizarin red staining by MSCs cultured in osteogenic differentiation medium (A), whereas no staining was observed in the

negative control (expansion medium) (**B**). Fat globules are confirmed by Oil Red O staining for MSCs cultured in adipogenic medium (**C**), but not in the negative control (expansion medium) (**D**). Staining with Alcian Blue for chondrocyte-associated glycosaminoglycan was positive within the aggregate cultured in chondrogenic medium (**E**), but was not seen in the negative control (expansion medium) (**F**).

4.3.2 Cell Number

There was no statistical difference in cell number for the CP21-HUVECs group over the course of the experiment. However, there was a significant increase ($p < 0.05$) in DNA content in both the CP21+HUVECs and CP21+HUVECs:MSCs groups from 1 week to 3 weeks into culture. The CP21+HUVECs:MSCs group had significantly higher DNA content than both the CP21-HUVECs ($p < 0.001$) and the CP21+HUVECs group ($p < 0.05$) at both 1 and 2 weeks into culture. Both the CP21+HUVECs and CP21+HUVECs:MSCs groups had significantly higher DNA content than the CP21-HUVECs ($p < 0.001$) after 3 weeks (see Figure 4.3).

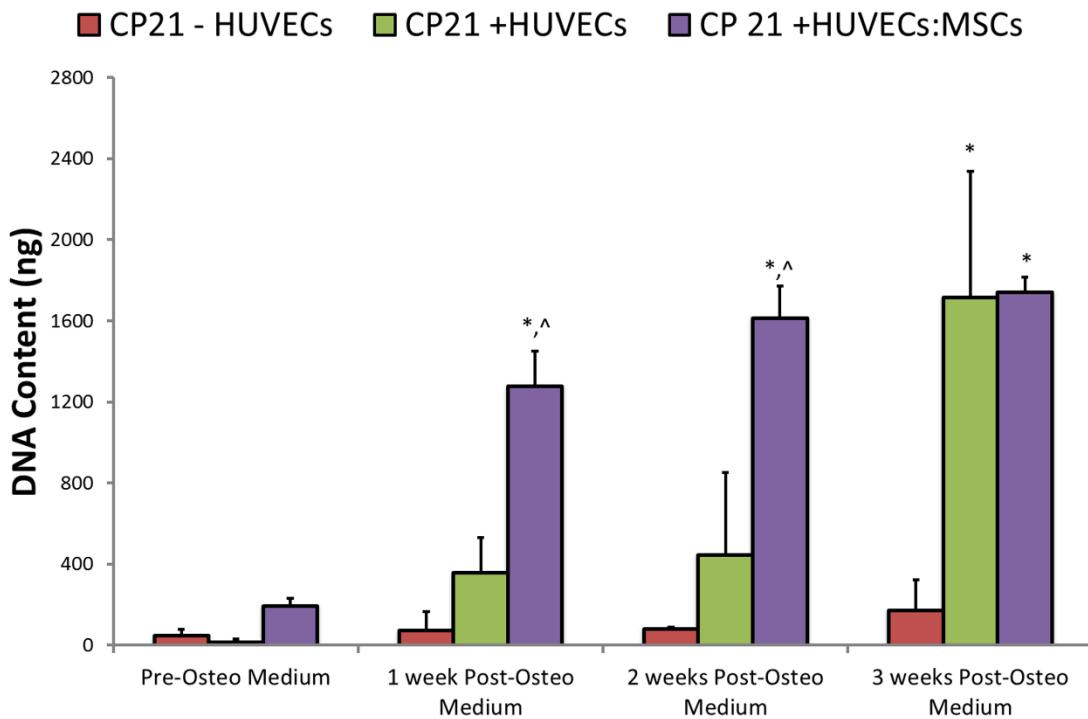


Figure 4.3: DNA content of the co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) compared with 21 days priming group alone (CP21-HUVECs) pre and 1, 2 and 3 weeks post the addition of HUVECs/MSCs ($n = 6$ samples per group per time point). $^{\wedge}p < 0.05$ vs. CP21 +HUVECs group and $*p < 0.05$ vs. CP21 -HUVECs. Error bars denote standard deviation.

4.3.3 Production of the Cartilage Template

4.3.3.1 Alcian Blue Staining

All three culture groups stained positive blue for sGAG 1 week into culture. HUVECs were still present around the periphery of the aggregate in the CP21+HUVECs group after 3 weeks in culture. However, an increase in size of the aggregate from 2 weeks ($105,914.426 \mu\text{m}^2$) to 3 weeks ($218,870.303 \mu\text{m}^2$) suggests that the HUVECs/MSCs were incorporated into the aggregate after 3 weeks in culture in the CP21+HUVECs:MSCs group (see Figure 4.4 (F) and Figure 4.4 (I)).

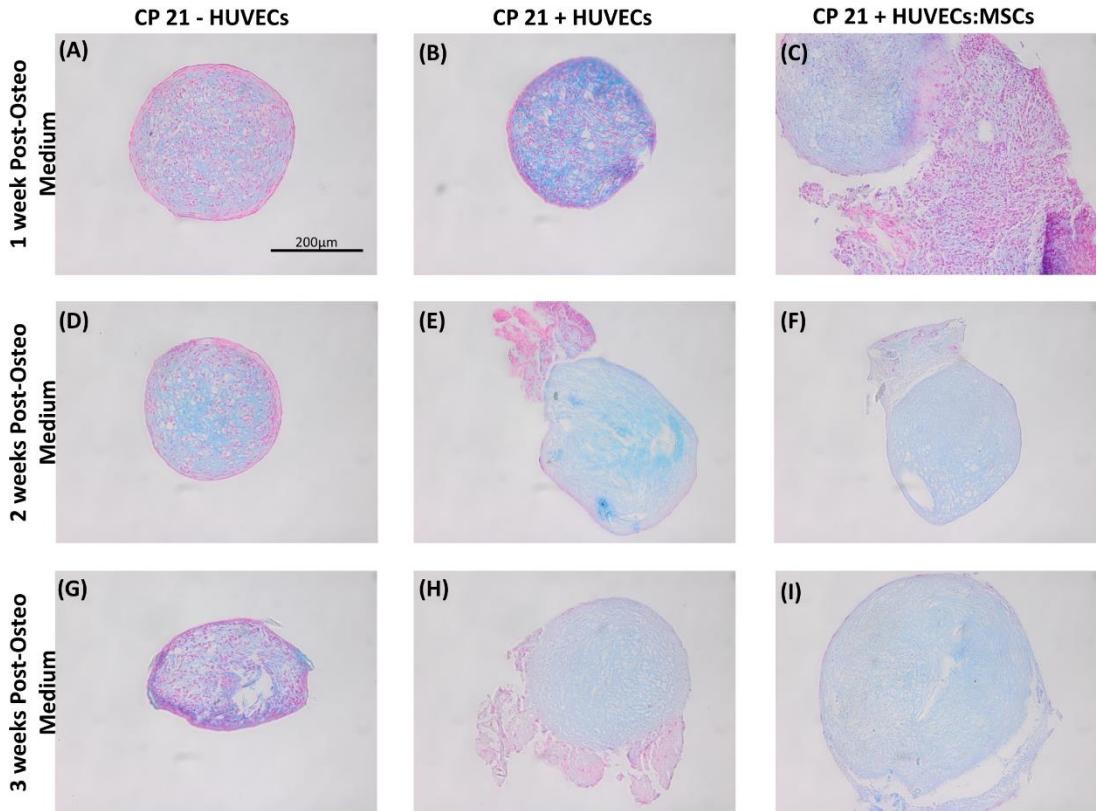


Figure 4.4: Alcian Blue staining of all co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) and 21 days priming group alone (CP21-HUVECs) over the course of the experiment. Each of the images was taken at a magnification of 20 X.

4.3.4 Mineralisation of the Cartilage Template

4.3.4.1 ALP Production

There was no statistical difference in ALP production between the groups at any time point (see Figure 4.5).

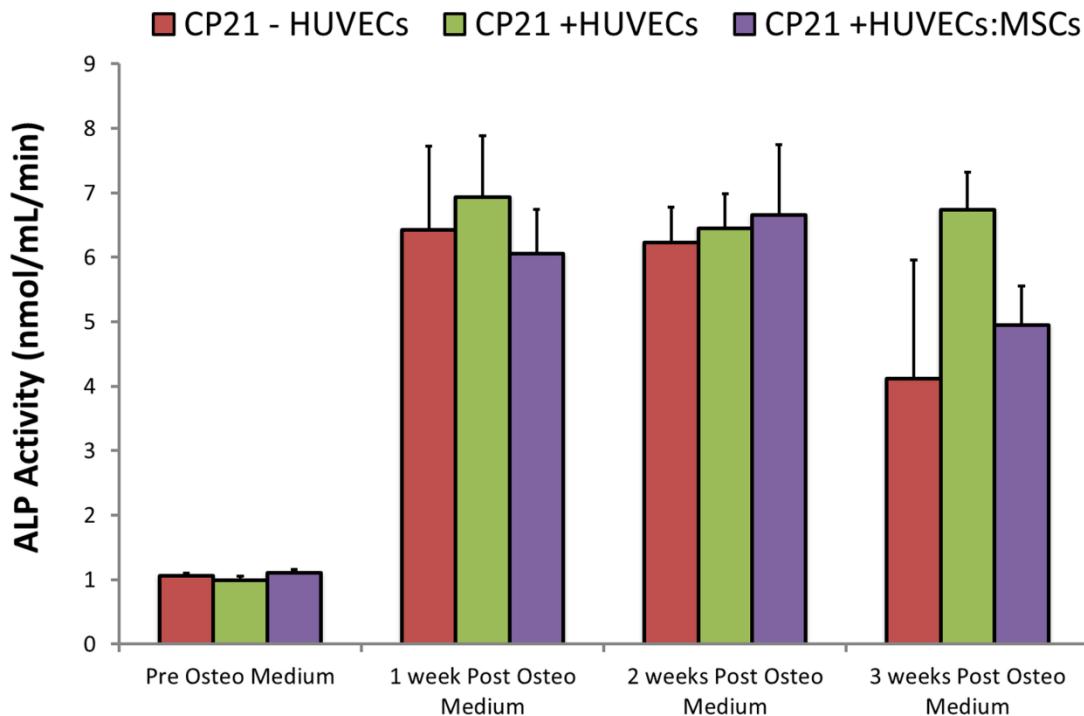


Figure 4.5: ALP Activity the co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) compared with 21 days priming group alone (CP21-HUVECs) pre and 1, 2 and 3 weeks post the addition of HUVECs/MSCs ($n = 6$ samples per group per time point). Error bars denote standard deviation.

4.3.4.2 Calcium Content

The CP21+HUVECs:MSCS group increased calcium production up to two weeks into culture, after which the calcium content began to plateau (see Figure 4.6). The CP21+HUVECs:MSCS group had significantly higher calcium content ($p < 0.05$) than both the CP21-HUVECs and CP21+HUVECs groups at both 1 week and 2 weeks into culture. By 3 weeks this significance was lost, however there was still a trend towards higher calcium content in both the CP21+HUVECs and CP21+HUVECs:MSCs groups compared to the control, although not statistically different ($p\text{-value} = 0.2$) (see Figure 4.6).

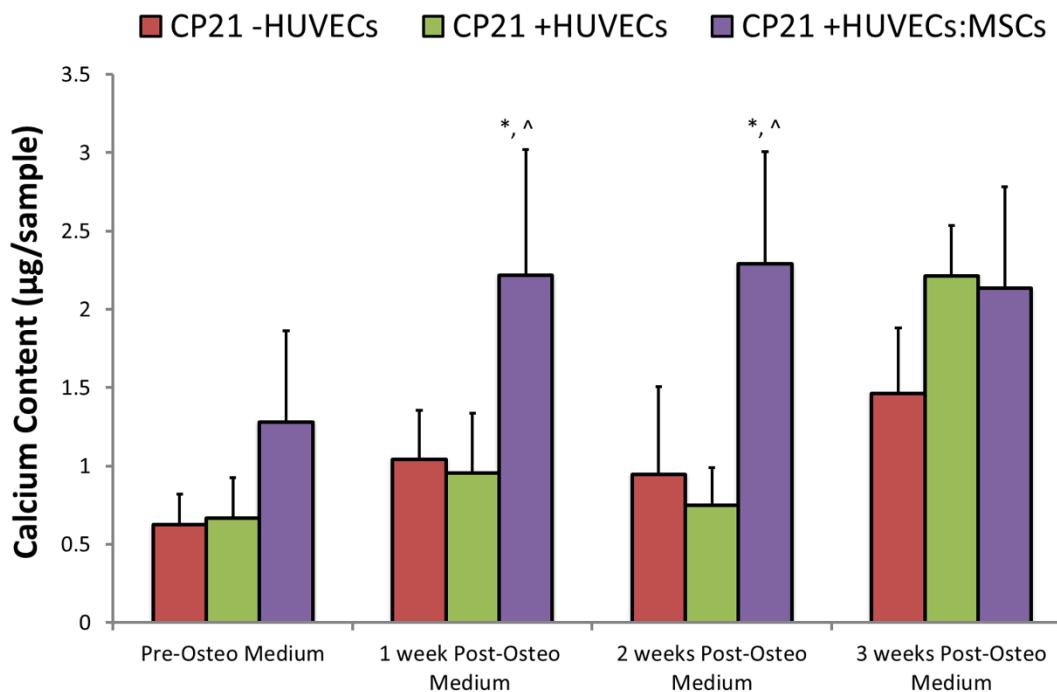


Figure 4.6: Calcium content for the co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) compared with 21 days priming group alone (CP21-HUVECs) pre and 1, 2 and 3 weeks post the addition of HUVECs/MSCs ($n = 6$ samples per group per time point). $^{\wedge}p < 0.05$ vs. CP21 + HUVECs group; $*p < 0.05$ vs. CP21 -HUVECs. Error bars denote standard deviation.

4.3.4.3 Alizarin Red

The highest amount of positive staining was seen in the CP21+HUVECs:MSCs group after 1 week into culture (see Figure 4.7 and Figure 4.8). By 3 weeks the highest amount of Alizarin Red staining was present in the CP21+HUVECs group. There was no significant difference in positive staining between any of the groups at any time point (see Figure 4.8). However, there was a difference in how the mineral was deposited. The mineral seen in the CP21 +HUVECs group was deposited throughout the aggregate, whereas in the CP21+HUVECs:MSCs group the mineralisation was

present in small discrete nodules throughout the aggregate. The least amount of Alizarin Red mineralisation at each time point was in the CP21–HUVECs group.

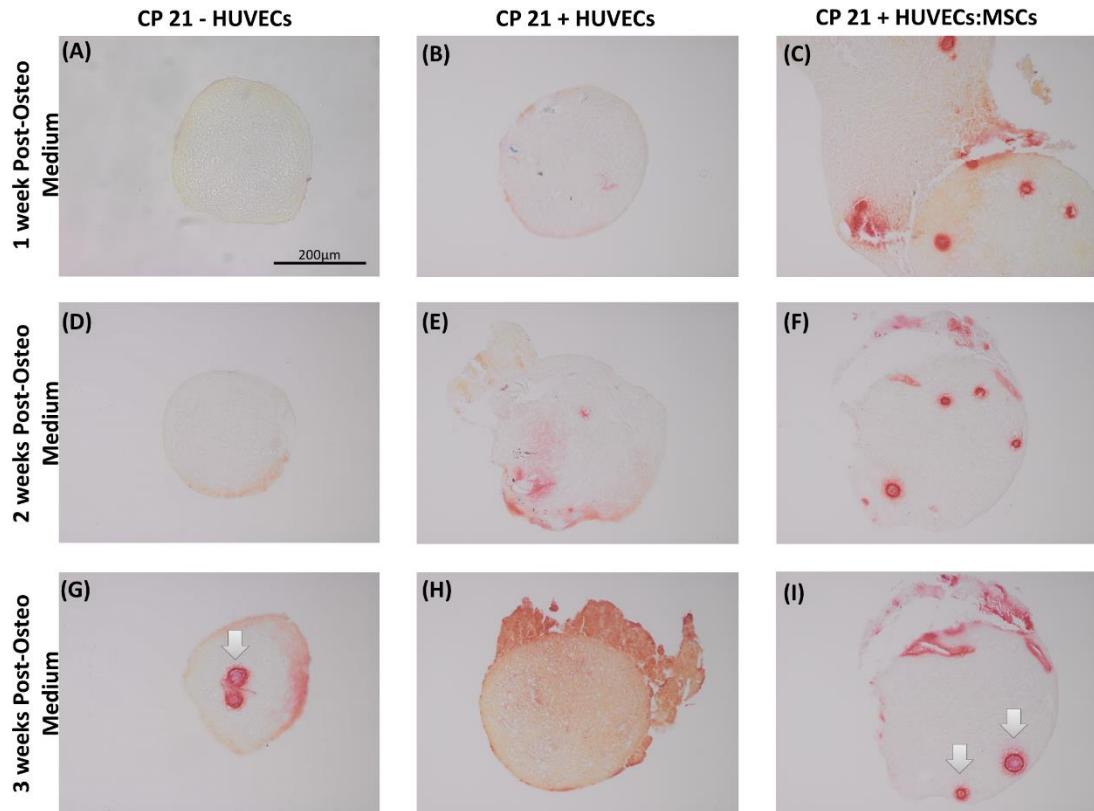


Figure 4.7: Alizarin Red staining of co-culture groups (CP21+HUVECs, CP21+HUVECs:MSCs) and 21 days priming group alone (CP21-HUVECs) over the course of the experiment. Arrows denote mineralisation nodules present in the cellular aggregates. Each of the images was taken at a magnification of 20 X.

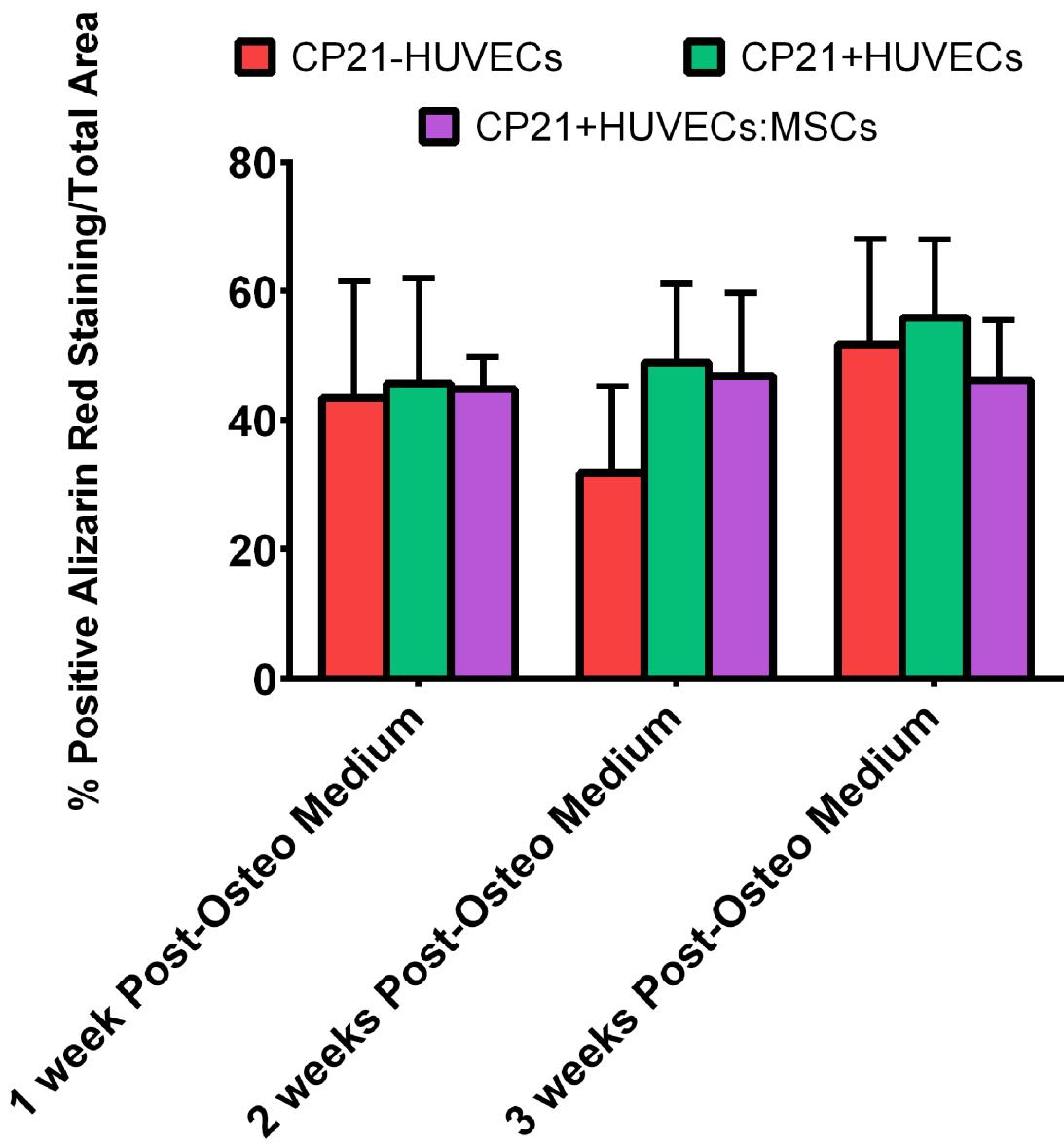


Figure 4.8: Quantitative Analysis of Alizarin Red Staining. Error bars denote standard deviation.

4.3.5 Vascularisation of the Cartilage Template

4.3.5.1 VEGF Production

The CP21+HUVECs:MSCs group had the highest amount of VEGF production at each time point. At both 2 and 3 weeks post addition of cells there was significantly ($p < 0.05$) higher VEGF content in the CP21+HUVECs:MSCs group compared to the

CP21+HUEVCs group. By 3 weeks the CP21+HUEVCs:MSCs group had significantly higher ($p < 0.01$) VEGF content compared to both of the other groups (See Figure 4.9).

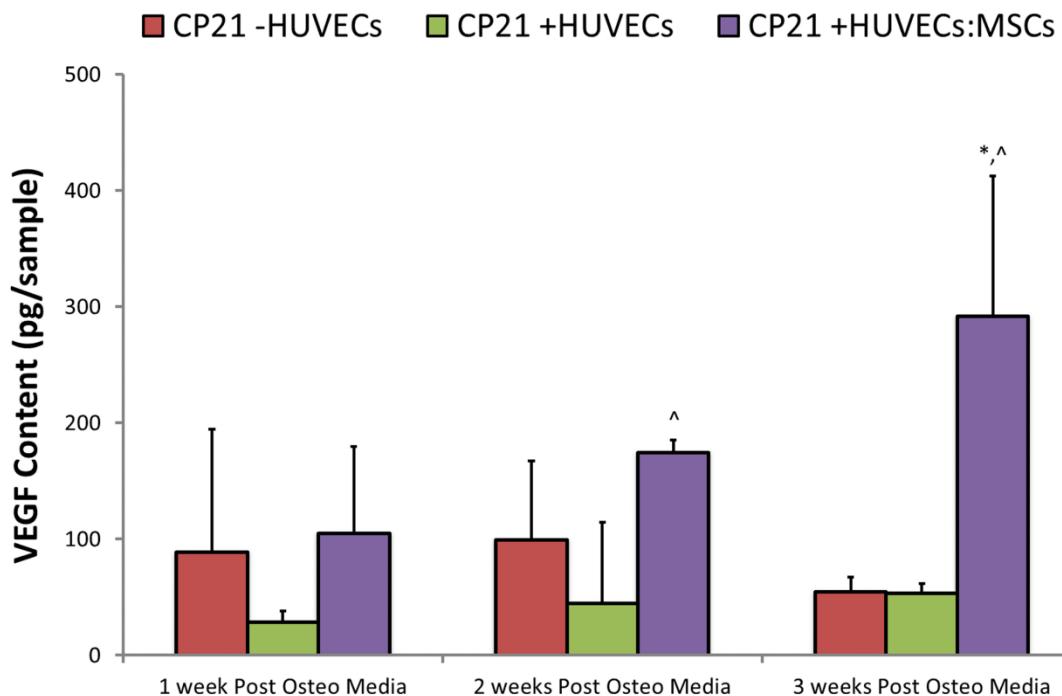


Figure 4.9: VEGF content the co-culture groups (CP21+HUEVCs, CP21+HUEVCs:MSCs) compared with 21 days priming group alone (CP21-HUEVCs) pre and 1, 2 and 3 weeks post the addition of HUEVCs/MSCs ($n = 6$ samples per group per time point). $^p < 0.05$ vs. CP21 +HUEVCs group; $*p < 0.05$ vs. CP21-HUEVCs. Error bars denote standard deviation.

4.3.5.2 CD31+ Staining

At all-time points there was a distinct lack of positive staining for CD31+ in the CP21-HUEVCs group, as expected there were no HUEVCs present. Positive staining for CD31+ was present around the periphery of the cartilage template in the CP21+HUEVCs group at all-time points (see arrows), but there was little infiltration into the cartilage template. However, with the CP21+HUEVCs:MSCs group there was

positive CD31+ staining on the periphery of the cartilage template 1 week into co-culture. By two weeks there was positive CD31+ staining within the template, and by 3 weeks into co-culture rudimentary vessels had formed within the template (see Figure 4.10). These rudimentary vessels were present in a series of sections throughout the aggregates (see Figure 4.11 (A-D)) with an average cross-sectional area of $220.89\mu\text{m}^2$ and average diameter of $3.79\mu\text{m}$ (see Figure 4.11 (E&F)).

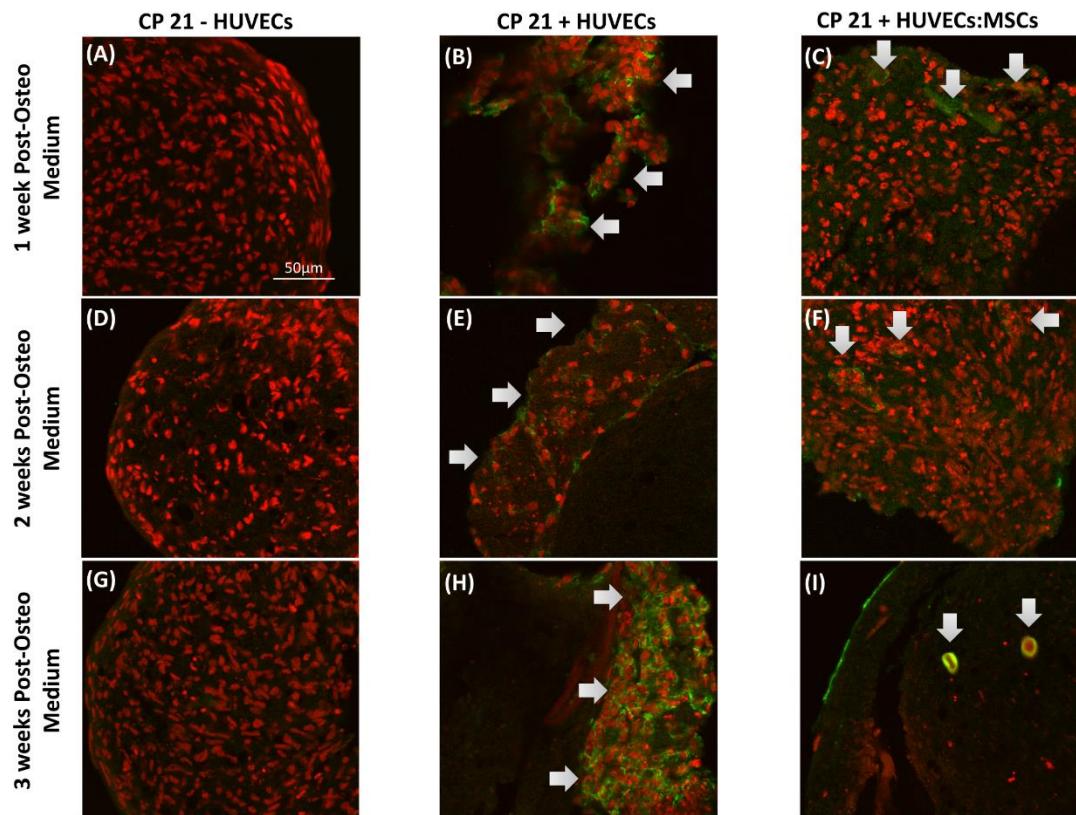


Figure 4.10: CD31 staining (green) of co-culture groups (CP21+HUEVCs, CP21+HUEVCs:MSCs) and 21 days priming group alone (CP21-HUEVCs) over the course of the experiment. Arrows denote positive CD31+ staining present in the cellular aggregates. Each of the images was taken at a magnification of 40 X. Nuclear counterstain: propidium iodide (red).

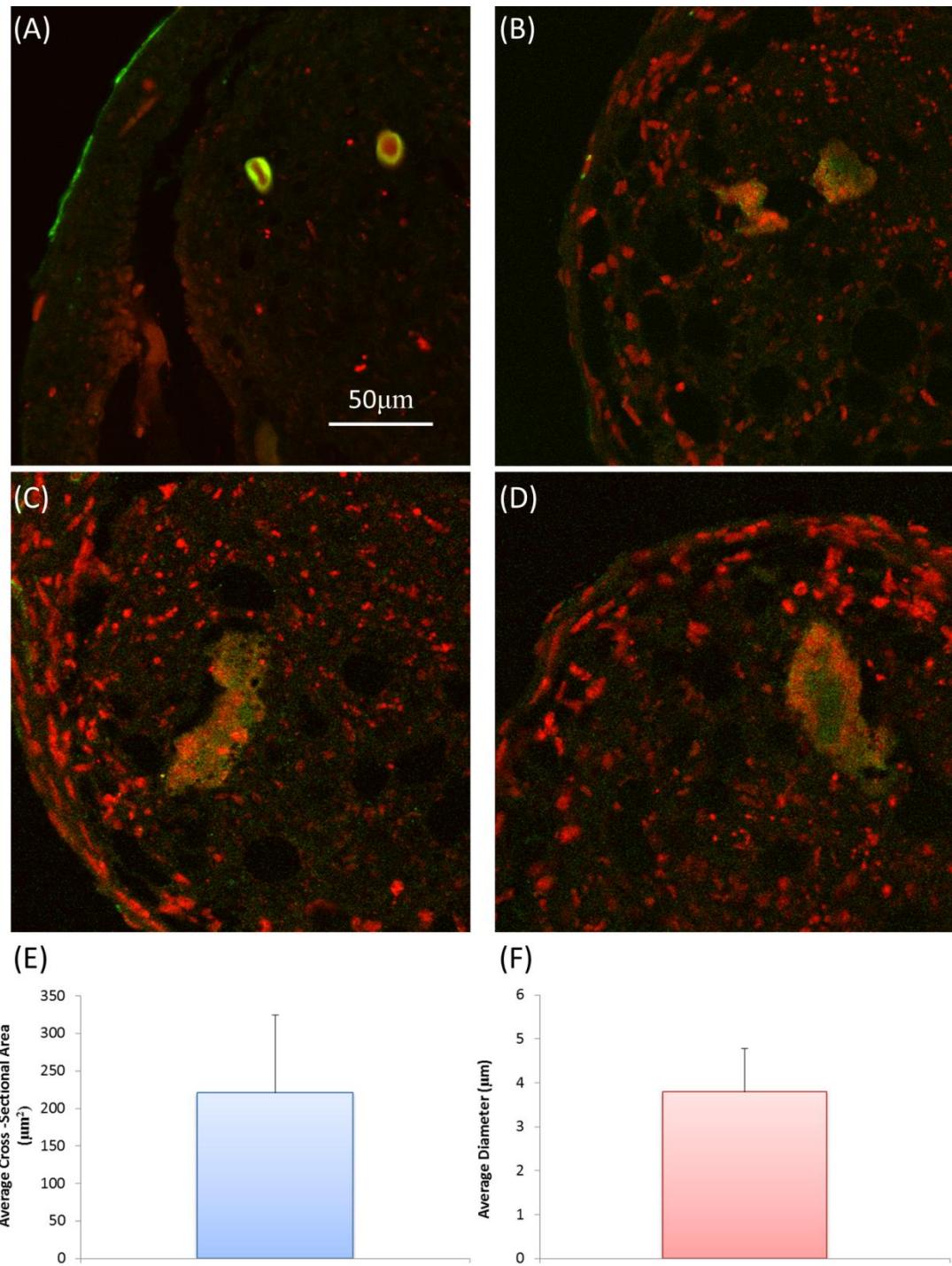


Figure 4.11: Representative images of CD31+ (green) stained sections showing the presence of rudimentary vessels (A-D). Each section is 8 μm thick and each of the images was taken at a magnification of 40X. Nuclear counterstain: propidium iodide

(red). Quantitative analysis of Cross-Sectional Area (**E**) and Vessel Diameter (**F**) of rudimentary vessels found. Error bars denote standard deviation ($n = 16$).

4.3.6 Production of Hypertrophic Cartilage Template

4.3.6.1 Collagen Type X Staining

There was a higher amount of positive staining for Collagen Type X in both CP21+HUEVCs and CP21+HUEVCs:MSCs groups compared to the CP21-HUEVCs group after 3 weeks into culture (Figure 4.12 (G-I), arrows). This collagen Type X was predominately seen around the periphery of the aggregates. The positive control stained chondrocytes were identified in the growth plate of long bones from a neonatal mouse model (Figure 4.12 (K)), and negative controls replacing primary antibody by normal serum were used (Figure 4.12 (J)) and did not show any specific staining.

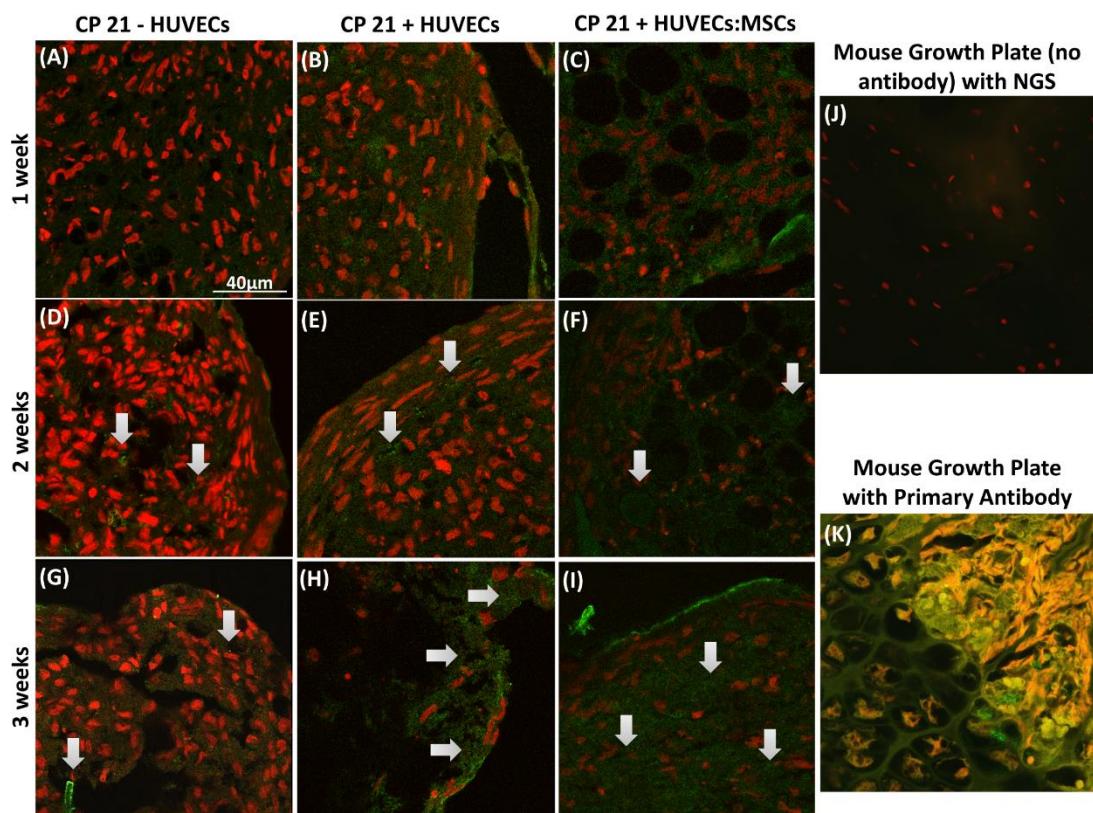


Figure 4.12: Collagen Type X staining (green) of co-culture groups (CP21+HUVECs (B, E, H), CP21+HUVECs:MSCs (C, F, I)) and 21 days priming group alone (CP21-HUVECs (A, D, G)) over the course of the experiment, compared to the positive control (neo-natal mouse long-bone) (K) and negative control (Blocking solution) (J). Arrows denote positive Collagen Type X staining present in the cellular aggregates. Each of the images was taken at a magnification of 40 X. Nuclear counterstain: propidium iodide (red).

4.4 Discussion

The results from this Chapter show that both chondrogenic priming (for a period of 21 days) and subsequent vascular priming can enhance the mineralisation potential of MSCs produced through chondrogenic priming alone. In particular, this Chapter

shows that a cartilage template provides a suitable platform for HUVECs/MSCs to attach, proliferate and infiltrate for up to 3 weeks in culture (as indicated by CD31+ staining). The results also show that an upregulation of VEGF expression occurs in the co-culture group when both MSCs/HUVECs are added compared to just HUVECs added and the non-co-culture group. This enhanced VEGF expression, seen with the MSCs/HUVECs added co-culture group, was further supported by the fact that it was the only group to have the formation of immature vessels. These rudimentary vessels were present in a series of sections throughout the aggregates (as seen through CD31+ staining). Interestingly, this Chapter also shows that the highest amount of mineralisation was seen when HUVECs alone were added to the cartilage template, whereas when both MSCs and HUVECs were added to the culture the mineralisation was reduced, albeit that vascularisation and the formation of rudimentary vessels was observed. Together these results suggest that there may be a trade-off between mineralisation and vascularisation for in vitro bone regeneration strategies for the durations investigated in this study.

One limitation of this study was that osteogenic and chondrogenic factors (ascorbic acid dexamethasone, β -glycerol and TGF- β 3) were introduced into the culture medium of MSCs to encourage MSC differentiation down specific pathways. As stated in Chapter 3 it was unlikely that these factors were present in the combinations used here during endochondral ossification *in vivo*. However, there is extensive research which has exposed MSCs to both chondrogenic and osteogenic differentiating agents for long durations (14 days to 5 weeks) and have demonstrated the long term viability and matrix production by MSCs (Thorpe et al., 2010; Farrell et al., 2011; Farrell et al., 2009; Vinardell et al., 2012; Haugh et al., 2011; Jaiswal et al., 1997b; Coleman et al., 2013; Cheng et al., 2009; Estes et al., 2010; Mauck et al., 2006;

Mauck et al., 2003). Furthermore, the control groups were exposed to the same factors, so the differences observed in osteogenesis are due to the combined strategy of chondrogenic priming and co-culture with HUVECs/MSCs. Another potential limitation is donor variability, specifically the age differences between the donors. While the age differences between the middle aged donors and young donor may confound these results, it was not feasible to obtain a large enough stock of human bone marrow MSCs from bone marrow aspirates alone to conduct all of the necessary experiments, involving numerous treatment groups and time-points. However, it should be noted that upon analysis of the data there was little variation between the aggregates formed from MSCs of middle aged donors and the young donor for any of the results (DNA content, Calcium content, etc.), and as such these findings of the importance of a combination of chondrogenic priming and vascular priming are deemed to be applicable to both younger and older donors. Moreover, the donors were chosen for their tri-potentiality potential. Therefore, the optimum conditions determined through this study were only optimum for bone marrow MSCs under the conditions mentioned. Future studies should investigate if the same is true for adipose derived MSCs or embryonic MSCs. Another limitation is the chosen cell ratio of HUVECs/MSCs to the already formed cellular aggregate. 250,000 cells were added to the already formed aggregate. Previous studies which looked at the direct co-culture of osteoprogenitor cells and endothelial cells found both mineralisation and angiogenic potential increased with decreasing MSC/HUVECs ratio till a ratio of 50:50 (Villars et al., 2002; Ma et al., 2011; Kaigler et al., 2005). However, in the CP21+HUVECs:MSCs group both MSCs and HUVECs were added to the already formed cartilage template, which lead to a ratio of 25:75. Other studies have found that the formation of prevascular networks was promoted through seeding 2% or fewer

(Rouwkema et al., 2006). This may account for the fact that prevascular networks were only found in the co-culture group with added HUVECs/MSCs however, further studies are needed to estimate the optimum co-culture ratios required for the promotion of prevascular networks and mineral formation.

The results from Chapter 3 showed that chondrogenic priming for 21 days lead to the formation of a cartilage-like template, and this is also further confirmed through the histological staining seen in this Chapter. However, the results also show that this cartilage template forms a suitable platform for HUVECs and MSCs to attach, proliferate and invade. Previous studies, have reported that chondrogenic priming of MSCs can enhance mineralisation both in vitro (Freeman et al., 2013; Farrell et al., 2009) and once implanted subcutaneously can enhance ectopic bone formation (Farrell et al., 2011; Farrell et al., 2009; Freeman et al., 2013; Scotti et al., 2013; Scotti et al., 2010; Visser et al., 2015; Gawlitta et al., 2015), however, some of these studies did not provide tissue appropriate for clinical use due to issues with core degradation and an uneven distribution of bone mineral throughout the construct (Farrell et al., 2009; Scotti et al., 2010; Farrell et al., 2011). Other studies have shown the clinical potential of chondrogenically primed rat MSCs for the repair of large bone defects, by implanting chondrogenically primed MSCs in either cellular aggregate form or seeded upon biomaterial scaffolds, and comparing the amount of bone formation, to that of constructs seeded with undifferentiated rat MSCs alone (Harada et al., 2014; van der Stok et al., 2014). None of these studies investigated both chondrogenic and vascular priming. The current Chapter shows for the first time that vascular priming also plays an important role in the mineralisation capacity of human MSCs. As such, the results of this Chapter suggest that a combination of chondrogenic and vascular priming is an effective strategy for osteogenic differentiation of human MSC aggregates in vitro.

Previous studies have shown that the co-culture of MSCs and HUVECs alone can enhance the osteogenic potential of MSCs when immediately put into direct co-culture (Fuchs et al., 2007; Saleh et al., 2011; Grellier et al., 2009a). However, these studies only looked at ALP activity, which is an early marker for bone formation and cannot be directly used to infer the likelihood of bone mineralisation, which is necessary to provide a construct with sufficient mechanical integrity. The results of this Chapter show for the first time the beneficial effect of vascular priming for encouraging the production of calcium, a crucial indicator of the formation of bone mineral in the aggregate. Interestingly, the results from this Chapter also show that the addition of both HUVECs and MSCs, compared to HUVECs alone, has a significant effect on mineralisation location within the aggregates. The highest amount of mineralisation is seen when HUVECs are added to the cartilage template alone, and it is the only aggregate to have mineralisation throughout the aggregate. Previous chondrogenic priming studies have been limited by mineralization only occurring around the periphery or in the core alone, but not throughout the aggregate (Farrell et al., 2009; Scotti et al., 2010). Interestingly, when HUVECs were added to the culture alone there was indeed mineralisation throughout the aggregate rather than just around the periphery, as was shown in Figure 4.7. However, when both MSCs and HUVECs were added to the culture the mineralisation was reduced, compared to the addition of HUVECs alone, and was characterised by the formation of discrete mineralised nodules rather than homogenous mineralisation throughout the aggregate. It is important to note that even though the co-culture group with MSCs and HUVECs had less mineralisation than the group with HUVECs added alone, it still had higher mineralisation and more nodules than the control. The author proposes that, mineralisation did not proceed during the process of vessel formation and as result

mineralisation did not initiate until later for this group. Therefore, it is possible that culturing this group *in vivo* after both the chondrogenic and vascular priming period *in vitro* will ultimately allow for enhanced mineralisation. This will be investigated further in Chapter 6.

Hypertrophy and the formation of vasculature networks, by a process known as quiescent angiogenesis, is the next stage of endochondral ossification and both events proceed bone formation by means of this process *in vivo* (Mackie et al., 2008; McNamara, 2011; Kronenberg, 2003; Gerber and Ferrara, 2000). In this Chapter the results show that both co-culture groups had begun to undergo hypertrophy as the highest amount of Collagen Type X is seen in these groups after 3 weeks in culture. Interestingly, the highest amount of Collagen Type X staining was present around the periphery of the aggregate and not in the centre. *In vivo* (Gerber and Ferrara, 2000), the onset of angiogenesis leads to the hypertrophy of the cartilage template so the addition of the HUVECs to the periphery of the cartilage template might attribute to the cells around the periphery to become hypertrophic. Previous studies (Leboy et al., 1989) have also found that the co-culture of human MSCs and chick chondrocytes reduces the potential of the chondrocytes to become hypertrophic. Therefore, the reduction of Collagen Type X in the co-culture group with HUVECs and MSCs seen in this study may be due to the addition of undifferentiated MSCs. However, further studies are needed to verify this.

The addition of the HUVECs or HUVECs and MSCs had a significant effect not only on the mineralisation potential of the MSCs but also on the VEGF expression. VEGF has been shown to be the marker to stimulate vascular cells to undergo the formation of early vessels (Gerber and Ferrara, 2000; Hans-Peter et al., 1999; Fiedler

et al., 2005; Mayr-Wohlfart et al., 2002; Nakagawa et al., 2000; Farrell et al., 2009) and previous studies have postulated that vasculogenesis should be induced prior to osteogenesis in vitro in order to obtain functional bone tissue in vivo (Correia et al., 2011). However, which cells are expressing the VEGF is still unknown, further studies are needed to elucidate this. Therefore, even though the co-culture group with MSCs/HUVECs had less mineralisation than that with HUVECS alone, it was the only group to form immature vasculature structures with an average diameter (Kusumbe et al., 2014) and structure (Buga et al., 2014; Izquierdo et al., 2009) similar to those seen in vivo of early arterioles. It also had the highest expression of VEGF, and as such might have the best potential for host integration and mineralisation once implanted in vivo, however this will be discussed further in Chapter 6.

4.5 Conclusions

The results of this Chapter show that both chondrogenic priming (for 21 days) and co-culture of MSCs and HUVECs can significantly increase the osteogenic potential produced through chondrogenic priming alone. Moreover, these results show that the formation a cartilage-like template provides a suitable platform for HUVECs/MSCs to attach, proliferate and infiltrate for up to 3 weeks. More importantly, the results show that both MSCs and HUVECs must be added to the formed cartilage template for the formation of immature vessels. Taken together, these results indicate for the first time, that the application of both chondrogenic and vascular priming of MSCs enhances the mineralisation potential of MSCs, compared to chondrogenic priming alone in vitro, whilst also allowing for vessel formation. This

co-culture model is the same model which will be further investigated both Chapter 5 and Chapter 6.

Chapter 5: Will both Chondrogenic and Vessel Priming obviate the need for osteogenic supplements to induce osteogenesis in vitro?

5.1 Introduction

The standard procedure to induce osteogenic differentiation of MSCs in vitro is through the culture of the cells in the presence of a cocktail of dexamethasone (dex), ascorbic acid (asc) and β -glycerolphosphate (β -glyc). (Song et al., 2009; Tenenbaum and Heersche, 1985; Cheng et al., 1994; Maniatopoulos et al., 1988; Jaiswal et al., 1997a; Leboy et al., 1991; Kamalia et al., 1992; Herbertson and Aubin, 1995; Peter et al., 1998). Dex is a steroid that causes MSC differentiation into osteoblasts by activating the signalling pathway (WNT/ β -catenin), which in turn activates Runx2 expression and induces the differentiation of MSCs into immature osteoblasts (Langenbach and Handschel, 2013; Komori, 2010; Hamidouche et al., 2008). However, dex alone is not sufficient to induce in vitro mineralisation (Langenbach and Handschel, 2013). Asc acts as a co-factor for enzymes that hydroxylate proline and lysine into collagen (Vater et al., 2011), participates in collagen chain formation (Franceschi and Iyer, 1992). It is the predominant regulator of collagen type 1 secretion (Langenbach and Handschel, 2013). β -Glyc is an inorganic phosphate needed to produce hydroxyapatite mineral and has been shown in many studies to play an important role in the osteogenic differentiation of MSCs (Tenenbaum, 1981;

Tenenbaum and Heersche, 1982; Ecarot-Charrier et al., 1983; Maniatopoulos et al., 1988). It also regulates expression of genes including osteopontin and BMP-2 (Foster et al., 2006; Fatherazi et al., 2009; Tada et al., 2011). Exposure of dex, asc, and β -glyc to rat MSCs (Kamalia et al., 1992; Leboy et al., 1991; Maniatopoulos et al., 1988; Herbertson and Aubin, 1995; Peter et al., 1998), human MSCs (Song et al., 2009; Cheng et al., 1994; Jaiswal et al., 1997a), or murine osteoblasts (Franceschi and Iyer, 1992; Franceschi et al., 1994) can significantly increase ALP activity in vitro. In vivo none of these growth factors naturally circulate, but bone progenitors and cells themselves produce various factors that can induce osteogenic differentiation. Recent studies have investigated the physical and chemical signalling that occurs due to the culture of MSCs with other cell types, including chondrocytes, endothelial cells, osteoblasts and osteocytes. One such study confirmed for the first time the synergistic relationship between osteocytes and osteoblasts in stimulating osteogenic differentiation of MSCs (Birmingham et al., 2012). However, to date the knowledge about MSC behaviour, particularly the interactions between MSCs and endothelial cells within the stem cell niche in vivo, remains largely unknown (da Silva Meirelles et al., 2008; Jones and McGonagle, 2008; Augello et al., 2010; Tsai et al., 2015; Chan et al., 2009). During endochondral ossification MSCs, endothelial stem cells, and chondrocytes all reside in close proximity within the cartilage template prior to the invasion by osteoblast bone cells and bone formation. Therefore an in vitro co-culture methodology involving MSCs, endothelial cells and chondrocytes might replace the need for osteogenic supplements to induce osteogenesis in vitro in a 3D culture environment, but this has not been established.

The anatomical location of MSCs and vascular endothelial cells suggests that these two cell types are in direct cell-cell interaction and/or paracrine signalling within

the stem cell niche during endochondral ossification. Previous in vitro studies have shown that direct co-culture of MSCs or osteoblasts with endothelial cells can upregulate production of the early osteogenic marker ALP (Villars et al., 2000; Villars et al., 2002; Guillotin et al., 2008; Zhao et al., 2012), without the presence of osteogenic supplements. Other studies have investigated whether co-culture of MSCs and endothelial cells can increase ALP production in 3D polymer scaffolds (Sun et al., 2007; Pedersen et al., 2013) or 3D and cellular aggregates (Rouwkema et al., 2006; Saleh et al., 2011; Liao et al., 2011; Fuchs et al., 2007), but the majority of these were in the presence of osteogenic growth supplements (Saleh et al., 2011; Rouwkema et al., 2006; Liao et al., 2011; Fuchs et al., 2007). The co-culture of MSCs or osteoblasts with chondrocytes have beneficial effects on ALP production in both 2D and 3D culture (Jiang et al., 2005; Giovannini et al., 2010; Nakaoka et al., 2006). One study investigated the effect of co-culture of human MSCs and chondrocytes without the use of osteogenic supplements, and found there was no ALP production/expression in 3D aggregate culture (Giovannini et al., 2010). However, direct 2D co-culture of rat osteoblasts and bovine chondrocytes reported a significant increase in ALP production over a period of 6 weeks and there was significantly higher ALP activity in the co-culture group compared to the osteoblast group alone (Nakaoka et al., 2006). Co-culture of MSCs and endothelial cells through transwell inserts has been shown to induce MSCs to undergo both osteogenesis and chondrogenesis through the endothelial-1 phosphatidylinositol 3-kinase/AKT (AKT) signalling pathway (Tsai et al., 2015). However, the exact signalling pathway that ensues due to the direct interaction of these cells during the endochondral ossification process *in vivo* has not been fully identified. In Chapter 4, a novel co-culture technique was investigated, which involved the co-culture of HUVECs and MSCs with MSCs that have been pre-

differentiated towards the chondrogenic lineage. This study showed that the HUVEC/MSC co-culture group did not produce as much mineral as co-culture with HUVECS alone. However, this approach involved the use of osteogenic supplements, which might interfere with the signalling occurring due to the direct co-culture. Therefore, an approach that implements co-culture of MSCs, HUVECS and chondrogenic cells in the absence of osteogenic growth factors requires exploration.

This study tests the hypothesis that an in vitro bone regeneration strategy that mimics the cellular niche of the endochondral template will provide an alternative strategy for in vitro mineralisation of MSCs, and thereby obviate the need for external osteogenic growth factors. The overall objective was to establish the mineralisation and vessel formation potential of **(a)** chondrogenic priming of MSCs, **(b)** addition of HUVECs alone to chondrogenic MSC aggregates, and **(c)** a novel methodology involving both chondrogenic priming and the co-culture of HUVECs and MSCs all without the use of any osteogenic supplements and compare them to **(d)** the same experimental groups, which were cultured in the presence of osteogenic supplements (results from Chapter 4) and to the **(e)** non co-culture group cultured in the presence of osteogenic growth factors alone. These studies involved the use of biochemical (DNA, ALP, Calcium and VEGF) assays and histological (Alcian Blue and Alizarin Red) and immunohistological (CD31+) staining.

5.2 Methods

5.2.1 Cell Culture

5.2.1.1 Human MSC Culture

Bone marrow-derived human mesenchymal stem cells (MSCs), harvested from two male donors 20-25 years old with established multi-potency, were purchased from the Texas A&M University Health Science Centre (Temple, TX). The human MSCs were expanded in Minimum Essential Medium alpha (α -MEM, Invitrogen, Carlsbad, CA) containing 16.7% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 100 units/mL penicillin, 100 μ g/mL streptomycin, 2 mM L-glutamine (PSL, Invitrogen) at 37 °C and 5% CO₂. For all cell culture performed in this study, cell culture medium was changed twice weekly unless stated otherwise. At passage 2, cells from each donor were detached using 0.25% trypsin-EDTA (Invitrogen) and combined 1:1 to produce a pooled human MSC population. MSCs were further cultured to passage 3-4.

5.2.1.2 HUVECs Culture

HUVECs were purchased from Lonza (Maryland, USA) and cultured in Clonetics Endothelial Growth Medium (EGM) SingleQuotes (Lonza). Medium was replaced every 3 days and upon reaching 80-90% confluency, cells were passaged using trypsin-EDTA solution. HUVECs were further cultured to passage 3.

5.2.2 Aggregate Formation

As described in Chapter 3 and 4 aggregates of 250,000 cells/aggregate were formed by centrifuging at 400 g for 5 minutes at 22 °C. For this study all aggregates

were formed from pooled cells. Carefully avoiding the newly formed aggregate, the medium was removed from each of the aggregates and 0.5 mL of Chondrogenic medium was added (as described in Chapter 3 and 4) [high glucose DMEM, 10 ng/mL TGF- β 3 (Invitrogen), 50 μ g/mL Ascorbic Acid, 4.7 μ g/mL Linoleic Acid, 100 nM Dexamethasone and 1x insulin-transferrin-selenium]. The EGM medium was the same medium that was used to culture the HUVECs (Lonza). For all experiments, aggregate cultures were fed twice per week by performing a 50% medium exchange. During each feed the aggregates were agitated so as to prevent them from adhering to the micro-tube.

These cells were further cultured under the same experimental conditions that were used in Chapter 4: (1) *CP21–HUVECs*: Chondrogenic priming (no cells added) for 21 days; (2) *CP21+HUVECs*: Chondrogenic priming for 21 days and addition of HUVECs (250,000 cells); (3) *CP21+HUVECs:MSCs*: CP for 21 days and co-culture of HUVECs and MSCs at a ratio of 1:1 (125,000:125,000 cells).

As described in Chapter 4, the HUVECs/MSCs were added by suspending the cells depending on experimental conditions so that there was 0.5×10^6 cells/mL. In the case of the CP21+HUVECs:MSCs, the ratio of cells was 1:1 HUVECs:MSCs. The cells were suspended in EGM medium alone (no osteogenic supplements were added) and 20% methocel from a stock solution that was generated by dissolving 6 g of carboxymethylcellulose (Sigma Aldrich) in 500 mL of DMEM as described in Chapter 4. After 24 hours the medium that contained methocel was removed and was replaced with EGM medium and the aggregates were cultured for a further 21 days.

5.2.3 Histochemical analysis and Biochemical Analysis

Aggregates were examined at Day 21 (pre-addition of cells), 1 week, 2 weeks and 3 weeks after the addition of the cells. Aggregates were prepared for either histochemical analysis or biochemical analysis. As described in Chapter 3 and 4, at each of the time points, the culture medium from the aggregates was collected, frozen and stored at -80°C until biochemical assays could be performed. The remaining aggregates were washed with PBS and then treated in one of the following two ways; (1) frozen and stored at -80°C for biochemical analysis or (2) fixed overnight in paraformaldehyde before being placed in PBS and refrigerated for histochemical analysis. For the above study, two independent experiments were carried out with at least two repeats in each experiment ($n = 4$ for histological analysis and $n = 10$ for biochemical analysis).

5.2.3.1 Quantitative Biochemical Analysis

5.2.3.1.1 DNA Content

To assess DNA content, aggregates were papain digested as previously described in Chapter 3 and Chapter 4. Briefly 500 µL of Papain digest was added to the aggregates and aggregates were placed in an oven at 60°C overnight. Once the aggregates were digested DNA content was performed using Picogreen DNA assay (BD Biosciences) with calf thymus DNA (Sigma Aldrich) as a standard. In minimal light 40 µL of papain digest of the sample/standard was added to a 96-well plate in triplicate. To this 200 µL of working solution (1X Tris EDTA (TE) Buffer and 5 µL/mL Picogreen solution (Bio-Sciences)) was added. The plate was incubated away from light for five minutes and then read on a microplate reader (Perkin Elmer HTS

7000 Microplate Reader) at an excitation wavelength of 485 nm and emission of 535 nm.

5.2.3.1.2 ALP Production

Extracellular ALP production was determined using a colorimetric assay of enzyme activity, which uses *p*-nitrophenyl phosphate (*p*NPP) as a phosphatase substrate with ALP enzyme (Sigma Aldrich) as a standard. Solutions of 2-Amino-2 Methyl-1-Propanol (AMP) (1.5 M with a pH 10.25), pNPP (20 mM), and Magnesium Chloride (MgCl₂) (10 mM) were made up. Each of these solutions was combined in a ratio of 1:1:1 to make up the substrate working solution (AMP-pNPP-MgCl₂). Next 50 µL of the medium was added to a 96-well plate in triplicate with 50 µL of substrate working solution. The samples were shielded from direct light at 37°C for one hour. After this, 100 µL of Stop Solution (1M NaOH) was added to the wells and the plate was read at 405 nm in a micro-plate reader.

5.2.3.1.3 Calcium Content

Calcium deposition within the aggregates was measured using Arsenazo III Calcium Reagent (Sekisui Diagnostics, UK) according to the manufacturer's protocol. Briefly aggregates were digested by adding 0.5 mL of 1 M Acetic Acid and the solution was rotated overnight in a cold room. If the samples were not fully digested, the samples were homogenised until fully digested. Next 25 µL of each of the digested samples and assay standard was added to a 96-well plate and 300 µL of the calcium reagent was added. The plate was incubated for 30 seconds at room temperature and analysed on a microplate reader at an absorbance of 650 nm.

5.2.3.1.4 Enzyme-linked Immunosorbent Assay (ELISA) for Vascular Growth Factor

As described in Chapter 4 an ELISA was used in order to quantify the levels of VEGF expressed by the aggregates. The cell culture medium was collected and analysed at the specific time points. Assays were carried out according to the manufacturer's protocol (R&D Systems) and analysed on a microplate reader at a wavelength of 450 nm.

5.2.3.2 Histology

All samples were sectioned with a thickness of 8 µm using a rotary microtome (Leica microtome, Leica). Sections were stained with 1% Alcian Blue 8GX solution for sGAG and 2% Alizarin Red solution for mineralisation as previously described in Chapter 3 and 4.

5.2.3.3 CD31 Immunohistochemical Analysis

Immunohistochemical analysis was used to detect CD31 staining, as previously described in Chapter 4. Briefly, sections were deparaffinised overnight before a series of rehydration steps. The samples were then treated with 40 µg/mL of proteinase K for 20 minutes at 37°C (Sigma Aldrich), rinsed with PBS-Tween and blocked with PBS with 1% w/v Bovine Serum Albumin (BSA) and 3% w/v Normal Goat serum (NGS) (Sigma Aldrich) for 60 minutes. Sections were then incubated overnight at 4 °C with rabbit polyclonal anti-CD31 (ab28364 Abcam, 1:50). After three washing steps with PBS with 1% w/v BSA the sections were incubated with Dylight488 goat anti-Rabbit secondary antibody (Jackson Immunoresearch, 115-485-209, 1/200), for one hour at room temperature in the dark. Finally samples were washed three times with PBS with 1% w/v BSA the sections were mounted using 4',6-diamidino-2-

phenylindole (DAPI) mounting medium (Sigma Aldrich). HUVECs provided a positive control for CD31 staining.

5.2.4 Statistical Analysis

Results are expressed as mean \pm standard deviation. For all the biochemical analysis two-way analysis of variance (ANOVA) with time and medium type as the independent factors followed by a pair-wise multiple comparison procedure (Tukey's HSD test) was used to test for significance. For quantitative analysis on vessel cross-sectional area a standard student t-test was performed. All analyses were performed with Graphpad. For all comparisons, the level of significance was $p \leq 0.05$.

5.3 Results

5.3.1 DNA Content

There was no statistical difference in cell number for the CP21-HUVECs (-OM) group over the course of the experiment (see Figure 5.1). However, there was a significant increase ($p < 0.05$) in DNA content in the CP21+HUVECs (-OM) group from 2 weeks to 3 weeks, and in the CP21+HUVECs:MSCs (-OM) group from 1 week up to 3 weeks into co-culture. The CP21+HUVECs:MSCs (-OM) group had significantly higher DNA content than both the CP21-HUVECs (-OM) ($p < 0.001$) and the CP21+HUVECs (-OM) groups ($p < 0.001$) at all-time points. The CP21+HUVECs (-OM) group had significantly higher DNA content than the CP21-HUVECs (-OM) group ($p < 0.01$) after 3 weeks.

There was no difference in DNA content in the CP21-HUVECs group cultured with osteogenic supplements compared to the same group cultured without relevant supplements. However, at both 2 and 3 weeks the CP21+HUVECs:MSCs group cultured with osteogenic supplements (+OM) had significantly higher ($p<0.05$, $p<0.0001$ respectively) DNA content than the same group cultured without osteogenic supplements (-OM). Moreover, the CP21+HUVECs group cultured with osteogenic supplements (+OM) had significantly higher ($p < 0.0001$) DNA content than its counterpart cultured without osteogenic supplements (-OM) after 3 weeks of co-culture.

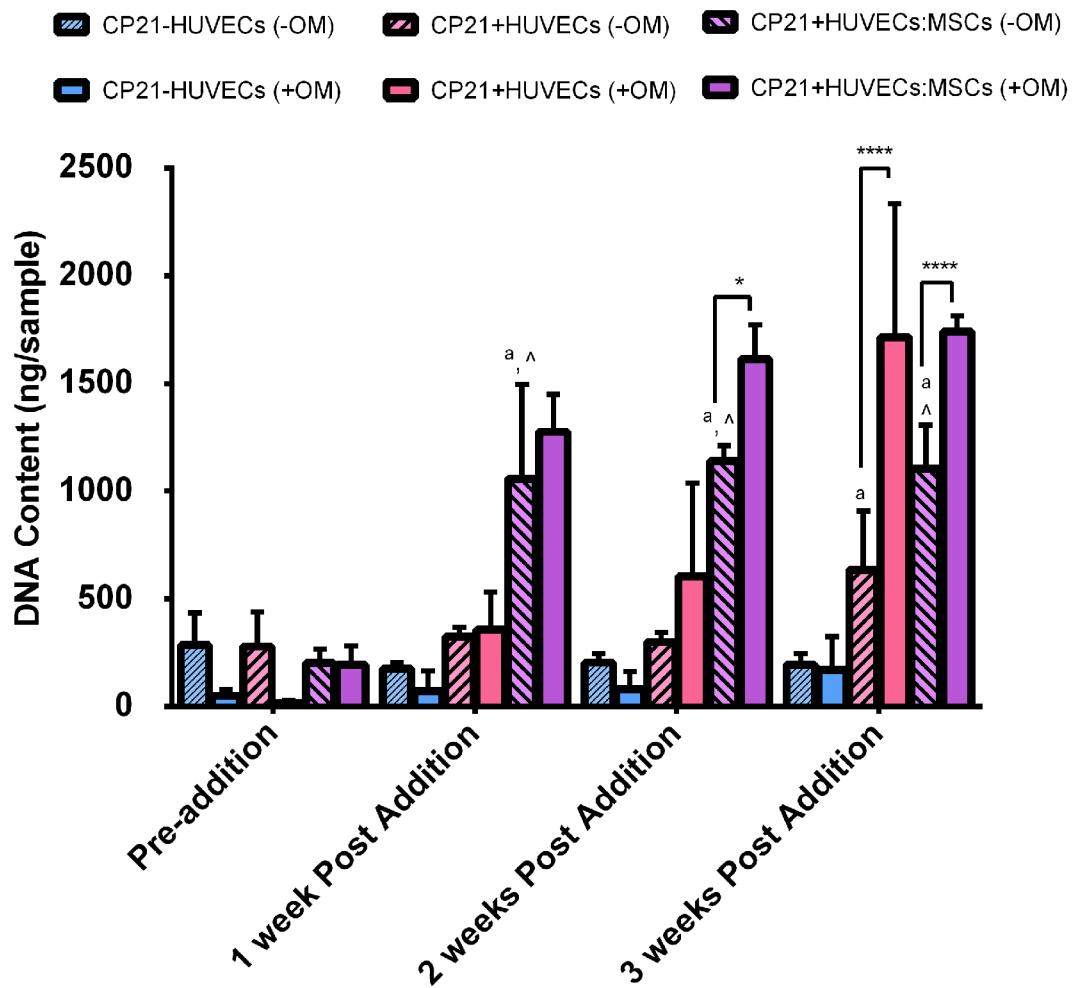


Figure 5.1: DNA content/sample of all three experimental conditions cultured without osteogenic supplements (-OM) compared to the results from Chapter 4 of the same groups cultured in the presence of osteogenic supplements (+OM). ^ap < 0.05 vs. CP21 +HUVECs group, [^]p < 0.05 vs. CP21 -HUVECs. *p < 0.05, and ****p < 0.0001. (n = 6 samples per group per time point). Error bars denote standard deviation.

5.3.2 Production of the Cartilage Template

5.3.2.1 Alcian Blue Staining

All three culture groups cultured without osteogenic factors stained positive blue for sGAG 1 week into culture and after 3 weeks of culture (see Figure 5.2). Alcian

blue staining also showed an increase in the size of the aggregate for both the CP21+HUVECs (-OM) ($82,456.717 \mu\text{m}^2$ to $92,934.557 \mu\text{m}^2$) and CP21+HUVECs:MSCs (-OM) ($103,440.097 \mu\text{m}^2$ to $118,857.341 \mu\text{m}^2$) groups from 1 week to 3 weeks of co-culture. This suggests that the HUVECs/MSCs that were added were incorporated into the aggregate after 3 weeks into co-culture.

In the CP21+HUVECs group cultured in osteogenic medium (+OM), HUVECs were present around the periphery of the aggregate throughout the course of the experiment (see arrows, Figure 5.2). However, in the CP21+HUVECs group cultured without osteogenic supplements (-OM) the HUVECs invaded the cartilage template by 2 weeks. The size of aggregates also differed, the CP21+HUVECs:MSCs aggregates cultured in osteogenic medium (+OM) had a larger cross-sectional area ($218,870.303 \mu\text{m}^3$) than its counterpart cultured without osteogenic supplements (-OM) ($118,857.341 \mu\text{m}^2$) after 3 weeks of co-culture.

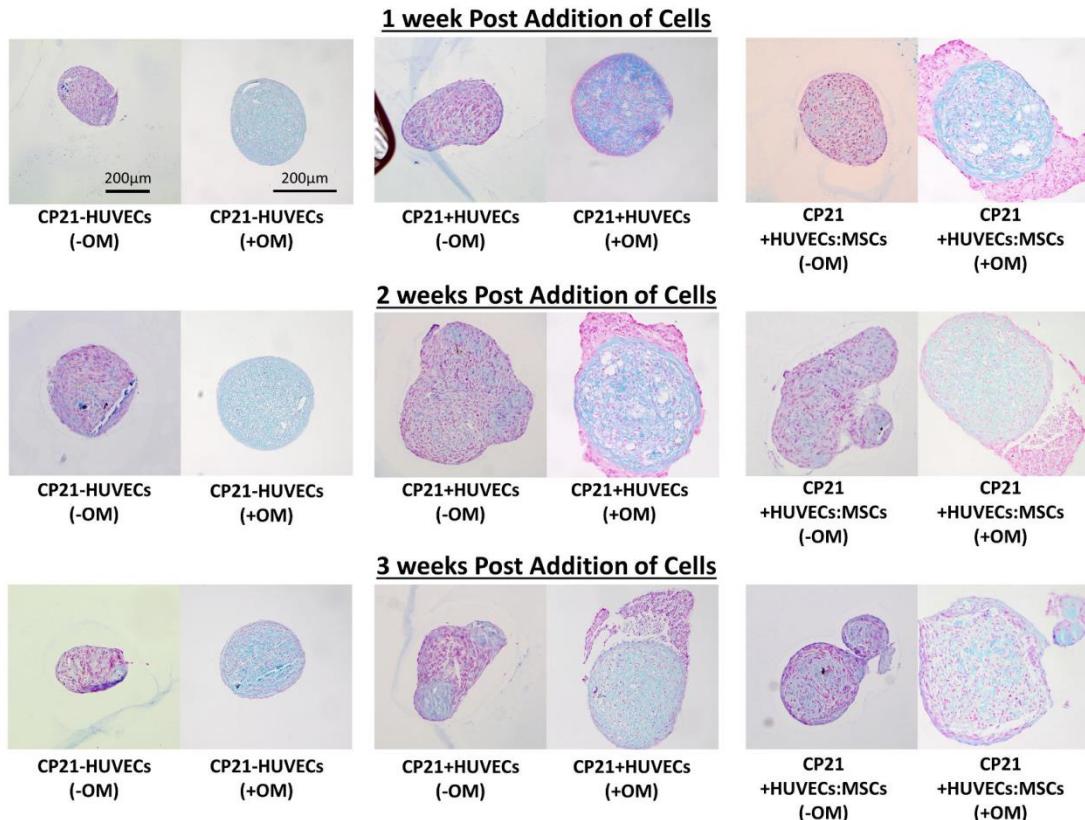


Figure 5.2: Alcian Blue staining of all three groups cultured with (+OM) and without osteogenic supplements (-OM) over the course of the experiment. Each of the images were imaged at a magnification of 20X (aggregates cultured with osteogenic supplements (+OM)) or at 10X (aggregates cultured without osteogenic supplements (-OM)).

5.3.3 Mineralisation of the Cartilage Template

5.3.3.1 ALP Production

There was no significant increase in ALP expression in either the CP21-HUVECs (-OM) or the CP21+HUEVCs (-OM) groups, without osteogenic supplements, over the course of the experiment (see Figure 5.3 (A)). However, there was a significant increase in ALP expression in the CP21+HUEVCs:MSCs (-OM) group from 1 week to 2 weeks ($p < 0.0001$) and from 2 weeks to 3 weeks ($p < 0.0001$)

into co-culture. Moreover, the CP21+HUVECs:MSCs (-OM) group had significantly higher ALP expression than the CP21+HUVECs (-OM) group at both 2 ($p < 0.0001$) and 3 weeks ($p < 0.0001$) into co-culture and significantly higher ALP expression than the CP21-HUVECs (-OM) group at all three time points ($p < 0.0001$). The CP21+HUVECs:MSCs (-OM) group also had significantly higher ALP expression than the Osteogenic group (MSCs cultured in osteogenic medium alone) at both 2 ($p < 0.001$) and 3 weeks ($p < 0.0001$) after the addition of cells.

Moreover, at all three time points the ALP expression in the CP21-HUVECs and CP21+HUVECs groups cultured in osteogenic medium (+OM) was significantly higher ($p < 0.05$) than the same groups cultured without (-OM) (see Figure 5.3 (B)). Interestingly, there was significantly higher ($p < 0.0001$) ALP expression in the CP21+HUVECs:MSCs (-OM) group compared to the same group cultured with osteogenic supplements as well as all other groups at both 2 and 3 weeks into co-culture.

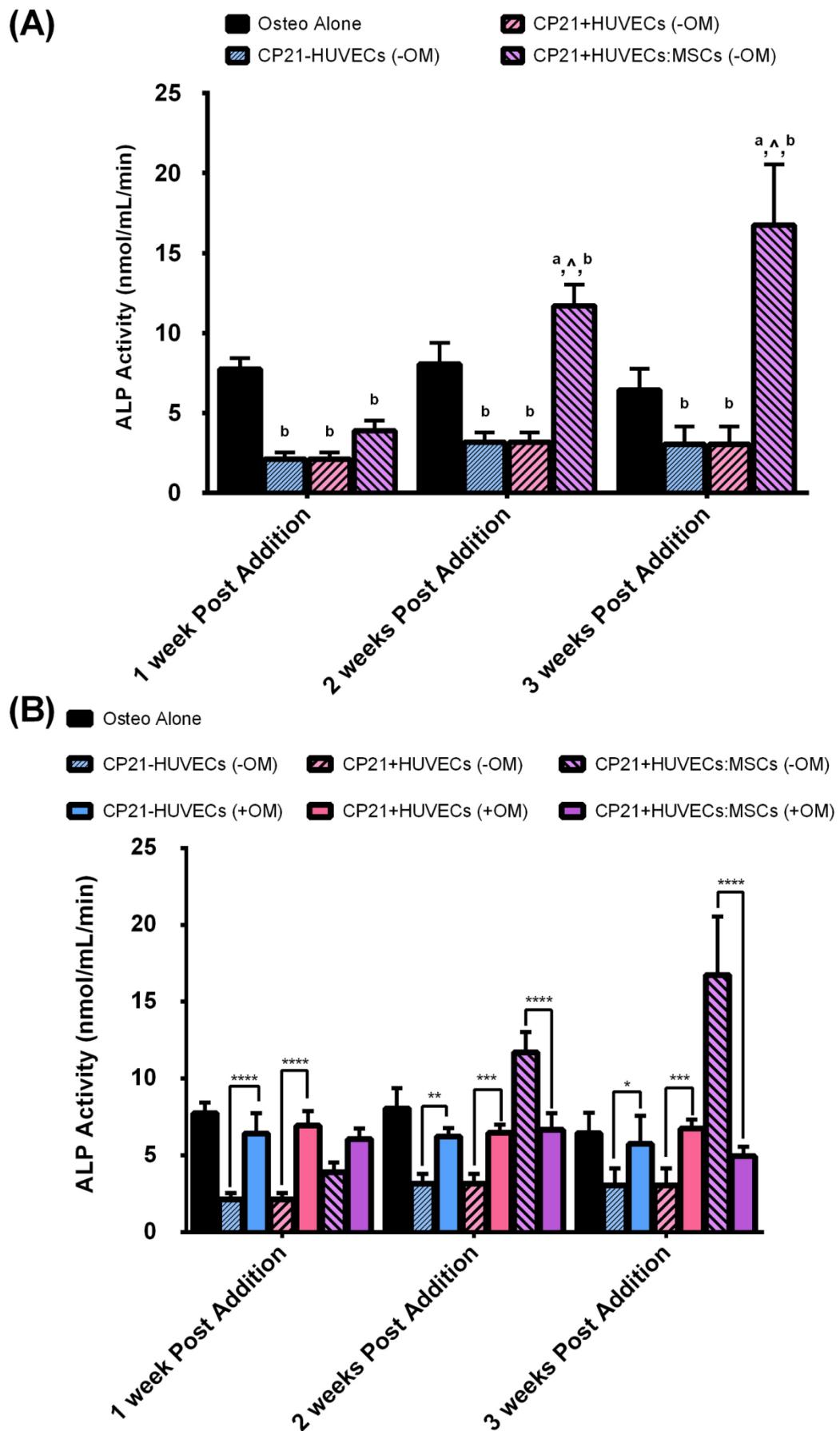


Figure 5.3: (A) ALP Activity of all of the experimental groups cultured without osteogenic supplements compared with osteogenic group alone 1, 2 and 3 weeks post the addition of HUVECs/MSCs. ^ap < 0.05 vs. CP21 +HUVECs group, ^ap < 0.05 vs. CP21 -HUVECs, and ^bp < 0.05 vs. Osteo alone. (B) ALP Activity of all three experimental groups cultured without osteogenic supplements compared to the same groups cultured in the presence of osteogenic supplements and the osteogenic group alone. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. (n = 6 samples per group per time point). Error bars denote standard deviation.

5.3.3.2 Calcium Content

There was no significant difference in calcium content in both the CP21-HUVECs (-OM) and the CP21+HUVECs (-OM) groups over the course of the co-culture period (see Figure 5.4 (A)). However, in the CP21+HUVECs:MSCs (-OM) group there was a significant increase in calcium content from 2 to 3 weeks into co-culture. Moreover, there was significantly higher (p < 0.05) calcium content in the CP21+HUVECs:MSCs (-OM) group compared to the Osteogenic group alone after 3 weeks of co-culture.

Interestingly, there was no significant difference in calcium content in both the CP21-HUVECs (-OM) and the CP21+HUVECs (-OM) groups, compared to their counterparts cultured in the presence of osteogenic supplements (+OM) at all-time points (see Figure 5.4 (B)). There was however, significantly higher calcium content in the CP21+HUVECs:MSCs group that was cultured in the presence of osteogenic supplements (+OM) compared to its counterpart cultured without osteogenic supplements (-OM) at both 1 (p < 0.0001) and 2 weeks (p < 0.0001) into co-culture. However, this significance was lost by 3 weeks into co-culture.

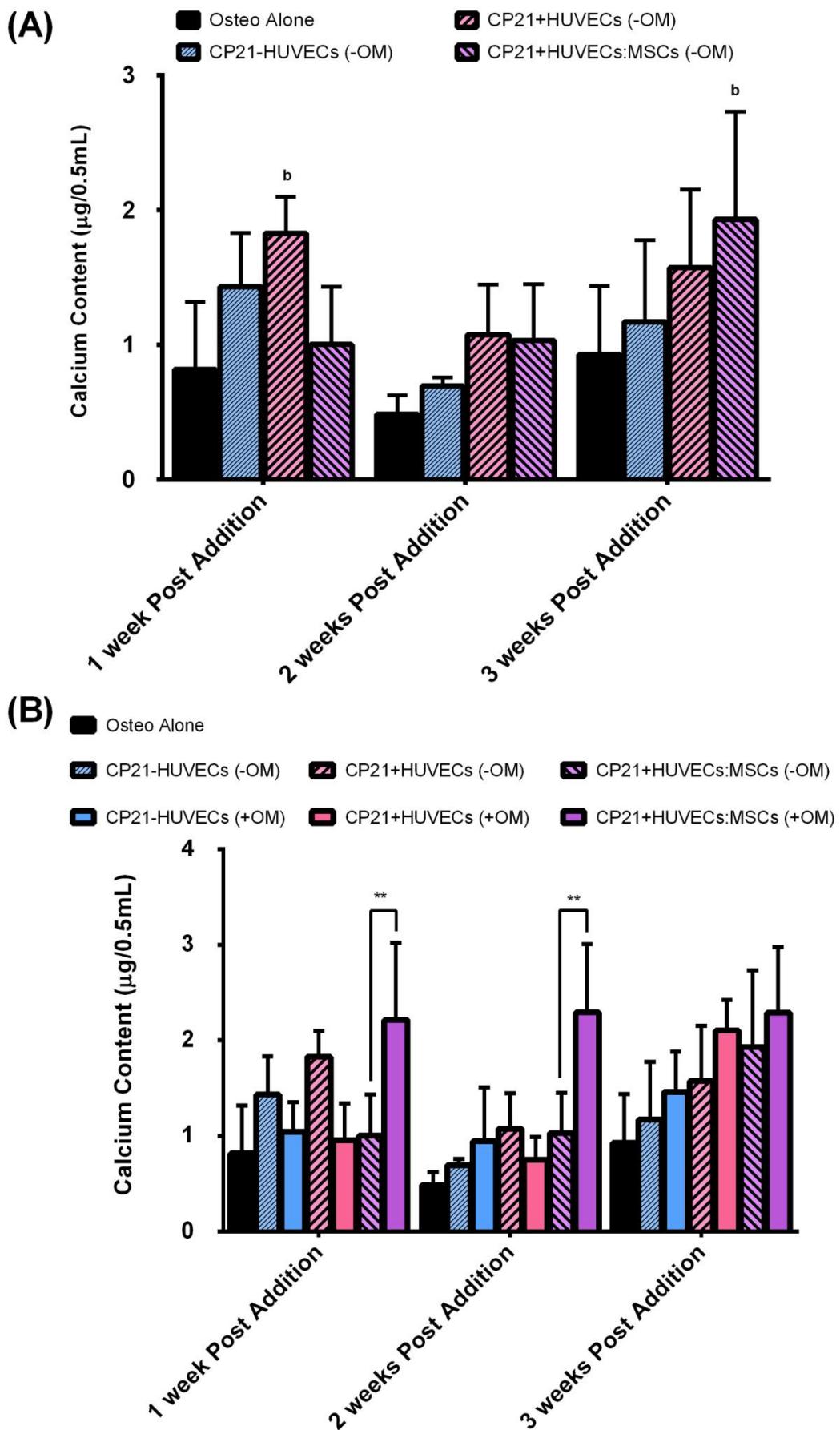


Figure 5.4: (A) Calcium content of all of the experimental groups cultured without osteogenic supplements (-OM) compared with osteogenic group alone 1, 2 and 3 weeks post the addition of HUVECs/MSCs. ^ap < 0.05 vs. CP21 +HUVECs group and ^bp < 0.05 vs. Osteo alone. (B) Calcium content of all three experimental groups cultured without osteogenic supplements compared to the same groups cultured in the presence of osteogenic supplements (+OM) and the osteogenic group alone. **p < 0.01, n = 6 samples per group per time point. Error bars denote standard deviation.

5.3.3.3 Alizarin Red

There was no positive Alizarin Red staining in any of the groups cultured without osteogenic supplements prior to the co-culture period, see Figure 5.5. No difference was seen between the co-culture groups cultured without osteogenic supplements and the CP21-HUVECs (-OM) group at both 1 week and 2 weeks into co-culture (Figure 5.5). However, positive red staining was seen throughout all the aggregates and by three weeks small mineralisation nodules were beginning to form in the CP21+HUVECs:MSCs (-OM) group, which were not seen in any of the other groups cultured without osteogenic supplements.

Interestingly, in the groups cultured without osteogenic supplements there was positive red staining throughout the aggregate after 1 week of co-culture, (see Figure 5.5). However, the same groups cultured in the presence of osteogenic supplements, mineralisation was only seen in the formation of nodules within the aggregate and the only group to have mineralisation throughout the aggregate was the CP21+HUVECs group 3 weeks after the addition of cells.

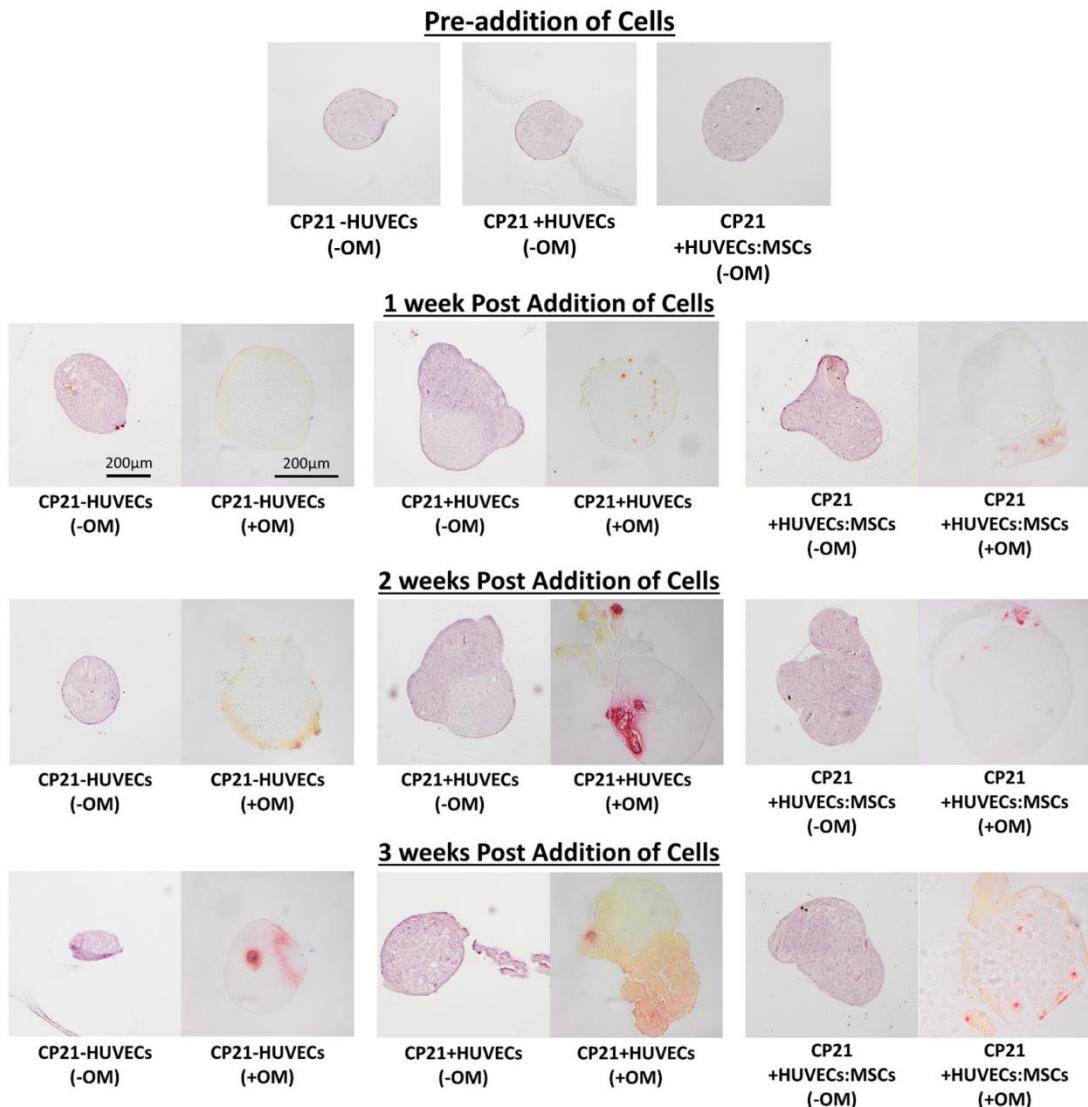


Figure 5.5: Alizarin Red staining of the aggregates cultured without osteogenic supplements prior to addition of cells and all three groups cultured with (+OM) and without osteogenic supplements (-OM) at 1, 2 and 3 weeks post the addition of cells. Each of the images were imaged at a magnification of 20X (aggregates cultured with osteogenic supplements (+OM)) or at 10X (aggregates cultured without osteogenic supplements (-OM)).

5.3.4 Vascularisation of the Cartilage Template

5.3.4.1 VEGF Production

There was no significant difference in VEGF content in both the CP21-HUVECs (-OM) and the CP21+HUVECs (-OM) groups over the course of the experiment (see Figure 5.6 (A)). However, there was a significant increase ($p < 0.01$) in VEGF content in the CP21+HUVECs:MSCs (-OM) group from 1 week to 2 weeks into co-culture, and a significant decrease ($p < 0.05$) in VEGF content from 2 to 3 weeks into co-culture. There was also significantly higher ($p < 0.0001$) VEGF content in the CP21+HUVECs:MSCs (-OM) group compared to the CP21+HUVECs (-OM) group at all-time points and significantly higher VEGF content ($p < 0.001$) than the CP21-HUVECs (-OM) group at both 2 and 3 weeks into co-culture. Moreover, the CP21-HUVECs (-OM) group had significantly higher ($p < 0.05$) VEGF expression 2 weeks into co-culture compared to the CP21+HUVECs group.

There was no significant difference in VEGF expression of both the CP21-HUVECs (-OM) and the CP21+HUVECs (-OM) groups compared to their counterparts cultured with osteogenic supplements (see Figure 5.6). However, at both 1 week and 2 weeks into co-culture there was significantly higher ($p < 0.01$) VEGF content in the CP21+HUVECs:MSCs (-OM) group compared to its counterpart cultured in the presence of osteogenic supplements. This significance was lost by 3 weeks into co-culture.

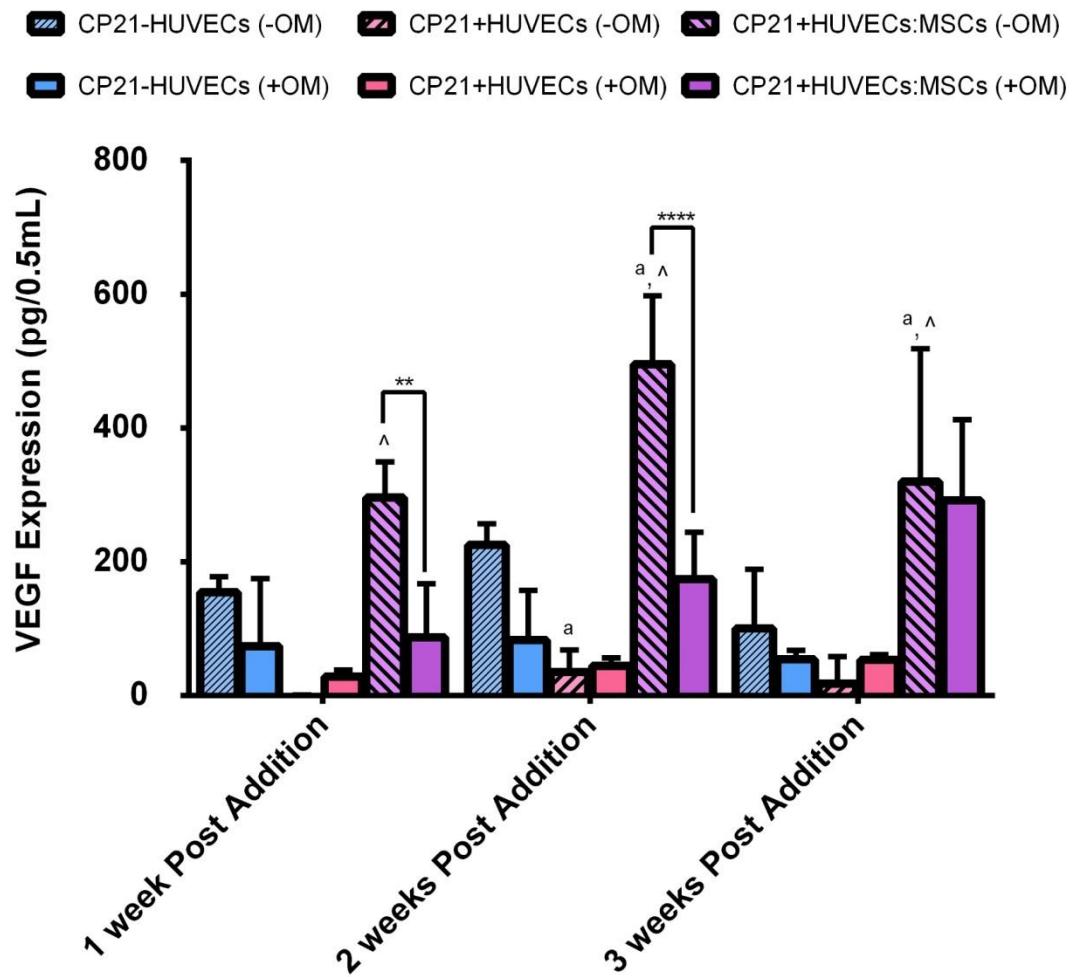


Figure 5.6: VEGF expression of all three experimental groups cultured without osteogenic supplements (-OM) compared to the same groups cultured in the presence of osteogenic supplements (+OM). $^{\wedge}p < 0.05$ vs. CP21 +HUVECs group, $^a p < 0.05$ vs. CP21 -HUVECs, $^{**}p < 0.01$ and $^{****}p < 0.0001$, $n = 6$ samples per group per time point. Error bars denote standard deviation.

5.3.4.2 CD31+ Staining

At all-time points there was a lack of positive (green) staining for CD31+ in the CP21-HUVECs (-OM) group cultured without osteogenic supplements, as there was no endothelial cells present (see Figure 5.7). However, for both the CP21+HUVECs (-OM) and the CP21+HUVECs:MSCs (-OM) groups there was positive (green)

staining seen around the periphery of both groups at 1 week and 2 weeks into co-culture. By 3 weeks into co-culture both groups had positive green staining present within the centre of the aggregates however, the CP21+HUVECs:MSCs (-OM) group was the only group to have rudimentary vessels beginning to form. The structure of these vessels were the same in all of the aggregates, circular CD31+ positive wall with irregularly shaped nuclei present within the lumen (indicated by arrows in Figure 5.8 (A)). Alpha smooth actin staining was also performed with no positive staining seen in any of the aggregates and as such results were not included.

At all-time points there was positive green staining around the periphery of the aggregate in CP21+HUVECs (+OM) group cultured in the presence of osteogenic supplements (see Figure 5.7). However, its counterpart cultured without osteogenic supplements had positive staining present both at the periphery and within the centre of the aggregates. Moreover, by three weeks preliminary vessels were present in the centre of the aggregates in the CP21+HUVECs:MSCs group irrespective of whether it was cultured with or without osteogenic supplements. Interestingly, the cross sectional area of the preliminary vessels present within the CP21+HUVECs:MSCs (-OM) group cultured without osteogenic supplements was significantly higher ($p < 0.001$) than the vessels present within its counterpart cultured with osteogenic supplements (see Figure 5.8 (B)).

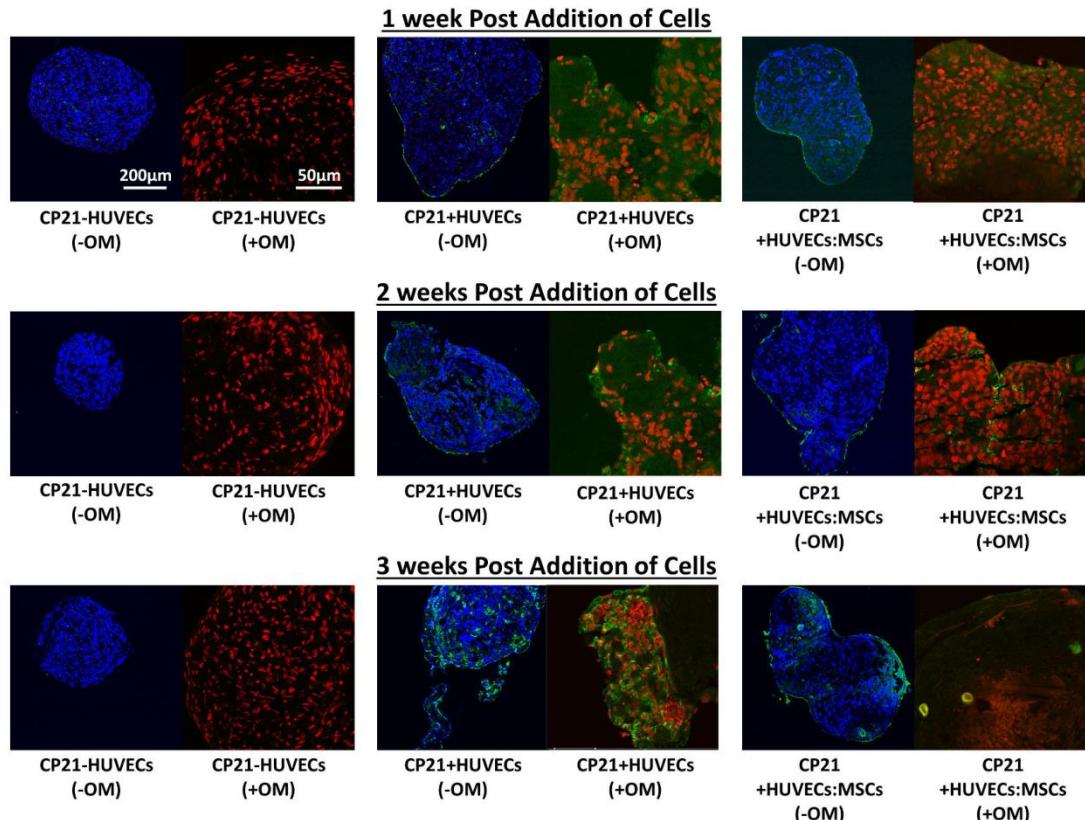


Figure 5.7: CD 31 staining (green) of all three experimental groups cultured with (+OM) and without (-OM) osteogenic supplements over the course of the experiment. Each of the images of the aggregates cultured without (-OM) osteogenic supplements were imaged at a magnification of 10X with a nuclei counterstain DAPI (blue). The aggregates cultured with osteogenic supplements (+OM) were imaged at a magnification of 40X, with a nuclei counterstain propidium iodide (red).

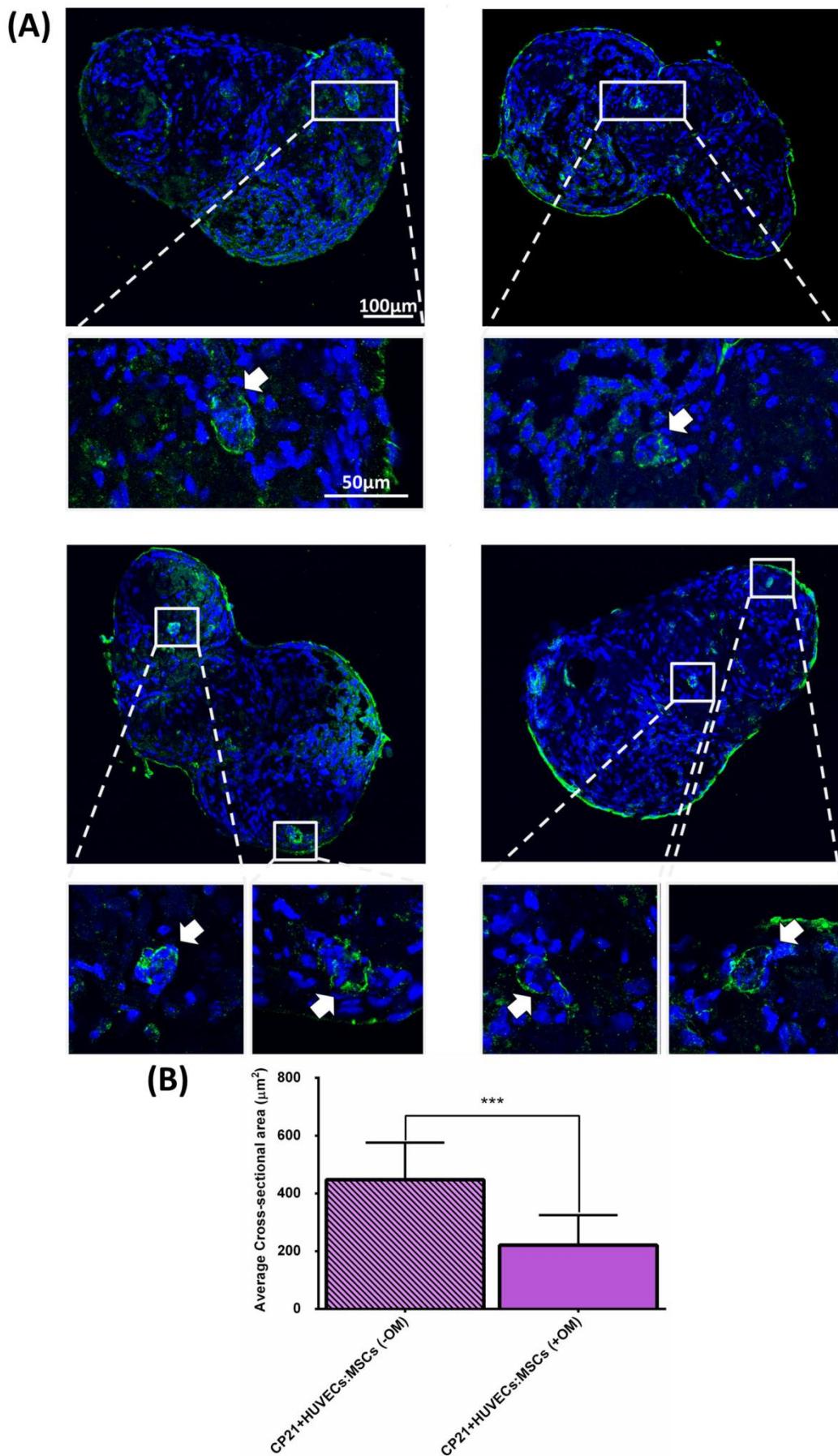


Figure 5.8: (A) Representative images of CD31+ (green) stained sections of the CP21+HUEVCs:MSCs group only, showing the presence of rudimentary vessels. Each section is 8 μ m thick and each of the images was taken at a magnification of 20X and 60X. Nuclear counterstain: DAPI (blue). (B) Quantitative analysis of cross-sectional area of the rudimentary vessels present in the aggregates. ***p < 0.001 and error bars denote standard deviation (n = 9).

5.4 Discussion

This Chapter investigated if a co-culture technique could obviate the need for osteogenic supplements to induce osteogenesis in a 3D cellular aggregate in vitro. The results from this Chapter showed that the application of both chondrogenic and vascular priming can significantly enhance the production of both early (ALP) and late (Calcium) osteogenic makers of MSCs, in the co-culture group with MSCs/HUEVCs added, even without the use of any osteogenic supplements compared to osteogenic culture alone. This increase in early osteogenic markers, seen with the MSCs/HUEVCs added co-culture group, was also found to be significantly higher than the same group cultured in the presence of osteogenic supplements. The results from this Chapter also show that the presence of osteogenic supplements has a significant effect on the way mineralisation is deposited within a construct. Without the presence of osteogenic supplements, mineral is deposited throughout the construct however, when osteogenic supplements are added, mineralisation is deposited through the formation of discrete mineralisation nodules. Interestingly, the addition of osteogenic supplements not only has an effect on the mineralisation potential of the constructs but also on the vascularisation potential. The results from this Chapter

found that without osteogenic supplements there was an upregulation in VEGF content in the co-culture group with both HUVECs and MSCs added to the cartilage template. Furthermore, the increase in VEGF content seen in the co-culture group with HUVECs/MSCs added, was significantly higher than the same group cultured in the presence of osteogenic growth supplements. Moreover rudimentary vessels in the co-culture group with HUVECs/MSCs added were significantly larger in cross-sectional area than those seen in the same group cultured in the presence of osteogenic supplements (as seen through CD31+ staining). Taken together, this Chapter suggests that the addition of osteogenic supplements inhibits the expression of early osteogenic markers (ALP), whilst promoting mineral deposition in the form of discrete mineralisation nodules, and also that there is an inhibitory effect on the vascularisation potential (VEGF and CD31) of in vitro 3D constructs.

A possible limitation of the study is that MSCs from two male donors were pooled and the study did not directly explore whether the human MSCs displayed a donor dependent response to mineral formation. However, the control groups also contained pooled cells, so the differences observed between the groups cannot be explained by donor variability. Another limitation of this study is the use of ascorbic acid and dexamethasone in the chondrogenic medium. As both ascorbic acid and dexamethasone are also used in osteogenic medium, the exposure to the osteogenic supplements during the chondrogenic priming period might have caused the MSCs to differentiate towards the osteogenic lineage. However, the use of dexamethasone and ascorbic acid in chondrogenic medium is well documented (Thorpe et al., 2010; Erickson et al., 2002; Meyer et al., 2011; El-Serafi et al., 2011; Sheehy et al., 2013; Mackay et al., 1998; Jukes et al., 2008; Farrell et al., 2011; Farrell et al., 2009; Scotti et al., 2013; Scotti et al., 2010; Harada et al., 2014; van der Stok et al., 2014; Dawson

et al., 2008; Tare et al., 2005; Meinel et al., 2004a) and has not been shown to cause mineralisation. Moreover, alizarin red staining of the aggregates prior to the co-culture showed no positive staining and the control groups were all exposed to the same chondrogenic medium, so any differences seen between the groups were not due to the exposure of these growth factors. Another limitation was the significant increase in DNA content in the CP21+HUVECs and CP21+HUVECs:MSCs groups cultured in the presence of osteogenic supplements, compared to the same groups cultured without. However, dexamethasone has been shown to induce MSC proliferation (Wang et al., 2012a; Langenbach and Handschel, 2013) and ascorbic acid has been shown to promote endothelial cell proliferation (Ulrich-Merzenich et al., 2002a; Ulrich-Merzenich et al., 2002b; Ulrich-Merzenich et al., 2007), which might account for this increase in DNA content. Moreover, the additional cells did not appear to influence mineralisation or vascularisation potential. Finally, in order to investigate if co-culture alone could induce osteogenesis in human MSCs the study only looked at the co-culture groups without all osteogenic supplements compared to with all osteogenic supplements. However, it is possible that the inhibitory effect seen in this study was only due to the β -glycerophosphate and not due to the dexamethasone and ascorbic acid. For the purpose of this study it was not within the scope to determine the optimum medium needed to induce osteogenesis through co-culture alone. Future studies could investigate the effect of ascorbic acid and dexamethasone on the co-culture of MSCs and HUVECs.

Various in vitro bone regeneration strategies have relied on the use of the osteogenic supplements (asc, dex, and β -glyc) to induce mineralisation in vitro (Farrell et al., 2009; Freeman et al., 2013; Freeman et al., 2015b; Jaiswal et al., 1997a; Ohgushi et al., 1993; Ohgushi et al., 1996; Yoshikawa et al., 1997; Maniatopoulos et

al., 1988; Kotobuki et al., 2005; Kotobuki et al., 2006; Oreffo et al., 1998; Dawson et al., 2008; Morgan et al., 2007; Bolland et al., 2008), and each supplement has been shown to be imperative to induce mineralisation of MSCs in vitro (Kamalia et al., 1992; Leboy et al., 1991; Maniatopoulos et al., 1988; Herbertson and Aubin, 1995; Peter et al., 1998; Song et al., 2009; Cheng et al., 1994; Jaiswal et al., 1997a; Franceschi et al., 1994; Franceschi and Iyer, 1992; Hofmann et al., 2007; Martin et al., 1998; Meinel et al., 2005; Meinel et al., 2004a; Meinel et al., 2004b). The main rationale behind the use of these supplements is to provide signalling proteins or enzymes to activate mineral production by MSCs. Previous in vitro 2D studies have shown that the direct co-culture of MSCs with endothelial cells (Villars et al., 2000; Villars et al., 2002; Guillotin et al., 2008; Zhao et al., 2012) or chondrocytes (Nakagawa et al., 2000) can provide the necessary factors to induce early mineralisation (ALP expression) without the need for these supplements. Other 2D studies have found indirect co-culture with osteocytes lead to increased calcium deposition compared to MSCs co-cultured with osteoblasts. Therefore, suggesting that osteocytes are more influential than osteoblasts at inducing osteogenesis in MSCs in vitro again without the use of any osteogenic supplements (Birmingham et al., 2012). However, in vivo, endochondral ossification involves the direct interaction of cartilage cells with both MSCs and endothelial stem cells, but a co-culture of such cells had not been investigated prior to the current study.

In Chapter 4 and this Chapter a novel co-culture technique was investigated, which involved not only the co-culture of HUVECs and MSCs, but also the co-culture of HUVECs and MSCs with MSCs that had been pre-differentiated towards the chondrogenic lineage. The results from this Chapter found that a combination of both chondrogenic priming and vascular priming obviated the need for osteogenic

supplements, and also enhanced both early (ALP) and late (calcium) osteogenic markers compared to MSCs cultured in osteogenic supplements alone. Moreover, similar to previous studies (Saleh et al., 2011; Rouwkema et al., 2006; Grellier et al., 2009b; Liao et al., 2011; Villars et al., 2002; Sun et al., 2007; Pedersen et al., 2013; Freeman et al., 2015b; Jiang et al., 2005; Giovannini et al., 2010; Nakaoka et al., 2006) the co-culture group with both HUVECs and MSCs added had significantly higher ALP production compared to the control groups (osteogenic and non-co-culture group) after 3 weeks of co-culture. However, unlike previous studies, there was also an increase in late osteogenic markers (Calcium) compared to the control group (osteogenic) and this increase in ALP and Calcium content was achieved in a 3D scaffoldless construct, without the use of any osteogenic supplements. Compared to the calcium content results from Chapter 4, this increase in calcium content in the co-culture group with added MSCs/HUVECs but no osteogenic supplements, was significantly higher than the same group cultured in osteogenic supplements. There was significantly higher calcium content in the group cultured with osteogenic supplements, compared to without, 1 and 2 weeks after the addition of the cells. However, although Calcium content was lower in the groups cultured without osteogenic supplements the mineral is deposited throughout the aggregate. In contrast, mineral deposition in the aggregates cultured with osteogenic supplements was found in the form of mineralisation nodules within the aggregate (as seen in Alizarin Red staining).

Bone tissue engineered constructs have had limited success post-implantation, which has been attributed to poor nutrient delivery and waste removal arising from a lack of vasculature (Amini et al., 2012; O'Brien, 2011; Lyons et al., 2010; Phelps and Garcia, 2009; Ko et al., 2007; Krishnan et al., 2014; Farrell et al., 2011; Farrell et al.,

2009). Previous studies have investigated whether pre-vascularising 3D constructs in vitro would eradicate this limitation, through the co-culture of endothelial stem cells with MSCs (Rouwkema et al., 2006; Saleh et al., 2011; Sun et al., 2007; Pedersen et al., 2013; Correia et al., 2011; Ghanaati et al., 2011; McFadden et al., 2013; Duffy et al., 2011; Scherberich et al., 2007) or osteoblasts (Fuchs et al., 2007; Hofmann et al., 2008; Kyriakidou et al., 2008), and found that prevascular networks are formed both in vitro and in vivo. In Chapter 4 rudimentary vessels were formed in vitro when both MSC and HUVECs were added to the already formed cartilage template. The results from this Chapter also show that the formation of rudimentary vessels was only seen in the aggregates with added HUVECs/MSCs (as seen by CD31+ staining). However, unlike the results seen in Chapter 4 the average vessel cross sectional area was significantly higher than those seen in the aggregates cultured in the presence of osteogenic supplements. Furthermore, unlike the results seen in Chapter 4, when HUVECs alone are added to an already formed cartilage template, in the absence of osteogenic supplements, they did not just attach and proliferate around the periphery, but also began to invade into the centre of the construct (as identified by CD31+ and Alcian Blue staining). Moreover, the omission of osteogenic supplements influenced vessel formation and VEGF expression. VEGF is well documented as a stimulator of vascular cells to undergo the formation of early vessels (Gerber and Ferrara, 2000; Hans-Peter et al., 1999; Fiedler et al., 2005; Mayr-Wohlfart et al., 2002; Nakagawa et al., 2000; Farrell et al., 2009). The results from both Chapter 4 and this Chapter corroborate this as the group with the highest VEGF content in both studies was also the only group to have rudimentary vessels present within the aggregate. However, unlike the results from Chapter 4, the peak VEGF content was seen after 2 weeks of co-culture and then it begins to plateau, whereas the group with osteogenic factors did

not peak until 3 weeks into co-culture. This suggests that the addition of osteogenic supplements to the medium also inhibits the vascularisation potential of in vitro bone constructs.

It has been widely documented that osteogenic supplements (asc, dex and β -glyc) are needed to produce mineralisation in vitro. However, the results from this Chapter show that a co-culture technique can actually provide all the necessary signalling to induce mineralisation of a 3D construct in vitro, whilst allowing for the formation of rudimentary vessels. Dex is needed to induce mineralisation in MSCs as it activates *Runx2* expression through the (WNT/ β -catenin) signalling pathway (Langenbach and Handschel, 2013; Komori, 2010; Hamidouche et al., 2008). However, in this co-culture model the *Runx2* expression (usually induced by dex) is likely produced by chondrocytes within the cellular aggregates, which undergo hypertrophy due to the addition of endothelial cells to the cartilage template (Bittner et al., 1998), as seen in the Collagen Type X staining from Chapter 4. Moreover, the formation of mineralisation nodules in the aggregates cultured in osteogenic supplements might be explained by the use of dex within the osteogenic medium, as similar result have been reported in costochondral chondrocyte cultures exposed to dex (Schwartz et al., 1995). Therefore, if dex is not supplemented, MSCs are free to form mineral throughout the aggregates rather than in discrete nodules. Asc is has been shown to be the main regulator of Collagen type X production (Langenbach and Handschel, 2013) and ALP expression. Studies have shown that co-culture of endothelial cells with chondrocytes induces both Collagen type X and ALP expression (Leboy et al., 1989), as was also shown in the Collagen type X straining presented in Chapter 4 of this Thesis. Finally β -glyc is the source of phosphate needed to produce hydroxyapatite (Foster et al., 2006; Fatherazi et al., 2009; Tada et al., 2011), but it is

proposed that in the co-culture model these phosphates are naturally provided by cartilage cells undergoing hypertrophy within the cartilage template (Bourne, 1972). The results suggest that a direct co-culture technique can obviate the need for osteogenic supplements to induce osteogenesis in vitro.

Although little is known about the direct interaction between hMSCs and endothelial cells *in vivo* (da Silva Meirelles et al., 2008; Jones and McGonagle, 2008; Augello et al., 2010; Tsai et al., 2015; Chan et al., 2009), due to their anatomical position these cells are either in direct cell-cell contact or communicate through paracrine signalling within the endochondral template. The results from this study show that the direct interaction between the two cell types has a significant effect on both the migration and the vascularisation potential of the endothelial stem cells, as well as the osteogenic potential of hMSCs even without the use of any osteogenic supplements. These results provide a novel insight into the symbiotic relationship between hMSCs and endothelial stem cells during the endochondral ossification process *in vivo*. It is proposed that the MSCs undertake a perivascular role during the vascularisation process within the cartilage template, as no vessels were formed unless both MSCs and HUVECs were added to the already formed cartilage template. Moreover, the addition of HUVECs to a cartilage template also seem to increase the osteogenic potential of the MSCs, even without the use of any osteogenic supplements, compared to MSCs only cultured in osteogenic supplements. Taken together, this study elucidates that the direct co-culture of MSCs and endothelial cells during endochondral ossification, induces endothelial cells to form preliminary vessels within the cartilage template, and also induces MSCs to begin mineralising the cartilage template. However, the exact signalling pathways, including the FGF, Wnt/β-catenin

or TGF- β signalling pathways, that occurs due to this direct cell-cell interaction need to be further investigated.

5.5 Conclusions

This Chapter shows that both chondrogenic priming (for 21 days) and subsequent vascular priming can induce osteogenesis of a 3D scaffoldless construct without the use of any osteogenic supplements. Most interestingly, the inclusion of osteogenic supplements *in vitro* actually inhibits the promotion of ALP content, Calcium content and VEGF content produced through co-culture alone. Moreover, the results show that chondrogenic and vascular priming not only increases the expression of ALP, Calcium content and VEGF expression, but also allows for the formation of rudimentary vessels, seen within all of the aggregates in which both MSCs and HUVECs were added. These rudimentary vessels were also larger in cross-sectional area than those seen in the same groups cultured in the presence of osteogenic growth factors. Taken together, these results indicate for the first time the beneficial effect that co-culture of MSCs, endothelial cells and chondrocytes, has on both osteogenesis and vasculogenesis of 3D scaffoldless bone tissue engineering construct *in vitro*. This Chapter also suggests that the application of both chondrogenic and vascular priming of human MSCs can obviate the need for osteogenic supplements to induce osteogenesis by human MSCs, whilst allowing for the formation of rudimentary vessels *in vitro* in 3D bone tissue engineered constructs.

Chapter 6: What effect will the in vitro endochondral priming and prevascularisation of cellular aggregates have in vivo?

6.1 Introduction

As stated in Chapter 2 of this Thesis one of the primary limitations of bone tissue engineering is the occurrence of core degradation and loss of cell viability due to hypoxia occurring within the constructs, which arise from lack of vascularisation (Amini et al., 2012; O'Brien, 2011; Lyons et al., 2010; Phelps and Garcia, 2009; Ko et al., 2007; Krishnan et al., 2014; Farrell et al., 2011; Farrell et al., 2009). As a result such bone tissue engineering strategies are not yet widely used for clinical treatment of large bone defects. However, the results from Chapter 3-5, provide evidence that one way of overcoming these limitations might be to mimic the endochondral ossification process in vitro, such that when the construct is implanted it will provide the necessary factors to induce bone formation in vivo.

Chondrogenically primed constructs seeded with embryonic stem cells (Jukes et al., 2008), chick embryonic stem cells (Oliveira et al., 2009b) and human MSCs (Farrell et al., 2011; Farrell et al., 2009; Scotti et al., 2013; Scotti et al., 2010; Gawlitta et al., 2015; Visser et al., 2015) were shown to mineralise, and in some cases formed bone marrow cavities (Scotti et al., 2013; Oliveira et al., 2009b; Gawlitta et al., 2015;

Visser et al., 2015) following subcutaneous implantation in rodent animal models. Chondrogenically primed rat MSCs cultured on a PLGA scaffold were also found to have increased bone healing in both a 5 mm and 15 mm rat femur defect (Harada et al., 2014). Similar rapid healing was also reported when chondrogenically primed human MSC cellular aggregates were implanted in a 6 mm rat femur defect (van der Stok et al., 2014). However, even with chondrogenic priming, core degradation and an uneven distribution of mineral have been reported in constructs after subcutaneous implantation (Farrell et al., 2009; Scotti et al., 2010; Farrell et al., 2011). The results from Chapter 3 of this Thesis found that chondrogenic priming of BALB/c mice MSCs and human MSCs in vitro for specific durations (14, 21 days) can produce an aggregate that is mineralised throughout the core (Freeman et al., 2013). However, it is well documented that without a suitable vascular supply, cells within a tissue engineered construct lack the necessary requirements to regenerate bone tissue and readily perish when implanted in vivo (Rivron et al., 2008; Kanczler and Oreffo, 2008).

In vitro co-culture studies have investigated whether pre-vascularising 3D tissue engineered constructs, through the co-culture of MSCs and HUVECs, would allow faster host integration post-implantation (Correia et al., 2011; Ghanaati et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Scherberich et al., 2007; Duffy et al., 2011). It has been shown that pre-vascular networks can be formed in a subcutaneous animal model in vivo when human MSCs (Correia et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Scherberich et al., 2007) are first co-cultured with HUVECs in vitro. Moreover, in vitro co-culture studies of HUVECs and MSCs have led to production of the early osteogenic growth factor ALP in both 2D and 3D culture (Villars et al., 2000; Sun et al., 2007; Liao et al., 2011; Rouwkema et al., 2006; Saleh et al., 2011). However, while various strategies have sought to separately incorporate

cartilage template formation or vascularisation to enhance bone tissue regeneration, no strategy has sought to incorporate both events simultaneously, even though both are crucial precursors for bone formation during endochondral ossification *in vivo*. The results from Chapters 4 and 5 of this Thesis showed that chondrogenic priming (for 21 days) together with co-culture of human MSCs and HUVECs significantly increased the osteogenic potential of the culture compared to chondrogenic priming alone (Freeman et al., 2015b). It was also shown that both MSCs and HUVECs must be added to the formed cartilage template for the formation of rudimentary vessels to occur *in vitro*. However, it was found that the addition of osteogenic supplements to the medium actually inhibits the expression of early osteogenic marker (ALP), whilst mineralisation in the form of discrete mineralisation nodules occurred. Moreover, the production of mineral also had an inhibitory effect on the vascularisation potential (VEGF and CD31) of *in vitro* 3D aggregates.

These results of Chapters 3-5 of this Thesis suggest that chondrogenic priming of MSC cellular aggregates and co-culture of human MSCs and HUVECs, in the absence of osteogenic supplements, might be an effective strategy to develop *in vitro* tissue engineered bone constructs. However, the *in vivo* viability, vascularisation and mineralisation potential of MSC aggregates that have been pre-conditioned *in vitro* by a combination of chondrogenic and vascular priming, has yet to be established. Therefore, in this Chapter, the author tested the hypothesis that “A tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage aggregates, through the co-culture of HUVECs *in vitro*, will serve as an effective *in vitro* bone regeneration approach”. The overall objective of this study was to assess the outcome of cell survival, vessel infiltration and thus mineral formation of the cellular aggregates

within a subcutaneous implantation nude rat model. The specific objectives of this study were to compare mineral and vessel formation by means of bioluminescent imaging (BLI), microcomputed tomography (μ CT), histology (Masson's trichrome and Alizarin Red) and immunohistochemistry (CD31, CD146, and α -smooth actin) to assess their functionality *in vivo*. This Chapter presents an adapted version of work previously published in the Journal of Stem Cell Research & Therapy (Freeman et al., 2015a).

6.2 Methods

6.2.1 Cell Culture

6.2.1.1 Human Donor MSCs

As previously described in Chapter 5, bone marrow-derived human MSCs harvested from two male donors 20-25 years old, with established multipotency, were purchased from the Texas A&M University Health Science Centre (Temple, TX). The human MSCs were expanded in Minimum Essential Medium alpha (α MEM, Invitrogen, Carlsbad, CA) containing 16.7% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and 100 units/mL penicillin/100 μ g/mL streptomycin/2 mM L-glutamine (PSL, Invitrogen) at 37°C and 5% CO₂. For all cell culture performed in this study, cell culture medium was changed twice weekly unless stated otherwise. At passage 2, cells from each donor were detached using 0.25%

trypsin-EDTA (Invitrogen) and combined 1:1 to produce a pooled human MSC population. MSCs were further cultured to passage 3-4.

6.2.1.2 Cell Labelling

Human MSCs were co-transduced using lentiviral vector containing green fluorescent protein (GFP) and firefly luciferase (Luc) downstream of the ubiquitin promoter, as previously described (Sheyn et al., 2011; Sun et al., 2009; Allen et al., 2014). Briefly, human MSCs were suspended in polybrene and a viral vector at a multiplicity of infection (MOI) of 20 and incubated in flasks at a density of 10,000 cells/cm² overnight (Sigma, St. Louis, MO). Medium was changed daily for 3 days, after which the labelling efficiency of GFP/Luc was determined using fluorescent microscopy. GFP/Luc labelled human MSCs were replated at a seeding density of 500 cells/cm² and were further cultured to passage 3-4. These GFP/Luc labelled human MSCs were only used in the constructs tested for BLI. In order to have the same amount of labelled cells in each group the only cells that were labelled were the MSCs that were chondrogenically primed (i.e. used to generate the cartilage template). The added HUVECs/MSCs were not labelled.

6.2.1.3 HUVECs Culture

As previously described in Chapter 5, HUVECs were purchased from Lonza (Maryland, USA) and cultured in Clonetics Endothelial Growth Medium (EGM) SingleQuotes (Lonza). Medium was replaced every 3 days and, upon reaching 80-90% confluence, cells were passaged using 0.25% trypsin-EDTA (Invitrogen). HUVECs were further cultured to passage 3.

6.2.2 Aggregate Formation

Once the human MSCs (labelled and unlabelled) reached a confluence of ~80% aggregates were formed. As stated in previous Chapters this was achieved dividing a cell suspension of 0.25×10^6 cells/mL into 1.5 mL tubes, having 250,000 cells in each tube, and these were then centrifuged for 5 minutes (Eppendorf Centrifuge 5430R; Vashaw Scientific, Norcross, GA) at 400 g to create cell aggregates. Carefully avoiding the newly formed aggregate, the medium was removed from each of the aggregates and 0.5 mL of Chondrogenic Medium was added. Chondrogenic medium was the same as described previously (high glucose DMEM, 10 ng/mL TGF- β 3 (Invitrogen), 50 μ g/mL Ascorbic Acid, 4.7 μ g/mL Linoleic Acid, 100 nM Dexamethasone and 1x insulin-transferrin-selenium) and the EGM medium was the same medium that was used to culture the HUVECs (Lonza). For all experiments aggregate cultures were fed twice per week by performing a 50% medium exchange. During each feed the aggregates were agitated, so as to prevent them from adhering to the micro-tube. This was achieved through aspirating the medium beneath the aggregate with a micro-pipette.

These cells were further cultured under the same experimental conditions that were used in Chapter 4: (1) *CP21-HUVECs*: Aggregates were chondrogenically primed for a period of 21 days and then cultured in EGM medium for a further 21 days (in the Results section this group will be hereafter known as the **Cartilage Template group**). (2) *CP21+HUVECs*: Aggregates were chondrogenically primed for 21 days after which 250,000 suspended HUVECs in EGM were added to the cellular aggregate and cultured in EGM for further 21 days (hereafter known as the **Co-Culture Cartilage Template group**). (3) *CP21+HUVECs:MSCs*: Aggregates were

chondrogenically primed for 21 days after which 250,000 suspended HUVECs and MSCs at a ratio of 1:1 (125,000:125,000 cells) in EGM were added and further cultured in EGM for 21 days (hereafter known as the **Prevascularised Cartilage Template group**) as seen in Figure 1.

For all groups the EGM Medium added at day 21 also contained 20% methocel from a stock solution that was generated by dissolving 6 g of carboxymethylcellulose (Sigma Aldrich) in 500 mL of DMEM as previously described (Korff and Augustin, 1998). After 24 hours the medium that contained methocel was removed and was replaced with EGM medium alone and EGM medium alone was used for the further 20 days.

6.2.3 Construct Preparation

After 42 days of in vitro culture, the primed aggregates were prepared for implantation. A dual syringe approach, previously described by Kolambkar et al. (Kolambkar et al., 2011), was adapted to imbed the cellular aggregates within hydrogels. Briefly functionalised alginate (FMC Biopolymer; Sandvik, Norway) containing BMP-2 (Pfizer, MA, USA) at a concentration of 1.6 µg/100 µL was cross-linked by adding calcium sulphate (Sigma) to a final concentration of 8.4 mg/mL. Constructs were prepared by injecting 100 µL into an electrospun, PCL nanofiber mesh tube (Kolambkar et al., 2011), and two cellular aggregates from each group were placed within each alginate/mesh, as seen in Figure 6.1. One group, which contained no aggregates within the mesh, was used as an acellular group (known as the **Alginate group**). These constructs were then incubated in culture medium within a 24-well ultra low-attachment plate (Corning, Lowell, MA, USA) for 2-6 hours prior to implantation.

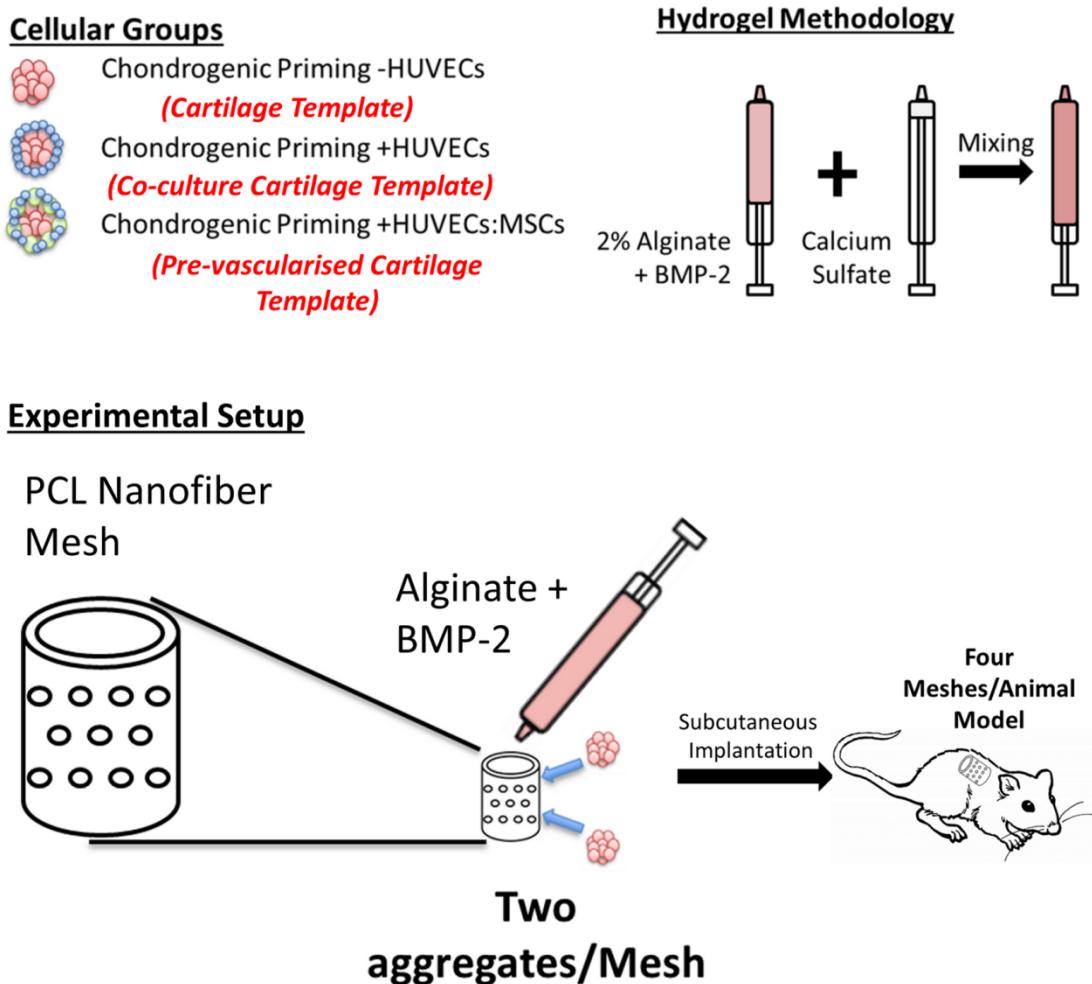


Figure 6.1: Schematic of the Cellular Groups, Hydrogel Methodology, and Experimental Setup.

6.2.4 Surgical Procedures

All animal procedures were conducted in accordance with the Georgia Institute of Technology Institutional Animal Care and Use Committee protocol (#A13023). Ten 11-week-old female, athymic nude rats (Charles River Labs; Wilmington, MA, USA) were anaesthetised using isoflurane. Two incisions were made in the skin slightly lateral to the spine of each animal and a custom made tunnelling device was used to create four subcutaneous pockets. One construct (from each of the four groups) was

placed in each pocket. Each construct was implanted in a balanced manner, such that each group contained an implant placed at each of the subcutaneous locations and samples were randomly distributed across the operated animals. Once the four constructs were implanted, incisions were closed using suture and wound clips.

6.2.5 Bioluminescent imaging (BLI)

Two rats received constructs with aggregates formed from GFP-Luc labelled human MSCs (as discussed above) and were maintained under anaesthesia to perform day 0 BLI.

BLI was performed on the animals at day 0, 7, 14, and 21, following a previously developed approach (Allen et al., 2014). Briefly rats were anesthetised using isoflurane and 300 µL luciferin was injected subcutaneously in close proximity to the construct site. After 30 minutes animals were positioned with their lateral side facing up and scanned using an IVIS Lumina machine (Caliper Life Sciences, Hopkinton, MA, USA). The animals were then repositioned so that their other side could be scanned. BLI images were evaluated by demarcation of a 4 cm² elliptical region of interest (ROI) centred on each construct using Living Image software version 3.2 (Caliper Life Sciences). BLI counts were normalised by exposure time and region of interest (ROI) for each sample.

6.2.6 Microcomputed Tomography Imaging

At 4 weeks post-surgery, eight rats (each rat contained the four groups) underwent a vascular perfusion protocol modified from that developed by Duvall et al. (Duvall et al., 2004; Allen et al., 2014). Briefly, the rats were put under anaesthesia

and maintained at 4% isoflurane. Once anesthetised, the thoracic cavity was opened to insert an 18 gauge catheter (SURFLO Teflon IV catheter; Terumo Medical; Somerset, NJ) through the left ventricle of the heart into the ascending aorta. The inferior cava was cut and 0.9% saline was perfused through the vasculature using a peristaltic pump (Masterflex, Cole Parmer, Vernon Hills, IL) until the vasculature system was completely flushed clear. A solution of 0.9% saline containing 0.4% (w/v) papaverin hydrochloride was then perfused followed by 10% Neutral Buffered Formalin (NBF) for 5 minutes. Animals received a final perfusion of 20-25 mL of radiopaque contrast agent Microfil (Flow Tech, Carver, MA) and were left at 4°C overnight. In this way, animals were euthanised by the combined effects of isoflurane overdose and exsanguination. Explants were extracted and incubated in NBF for 24 hours before being imaged via micro-computed tomography (μ CT) scans on a MicroCT42 (Scanco Medical, Brüttisellen, Switzerland) at 55 kVp, 145 μ A, and a 12 μ m voxel size. The volume of interest was defined as the construct and the minimal tissue surrounding the construct. Microfil has the same threshold as bone mineral and therefore to segment perfused vasculature from mineralised tissue within each construct two scans were analysed; calcified construct versus decalcified construct. The calcified constructs were scanned and post-processed using a threshold value that accurately depicted both the mineral content and the vessel volume by visual inspection of the 2D grayscale tomograms (Scanco Medical MicroCT42). Noise was removed using a low-pass Gaussian filter ($\sigma=1.2$, support=2). Next, samples were decalcified in Immunocal (Formic acid bone Decalcifier, Decal Chemical Corporation) for 1 week with the decalcification solution replaced every day (decalcified constructs). After one week these decalcified constructs were scanned using the same settings, and post-processed at the same threshold as the calcified

constructs to determine mineral content. Mineralised tissue content was determined by subtracting the bone volume of the decalcified scans from the calcified scans. Next, the decalcified scans were post-processed at a threshold value that accurately depicted just the vessel volume upon visual inspection of the 2D grayscale tomograms.

6.2.7 Histochemical analysis

Following μ CT scanning the samples were dehydrated and embedded in paraffin using an automatic tissue processor (Excelsior ES tissue processor, Thermo Scientific; Austin, TX). All samples were sectioned with a thickness of 8 μ m using a rotary microtome (Leica Microtome RM2235, Leica). Sections were stained with Masson's Trichrome and Alizarin Red (all Sigma Aldrich).

6.2.8 Immunohistochemical Analysis

Immunohistochemical analysis was used to detect CD31, CD146 and α -smooth muscle actin. Sections were deparaffinised overnight before a series of rehydration steps through varying ethanol grades (100-50%). The samples were then treated with 40 μ g/mL of proteinase K for 20 minutes at 37 °C (Sigma Aldrich), rinsed with PBSTween and blocked with PBS with 1% w/v Bovine Serum Albumin (BSA) and 3% w/v Normal Goat serum (NGS) (Sigma Aldrich) for 60 minutes. Sections were then incubated overnight at 4°C with either rabbit polyclonal anti-CD31 (ab28364 Abcam, 1:50) or rabbit monoclonal anti-CD146 (ab75769 Abcam, 1:250). After an additional three washing steps with PBS containing 1% w/v BSA the sections were incubated with Dylight488 goat anti-Rabbit secondary antibody (Jackson Immunoresearch, 115-485-209, 1/200), for one hour at room temperature in the dark.

The samples were washed three times in PBS with 1% w/v BSA, and the slides were then incubated overnight at 4 °C with mouse monoclonal anti-alpha smooth muscle actin antibody (ab7817 Abcam, 1:50). After an additional three washing steps with PBS with 1% w/v BSA the sections were incubated with Dylight549 goat anti-mouse secondary antibody (Jackson Immunoresearch, 115-505-062, 1/200), for one hour at room temperature in the dark. Finally, samples were washed three times with PBS with 1% w/v BSA and the sections were mounted using 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Sigma Aldrich). HUVECs provided a positive control for CD31 and CD146 staining and blood vessels provided positive control for α -smooth actin staining.

6.2.9 Statistical Analysis

Results are expressed as mean \pm standard error. All CT quantitative analyses were examined using one-way analyses of variance (ANOVA) with the addition of Tukey's correction for multiple comparisons testing. BLI quantitative analysis was examined using two-way analyses of variance (ANOVA) with the addition of Tukey's correction for multiple comparisons testing. All analyses were performed using Graphpad. For all comparisons, the level of significance was $p \leq 0.05$.

6.3 Results

6.3.1 Construct Morphology

All aggregates were identifiable as a clear circular bundle of cells present within the nanofiber mesh (as indicated by the letter A in Figure 6.2) after 4 weeks *in vivo*.

The aggregates were predominately surrounded by alginate and host cells (see Figure 6.2). As expected there was some degradation in the constructs, which was very evident in the Cartilage Template and the Co-culture Cartilage Template groups, as indicated by the channels present within the centre of the aggregates (indicated by the letter D), but in the Prevascularised Cartilage Template group the degradation was minimal (Figure 6.2). There was also positive sGAG staining present in all of the aggregates. Both the Cartilage Template and Co-culture cartilage groups also had a layer of tissue rich in sGAG surrounding the aggregate (see Figure 6.2, as indicated by the letter C), that was negligible in the Prevascularised Cartilage Template group. There was also a fibrous tissue layer surrounding the aggregates in both the Cartilage Template and Co-culture Cartilage template layer (as indicated by the red staining in Figure 6.2) which was not seen in the Prevascularised Cartilage Template group.

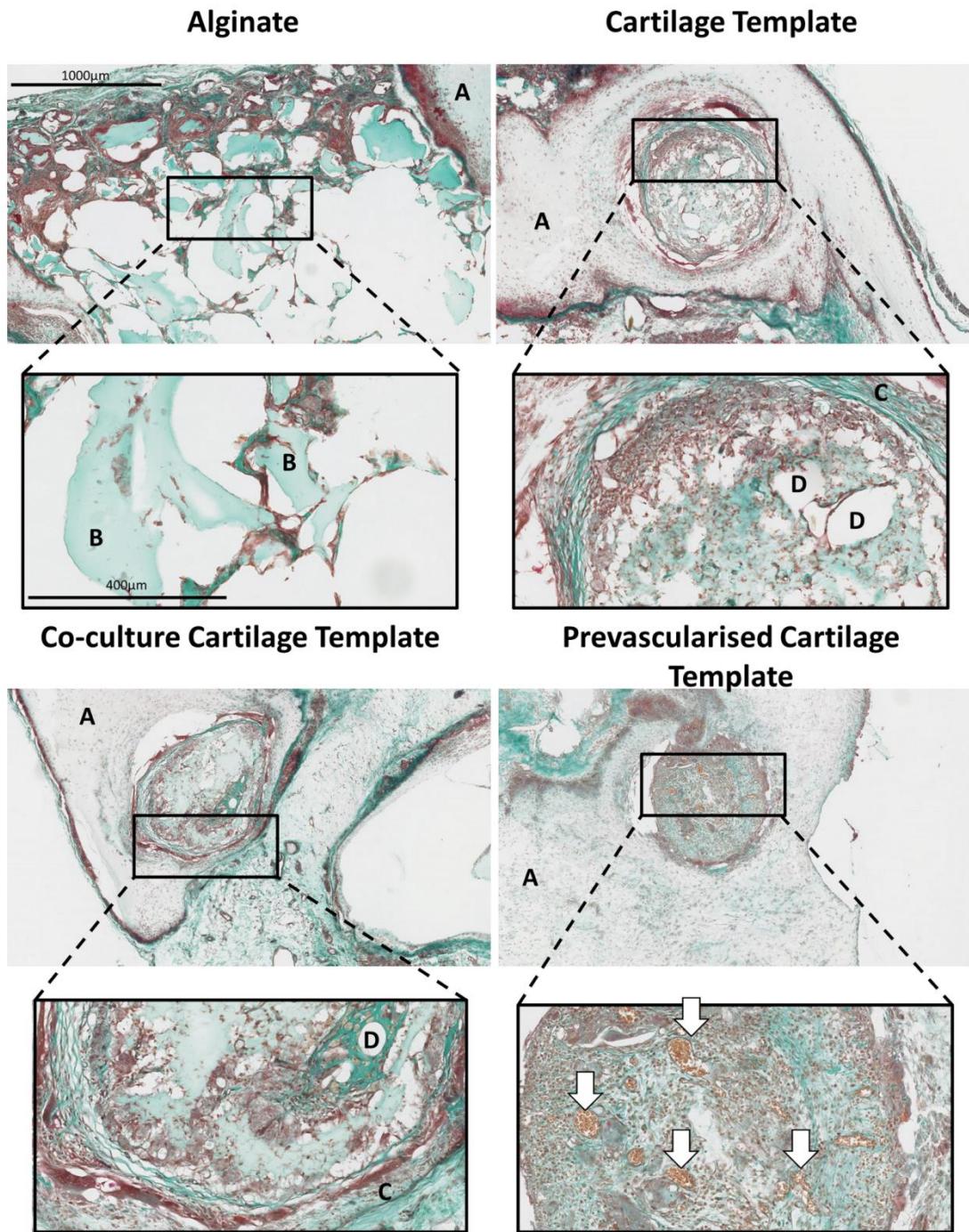


Figure 6.2: Masson's Trichrome of samples from each of the groups after 4 weeks implantation. A denotes nanofiber mesh, B denotes islands of alginate, C denotes sGAG rich encapsulation, D denotes area of degradation and arrows denote vessels complete with red blood cells. Images were taken at 5X and 20X.

6.3.2 Mineral Formation

Quantitative mineralisation of the constructs and the surrounding tissue in the hydrogel was analysed from the reconstructed μ CT data to determine Mineral Volume. All groups produced mineral volume between 0.5-0.8 mm³, however there was no significant difference between any of the groups after 4 weeks of implantation (See Figure 6.3).

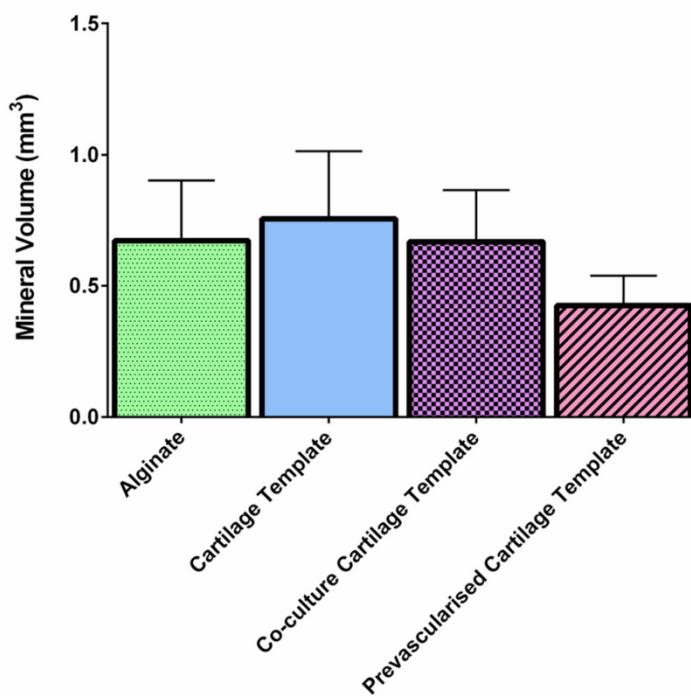


Figure 6.3: Total Mineral Volume. Error bars denote standard error ($n = 8$).

Positive Alizarin Red staining was present in all of the groups, however the location of the mineral differed by group. The only group to have mineralisation nodules present within the aggregate itself was the Prevascularised Cartilage Template group. All of the other groups only had mineralisation nodules present in the surrounding alginate, as seen in Figure 6.4. Some of these mineralisation nodules were

present in close proximity to mature blood vessels, as indicated in Figure 6.4 by the arrows.

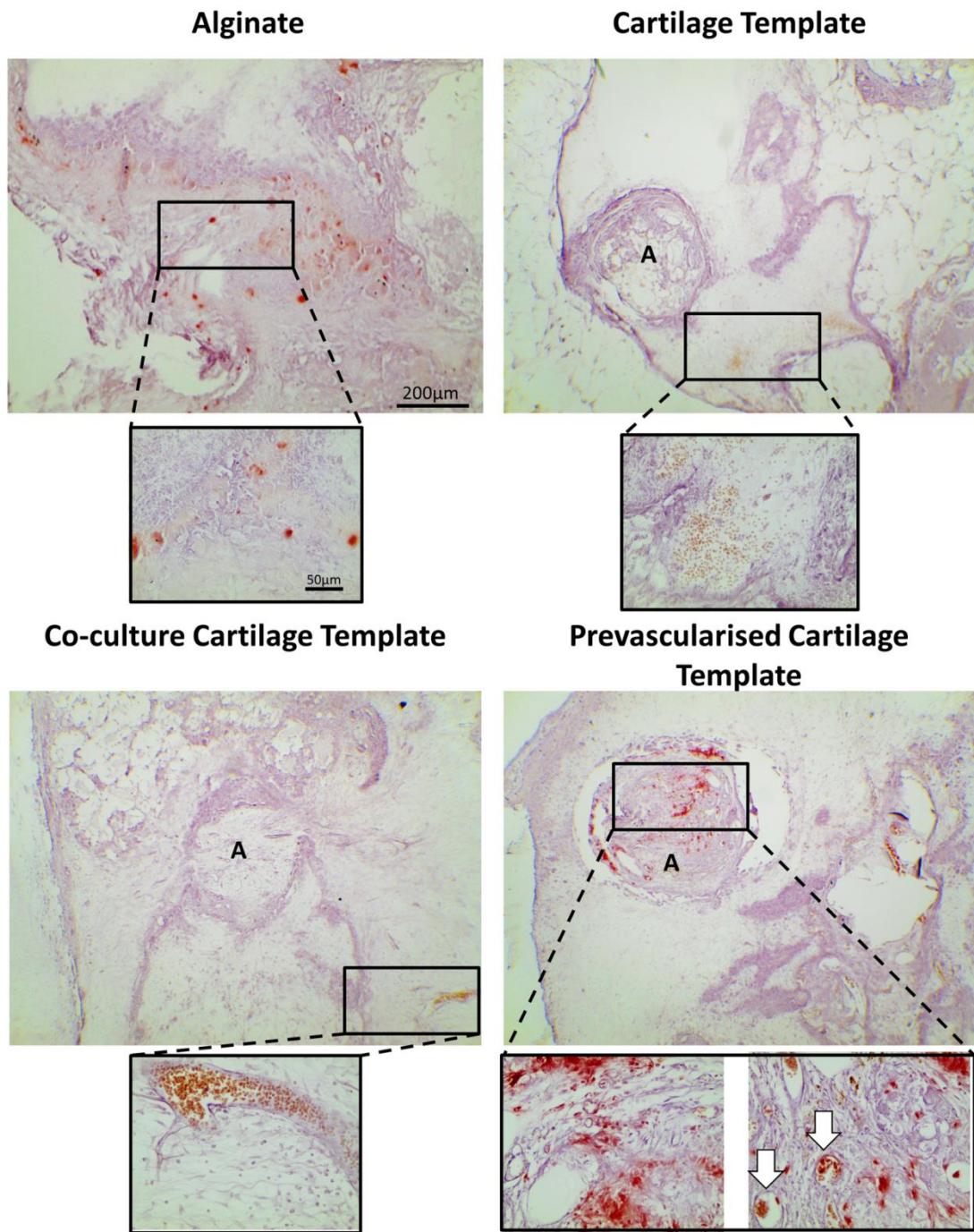


Figure 6.4: Alizarin Red staining of samples from each of the groups after 4 weeks implantation. Red Staining denotes mineralisation nodules present, A denotes

aggregates present within the alginate, and arrows denote vessels present. Images were taken at 4X and 40X.

6.3.3 Cell Viability

BLI data obtained over the course of the study showed that the live cell number from the original cellular aggregate decreased in all groups from the day of surgery to 2 weeks after implantation (see Figure 6.5 (A)). However, there was significantly higher BLI signal in the Prevascularised Cartilage Template group and Co-culture Cartilage Template group ($p < 0.05$) compared to the Alginate group at both day 0 and day 7. The Prevascularised Cartilage Template group also retained more cells compared to the other groups at day 7 (88% vs. 82-20.5%) and day 14 (27.4% vs. 18.3-1.7%) and by day 21. There were more viable human MSCs present in the Prevascularised Cartilage Template group compared to both the Alginate ($p = 0.1$) and Co-culture Cartilage Template groups ($p = 0.13$) (see Figure 6.5 (B)).

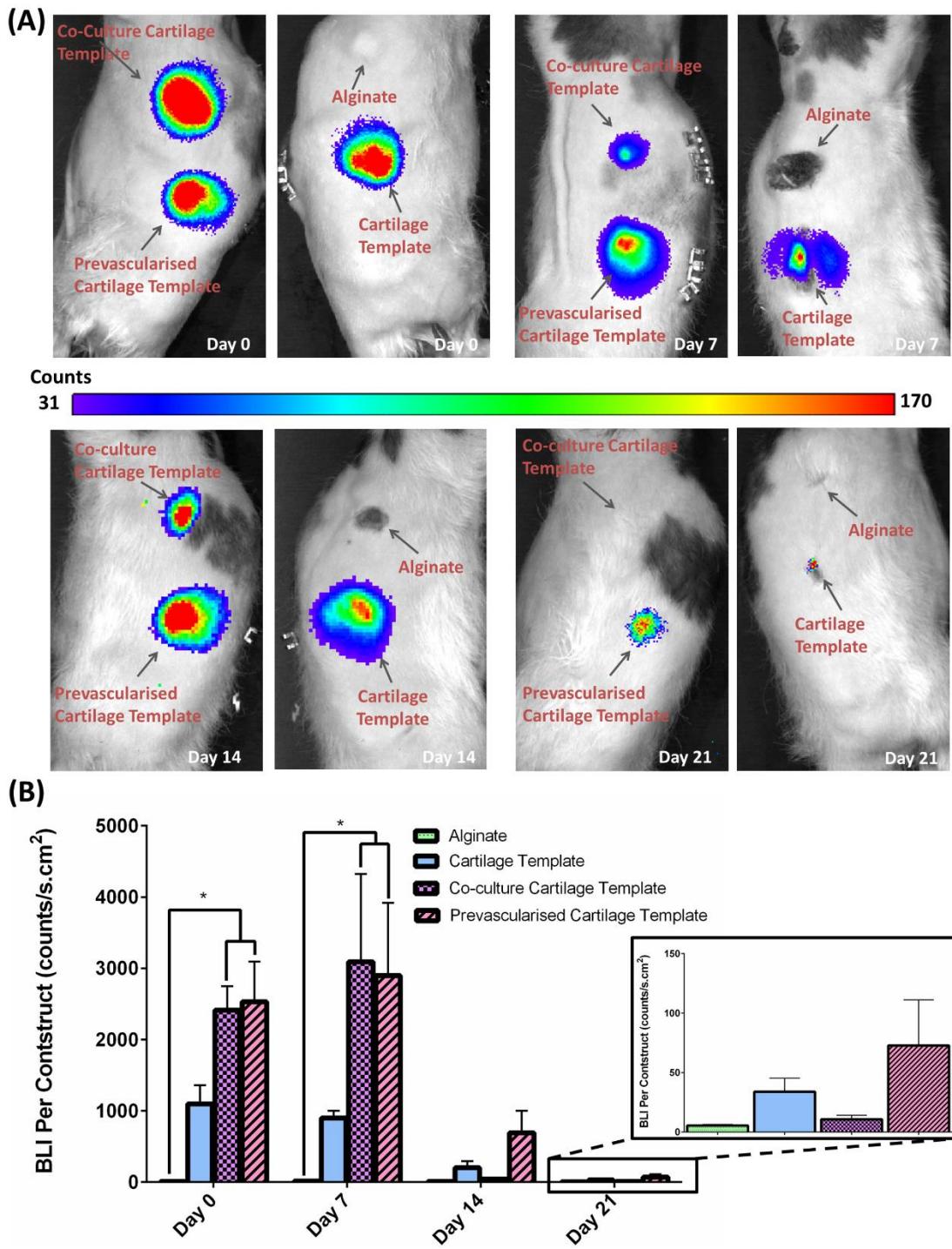


Figure 6.5: (A) Representative BLI heat-maps for representative rat over the time course of the study (B) Total BLI count of all the groups over the course of the study.

6.3.4 Vessel Infiltration

μ CT reconstruction of the explant vasculature illustrated the presence of host blood vessels surrounding the construct and infiltrating the construct through the holes present within the nanofiber mesh (Figure 6.7 (A)). Vessel volume was quantified in two ways; (1) Total Vessel Volume and (2) Average Vessel Diameter. After four weeks *in vivo* there was no significant difference in Total Vessel Volume or Average Vessel Diameter between any of the groups (Figure 6.6 (A, B)). However, for both the Cartilage Template and the Prevascularised Cartilage template groups a large proportion of the vessels were thicker than 0.15 mm. When only vessels with a diameter greater than 0.15 mm are considered, in both the alginate and the Co-culture Cartilage Template group only 3 out of 8 rats had vessels greater than 0.15 mm. These vessels only accounted for 7% of the overall vessels within the construct. However, in both the Cartilage Template and Prevascularised Cartilage Template group 5 out of 8 rats had vessels present with a greater diameter than 0.15 mm and these vessels accounted for up to 14% of the overall vessels seen within the construct (see Figure 6.7 (B)).

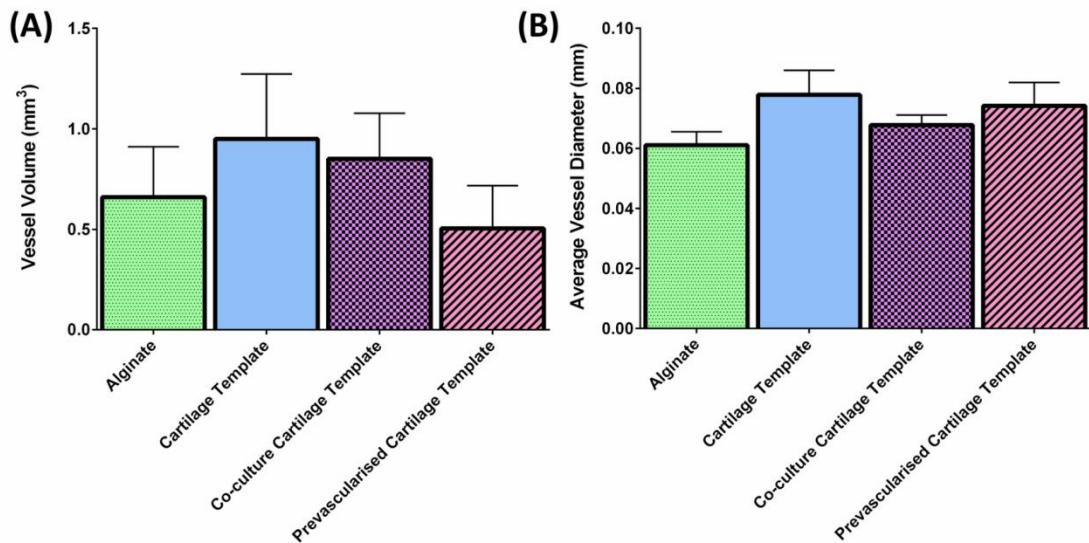


Figure 6.6: (A) Total Vessel volume, and (B) Average Vessel Diameter demonstrating the level of vessel formation within the implanted constructs after 4 weeks ($n = 8$).

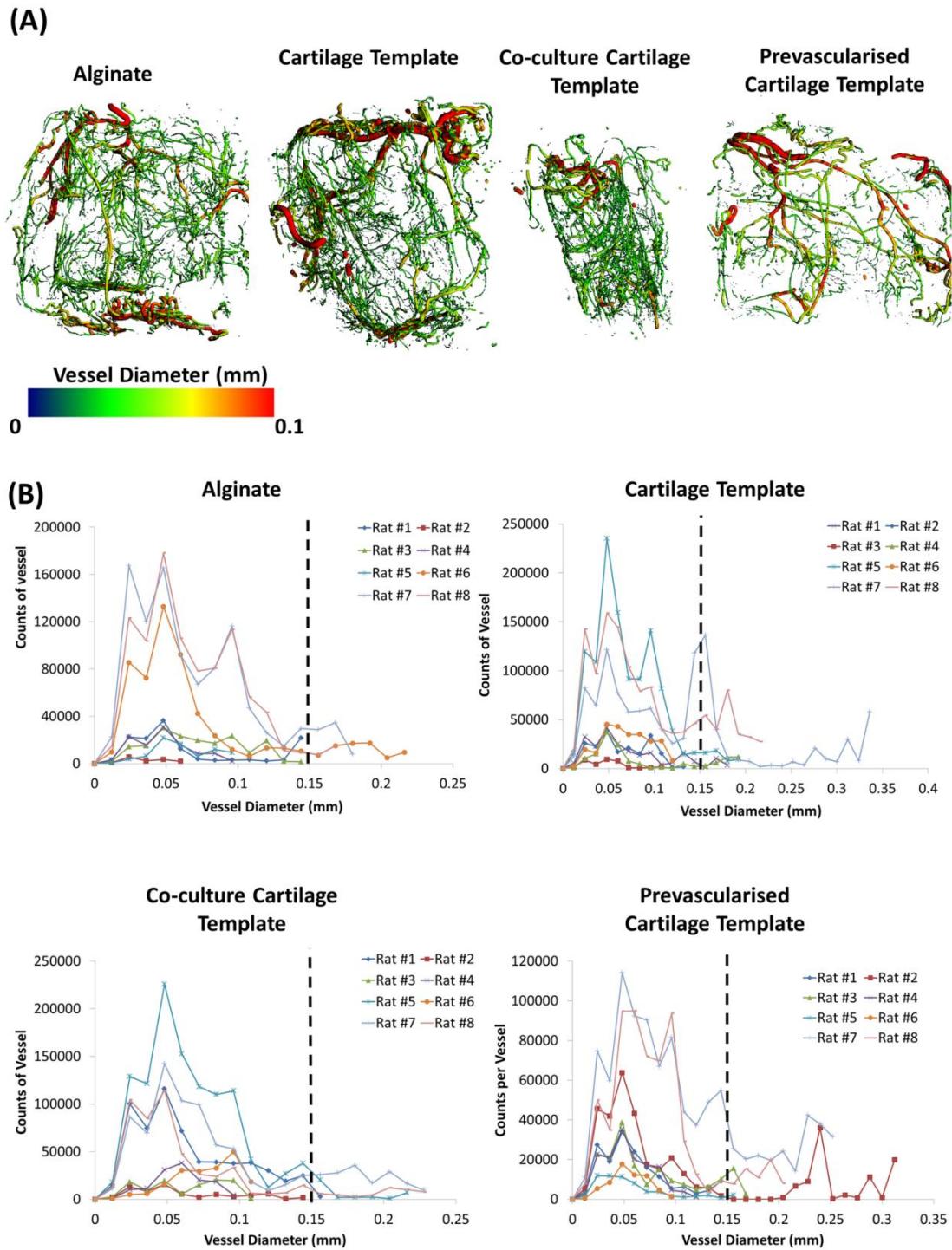


Figure 6.7: (A) Microcomputed tomography angiography representative images of vessel diameter and (B) Histograms of Vessel Diameter for each group demonstrating location of the varying vessel thickness of the vessels present within each of the implanted constructs after 4 weeks.

Histological staining revealed that there was little vessel formation present within the Cartilage Template and Co-culture Cartilage Template aggregates, but most of the vessels were found outside the aggregates within the alginate (see Figure 6.2). However, the Prevascularised Cartilage Template group was the only group to contain vessels within the aggregate itself complete with red blood cells (see Figure 6.2 denoted by the arrows).

Immunohistochemical analysis confirmed that mature vessels were present in the surrounding alginate in all of the groups, as indicated by the α -smooth actin staining. In contrast, the Prevascularised Cartilage Template group had mature vessels present within the centre of the aggregates, rather than around the periphery in the surrounding alginate. CD146 and CD31 staining, which are an endothelial cell marker and pericyte marker respectively, revealed that, for both the Co-culture Cartilage Template and the Prevascularised Cartilage Template groups, endothelial cells and pericytes were involved in the formation of these vessels (see Figure 6.8 and Figure 6.9). Moreover, CD31 positive stained cells were present within the structure of some of the vessels (Figure 6.8) indicating that the implanted human endothelial stem cells were involved in the formation of these vessels as CD31 is only present in human endothelial cells. The staining also showed CD31 positive stained cells were not only present on the periphery of the aggregates but were also present within the surrounding alginate.

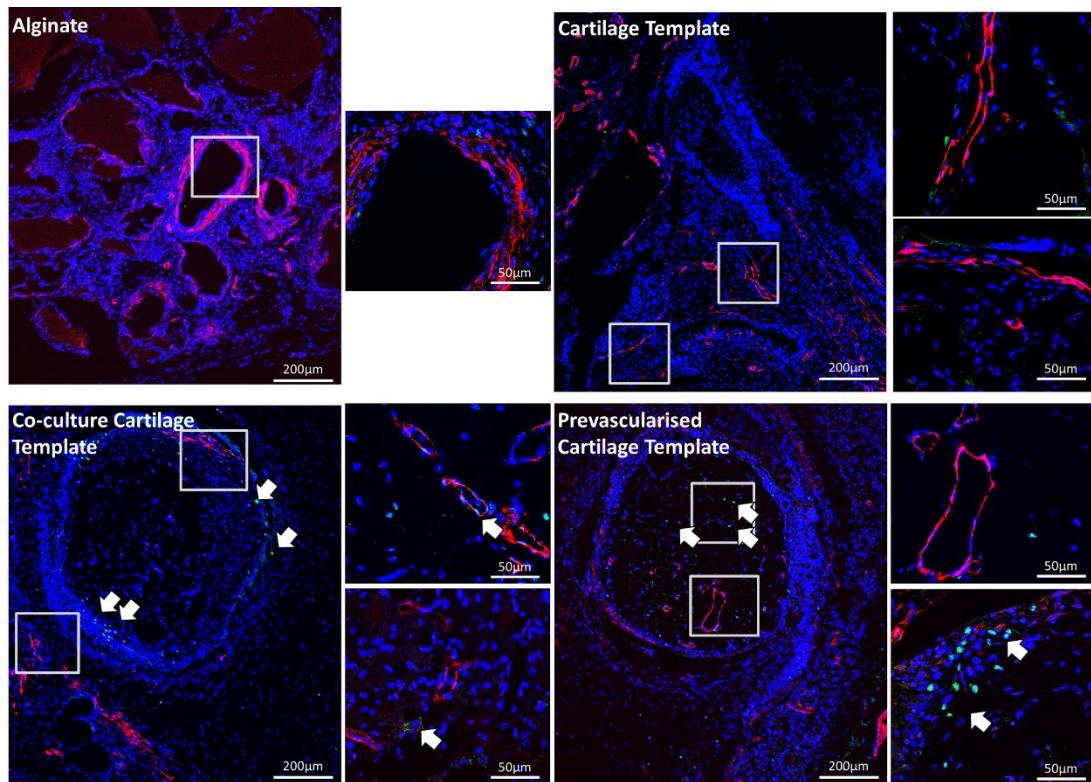


Figure 6.8: Immunohistochemical staining of the groups after 4 weeks implantation.

CD31 stained in green, Nucleus stained in blue, α -smooth actin stained in red. Boxes denote area of magnification. Arrows denote presence of CD31 (green) within vessel formation. Images were taken at 10X and 60X.

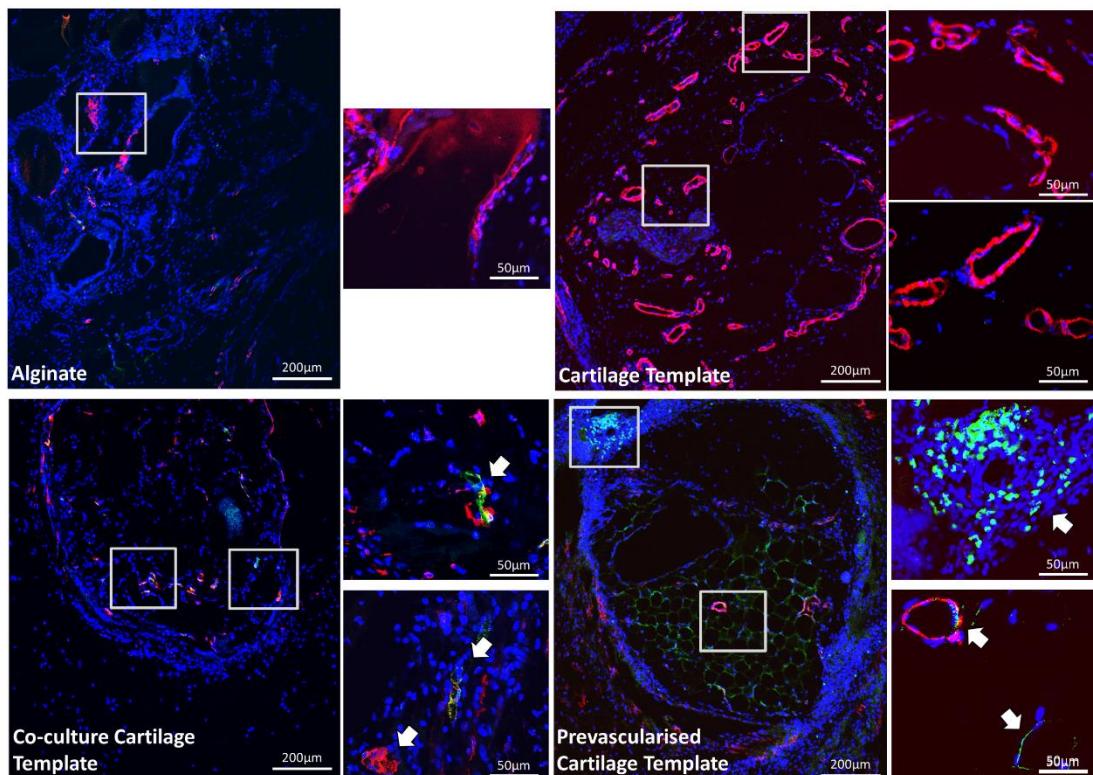


Figure 6.9: Immunohistochemical staining of the groups after 4 weeks implantation.

CD146 stained in green, Nucleus stained in blue, α -smooth actin stained in red. Boxes denote area of magnification. Arrows denote presence of CD146 within vessel formation. Images were taken at 10X and 60X.

6.4 Discussion

This Chapter investigated whether a tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage constructs, through the co-culture of HUVECs in vitro, would improve cell survival, vessel infiltration and thus mineral formation once implanted subcutaneously *in vivo*. The results from this Chapter showed that prevascularised cartilaginous aggregates successfully developed mature vessels (as indicated by α -smooth muscle actin walls and erythrocytes) within the

aggregates and retained viable cells from the original aggregate (as indicated by BLI signalling) 21 days after subcutaneous implantation. The prevascularised cartilaginous aggregates were also the only aggregates to have mineralisation nodules present within the aggregates. In contrast, chondrogenically primed aggregates, with and without HUVECS, did not have viable cells remaining from the original aggregate after 14 days, had a high vessel volume but these vessels were not present within the aggregate, and only had mineralisation nodules present in the alginate surrounding the aggregates.

One limitation of the study is the use of pooled MSCs from two male donors and the fact that the author did not directly explore whether the human MSCs displayed a donor dependent response to mineral formation. However, as the control groups also contained pooled cells, the differences observed between the groups cannot be explained by donor variability. A second potential limitation was the length of time the samples were examined for mineral formation (4 weeks). Previous ectopic bone formation models indicate that little to no bone formation will occur until approximately 8 weeks (Farrell et al., 2011; Farrell et al., 2009; Jukes et al., 2008; Oliveira et al., 2009b; Scotti et al., 2013; Scotti et al., 2010). The choice of the 4 week time point permitted the observation of both early mineral formation and vessel infiltration, and differences were distinguishable at this early time point. Future studies should investigate the long-term effect of subcutaneous implantation of the prevascularised cartilaginous aggregates in order to fully understand their mineralisation potential. Another possible limitation was the nanofiber mesh/alginate delivery system with osteogenic growth factors (BMP-2) used to ensure the retrieval of the aggregates after 4 weeks *in vivo*, which has proved challenging in other subcutaneous implantation studies (Farrell et al., 2011; Farrell et al., 2009; Scotti et

al., 2010). However, due to the fact that an acellular control group was included, the results obtained could not be explained by the addition of BMP-2 alone, but rather the addition of the cells leads directly to the differences seen between the groups. Finally, the animal model chosen for this study was a subcutaneous delivery method. This animal model has its advantages and disadvantages. The main advantages to using the subcutaneous model was that it was a relatively non-invasive surgery for the animals, all four groups could be placed in each animal which reduced animal numbers, and it provided an initial understanding of the in vivo potential of the experimental groups. There are also some disadvantages to subcutaneous implantation, it has large animal variability, the exact location of the construct cannot be kept constant and as such some constructs could be placed closer to host vessels than other and therefore further increase the variability between the groups. However, for the purpose of this study, the subcutaneous model provided an initial understanding of the in vivo potential of my aggregates. Further studies are needed to investigate the bone regeneration potential in a large bone segmental defect. As stated previously, current bone tissue engineering strategies are limited by the lack of nutrient delivery and waste removal arising from the lack of vasculature (Amini et al., 2012; O'Brien, 2011; Lyons et al., 2010; Phelps and Garcia, 2009; Ko et al., 2007; Krishnan et al., 2014; Farrell et al., 2011; Farrell et al., 2009). The results from Chapter 4 and 5 reported that a combination of chondrogenic priming and co-culture of human MSCs and HUVECs can lead to the formation of rudimentary vessels and significantly increased the in vitro osteogenic potential of MSC aggregates (Freeman et al., 2015b). Other studies have investigated whether pre-vascularising trabecular bone (Correia et al., 2011), PCL (Ghanaati et al., 2011), poly(LLA-co-DXO) (Pedersen et al., 2013), collagen GAG (McFadden et al., 2013; Duffy et al., 2011), and hydroxyapatite (Scherberich et al.,

2007) scaffolds in vitro would allow faster angiogenesis in vivo and have reported that microvascular networks established in vitro can be maintained when implanted in vivo (Correia et al., 2011; Scherberich et al., 2007; Pedersen et al., 2013; Duffy et al., 2011; McFadden et al., 2013; Ghanaati et al., 2011). In this Chapter the author investigated whether prevascularisation of chondrogenically primed constructs in vitro prior to implantation could overcome limitations of vascularisation and thus core degradation and uneven mineral distribution. The results from this Chapter show that vessel formation was achieved within the constructs of all groups after 4 weeks implantation (as indicated by μ CT angiography, Masson's Trichrome and α -smooth actin staining). However, the only group to have vessel formation within the aggregates, and not just in the surrounding alginate or in the periphery of the aggregate, was the prevascularised cartilaginous aggregates. This may be due to the fact that the vessels present within the centre of the constructs were mature vessels complete with a smooth muscle lining (as indicated by α -smooth actin staining) and red blood cells (as indicated by Masson's Trichrome). The prevascularised cartilaginous aggregates also had the thickest vessel diameters present within the constructs as a whole (as indicated by μ CT angiography), with 5 out of 8 of the rats having vessels with diameters between 0.15 – 0.35 mm and these vessels accounted for up to 14% of the overall vessels present within the constructs. Moreover, the prevascularised cartilaginous aggregates were the only group to have significantly more viable cells 21 days after implantation than other groups. Previous studies have only shown maintenance of viable MSCs to 7 days (Allen et al., 2014). These results indicate that prevascularisation of the cartilaginous aggregates prior to implantation exerts a positive effect on maintenance of MSC viability in aggregates implanted for 4 weeks.

in vivo, and this is directly associated with the formation of mature vessels present within the centre of the aggregates.

Previous studies have shown that MSCs themselves are a perivascular cell type (Caplan, 2008; Crisan et al., 2008; Melero-Martin et al., 2008), and have been shown to have pro-angiographic effects on endothelial cells when co-cultured in vitro (Hung et al., 2007; Freeman et al., 2015b). The results presented in this Chapter show the perivascular role of MSCs in vivo, through the CD146 staining, known surface marker for pericytes. The results also show MSCs have a pro-angiographic effect on endothelial cells, as the only group to form vessels within the cellular aggregates were the prevascularised cartilaginous group, which had both MSCs and HUVECs added to the cartilage template. Interestingly, immunohistochemical staining also revealed that the HUVEC cells that were added to the already formed cartilage template (in the Co-culture Cartilage Template group and the Prevascularised Cartilage Template group) were not just present around the periphery of the aggregates but were also present within the surrounding alginate. Moreover these HUVECs were shown to play a role in the formation of the mature vessels and integrate with the host cells to form vessels (as indicated by CD31 staining). However, whether it is the human MSCs added during the co-culture or the human MSCs used to form the cartilage template, that are involved in the formation of these vessels is still unknown. The CD31 stain used was specific for human cells and the persistent staining by 4 weeks after implantation confirms that human cells did persist and may be involved in the formation of the vascular networks but further studies are needed to elucidate exactly which exact cells are involved in forming the vessels observed here.

Unlike other studies (Correia et al., 2011; Scherberich et al., 2007; Pedersen et al., 2013; Duffy et al., 2011; McFadden et al., 2013; Ghanaati et al., 2011) a scaffold was not used in this study. The current limitations to scaffold tissue engineering studies include inhomogeneous distribution of cells within the construct (Chan and Leong, 2008). This uneven distribution can then lead to heterogeneous properties, fibrous tissue encapsulation (Lyons et al., 2010), and core degradation of the construct, which ultimately leads to the degradation of the scaffold itself (Amini et al., 2012; O'Brien, 2011; Lyons et al., 2010; Phelps and Garcia, 2009; Ko et al., 2007; Krishnan et al., 2014; Farrell et al., 2011; Farrell et al., 2009). The approach presented in the Chapter allows the cells to form their own scaffold, mimicking native endochondral ossification, therefore ameliorating inhomogeneous distribution of cells. The results from Chapter 4 and 5 showed that chondrogenically priming MSCs in vitro, to form a cartilage template, provides a suitable scaffold for HUVEC and MSC cells to attach, proliferate, infiltrate, and ultimately form rudimentary vessels (Freeman et al., 2015b). This study not only verifies the benefits of this scaffoldless approach but shows that, even after being implanted for 4 weeks, there was minimal core degradation in the cartilage template of the prevascularised cartilaginous aggregates. In contrast, the non-prevascularised groups had fibrous tissue present surrounding the aggregates, which can lead to hypertrophy of the cells in the centre of the aggregate and hence core degradation (seen in the Masson's Trichrome).

As previously described, angiogenesis only occurs once the cartilage template has formed, during endochondral ossification. This process involves endothelial cells invading through the cartilage canals already present in the developing bone tissue (Mackie et al., 2008; McNamara, 2011; Kronenberg, 2003; Gerber and Ferrara, 2000), and typically occurs between 14 and 18 days of embryogenesis of mice (Gerber and

Ferrara, 2000; Carlevaro et al., 2000). Therefore, in order for mineralisation to occur, the cartilage template must be formed, and vessel infiltration must then occur. Previous studies, which have looked at just the formation of the cartilage template through the subcutaneous implantation of either chondrogenically primed construct (Jukes et al., 2008; Farrell et al., 2011; Farrell et al., 2009; Oliveira et al., 2009b) or hypertrophic constructs (Scotti et al., 2013; Scotti et al., 2010), have found little to no mineral formation before 8 weeks *in vivo*. This study found that there was mineralisation present in all of the groups after 4 weeks in this ectopic bone model. Alizarin Red staining of the groups also shows that mineralisation nodules were present predominately in the surrounding Alginate. As the alginate contained BMP-2 this was to be expected. However, the only group that had mineralisation nodules present within the centre of the aggregates was the prevascularised cartilaginous aggregates. The results from Chapter 4 found that when both MSCs and HUVECs were added to a chondrogenically primed aggregate, mineralisation was characterised by the formation of discrete mineralised nodules (Freeman et al., 2015b). The results from Chapter 5 found that the same group cultured without osteogenic supplements showed mineralisation present throughout the aggregate. Even though the aggregates used in this study were co-cultured without the presence of osteogenic supplements, the mineral was found to deposit in the formation of discrete mineralisation nodules similar to those seen in Chapter 4, rather than homogenous mineralisation deposition seen in Chapter 5. Previous studies (Correia et al., 2011; Gerber and Ferrara, 2000; Freeman et al., 2015b) have postulated that, in order to mimic bone formation that occurs naturally during the early fetal development, vasculogenesis should be induced prior to osteogenesis *in vitro* in order to obtain functional bone tissue when implanted *in vivo*. The results from this study are in agreement as the only aggregates to have

mineralisation nodules present within the aggregates were also the only group to have mature vessels present within the aggregate. The author proposes that during the process of vessel formation mineralisation did not proceed, and as a result mineralisation was not seen in the chondrogenically primed aggregates, without and with HUVECS alone, as vascularisation was not induced in these aggregates. However, mineralisation nodules were beginning to form in the prevascularised group but only once mature vessels had formed within the aggregates. Moreover, mineralisation only occurred within close proximately of these vessels. Therefore, it is possible that culturing this group *in vivo* for longer than 4 weeks will ultimately allow for enhanced mineralisation, but this cannot be verified from the results of the current study and future *in vivo* investigations are required.

6.5 Conclusions

This Chapter shows for the first time that a tissue regeneration approach incorporating both chondrogenic priming of MSCs, to first form a cartilage template and subsequent pre-vascularisation of the cartilage constructs, through the co-culture of HUVECs and MSCs *in vitro*, improves cell viability, vessel infiltration and mineral formation once implanted *in vivo*. Specifically, the results from this study show that the only group to have mature vessels present within the aggregates after 4 weeks *in vivo* was the prevascularised cartilaginous aggregates. The author proposes that this vascularisation exerted a positive effect on the viability and mineralisation potential of the aggregate, as it was also the only group to have both viable cells 21 days after implantation and mineralisation nodules present within the aggregates. Taken together, these results indicate that, endochondrally priming constructs might have a

beneficial role on the constructs once implanted *in vivo*, however further studies are needed to fully verify this. Future bone tissue engineering strategies could be designed with these conditions in mind such that the factors needed to mimic the endochondral ossification process are incorporated to the point where the constructs themselves can autonomously progress to engraftment, remodelling and ultimately tissue regeneration.

Chapter 7: Discussion and Conclusion

7.1 Introduction

This Chapter summarises the main findings of this Thesis, drawing together the insight gained from the *in vitro* and *in vivo* studies performed to provide a greater understanding of whether an *in vitro* tissue regeneration approach that mimics endochondral ossification could serve as effective bone tissue engineering approach. The results of the individual Chapters of this Thesis are summarised in Section 7.2 and are graphically represented in Figure 7.1. This figure also illustrates how these results build upon the current understanding of this topic from studies conducted by other researchers prior to this Thesis, as is discussed in detail in Section 7.3. The key findings of the Thesis are discussed in the context of other relevant studies, and the implications of the findings of this Thesis for the field of bone tissue regeneration, and clinical treatment of non-union fractures are explored. Finally, recommendations for further work and future perspectives in the bone tissue engineering field are discussed.

7.2 Main Findings of the Thesis

The research described in this PhD Thesis has sought to identify whether an *in vitro* priming approach, which mimics certain aspects of the endochondral ossification process, can enhance the mineralisation potential of MSCs both *in vitro* and *in vivo*. In particular the results from this Thesis sought to determine the optimum priming conditions (timing, type of culture medium) for enhancing osteogenesis, angiogenesis and survivability once implanted *in vivo*. *In vitro* cell culture experiments were conducted to determine the optimum chondrogenic and vascular priming conditions

in vitro. An in vivo model was then used to investigate whether these optimum conditions had an effect on the survivability and thus vascularisation and mineralisation potential once implanted in vivo. The key contributions of each hypothesis are summarised below.

Hypothesis 1: There is an optimum duration for chondrogenic priming of MSCs that will enhance osteogenic differentiation of MSCs in vitro.

The first study, presented in Chapter 3 of this Thesis, demonstrated that chondrogenic priming for specific durations (14, 21 days), prior to being exposed to osteogenic supplements, can enhance the osteogenic differentiation of both murine MSCs and human MSCs. Chondrogenic priming also induced more mineralisation by MSCs compared to culture with osteogenic supplements alone. As such, the results of this study provided evidence that supported Hypothesis 1. Furthermore, it was demonstrated that chondrogenic priming for 21 days enhanced the distribution of mineral and resulted in an aggregate with mineralisation throughout, rather than around the periphery. By investigating different time periods for chondrogenic priming, this Chapter provided an insight into the optimum chondrogenic priming conditions needed to enhance osteogenic differentiation and thus produce a construct mineralised throughout the construct rather than just around the periphery. The optimum time (21 days) for chondrogenic priming determined from this Chapter was implemented in all of the subsequent studies investigated in this Thesis.

Hypothesis 2: A tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage aggregates, through the co-culture of HUVECs in vitro, will serve as an effective in vitro bone regeneration approach.

The second study, presented in Chapter 4, demonstrated that allowing MSCs to first form a cartilage template, not only enhances the mineralisation potential of the MSCs, but also provides a suitable template for both MSCs and HUVECs to attach, proliferate and invade. Furthermore, the results from this Chapter also showed that the highest amount of mineralisation was seen when HUVECs alone were added to the cartilage template, whereas the addition of both MSCs and HUVECs led to lower levels of mineralisation albeit that these were higher than the condition of chondrogenic priming alone. However, rudimentary vessels were only formed within the aggregates in which both MSCs and HUVECs were added to the already formed cartilage template. Together, the results obtained from this Chapter indicated that the application of both chondrogenic and vascular priming of MSCs enhanced the mineralisation potential of MSCs, compared to chondrogenic priming alone, whilst also allowing for vessel formation, and as such validated Hypothesis 2. More importantly, the results from this study indicated for the first time that there may be a trade-off between mineralisation and vascularisation for in vitro bone regeneration strategies, whereby osteogenic differentiation does not proceed during vascularisation of the template, at least for the time periods investigated during the course of this Thesis.

Hypothesis 3: An in vitro bone regeneration strategy that mimics the cellular niche of the endochondral template will provide an alternative strategy for in vitro mineralisation of MSCs, and thereby obviate the need for external osteogenic growth factors.

The third study of this Thesis, presented in Chapter 5, demonstrated that the application of both chondrogenic priming and vascular priming not only enhanced the mineralisation potential of MSCs in vitro, but also induced osteogenesis in MSCs

without the need for the addition of osteogenic supplements, and as such, the results of this study provided evidence in support of Hypothesis 3. Furthermore, similar to the results of Chapter 4, rudimentary vessels were only present in the aggregates in which both MSCs and HUVECs were added, even without the addition of osteogenic supplements. Unlike the results of Chapter 4, the results from this Chapter also showed that without the addition of osteogenic supplements the highest amount of mineralisation was seen in the group in which both HUVECs and MSCs were added. Moreover, the results actually indicated that the addition of osteogenic supplements inhibits early osteogenic markers (ALP expression), promotes late osteogenic markers (calcium content), and produces mineral deposition in the form of discrete mineralisation nodules. The results from this Chapter provided further insight into co-culture effect as the results also showed that this promotion of late osteogenic markers (Calcium content) also has an inhibitory effect on the vascularisation potential of the aggregates. This was seen in both VEGF expression, and the fact that the cross-sectional areas of the rudimentary vessels present within the aggregates were significantly smaller than those found in the same group without osteogenic supplements. Together, these results indicate that it is more beneficial to co-culture without osteogenic supplements as both vasculogenesis and osteogenesis is improved.

Hypothesis 4: A tissue regeneration approach that incorporates both chondrogenic priming of MSC aggregates and subsequent pre-vascularisation of the cartilage aggregates, will improve cell viability, vessel infiltration and thus mineral formation once implanted in vivo.

The final study of this Thesis, presented in Chapter 6, validated the in vivo potential of the endochondrally primed aggregates from Chapter 5, in an ectopic bone formation model. Specifically, the results from this Chapter demonstrated that

aggregates that had been both chondrogenically primed and prevascularised had enhanced cellular viability. Moreover, the results showed that, when the constructs were prevascularised in vitro prior to implantation, enhanced vessel infiltration and formation of mature vessels (smooth actin walls and erythrocytes) and mineralisation nodules were observed within the prevascularised aggregates, and as such corroborated Hypothesis 4.

These findings are further considered in the context of the current understanding of bone tissue engineering, and the optimum conditions needed to design a construct that will rapidly heal a large bone defects.

7.3 Implication for the Bone Tissue Engineering Field

The findings of this Thesis provide a novel insight into the priming conditions needed to produce constructs that can autonomously engraft, remodel, and ultimately lead to enhanced bone healing in a non-union defect. To date, many bone tissue engineering strategies have either designed scaffolds that mimic the mechanical properties of natural bone tissue (Heise et al., 1990; Sartoris et al., 1991; Marcacci et al., 1999; Elsinger and Leal, 1996; Ge et al., 2004; Mastrogiamomo et al., 2006; Kruyt et al., 2004; Rezwan et al., 2006; Ramay and Zhang, 2004; Quarto et al., 2001; Marcacci et al., 2007; Hibi et al., 2006; Meijer et al., 2007; Lee et al., 2010; Meijer et al., 2008; Shayesteh et al., 2008) or have designed scaffolds that incorporate the cells or necessary growth factors to induce osteogenesis and then degrade (Rose and Oreffo, 2002; Burg et al., 2000; Liu and Ma, 2004; Athanasiou et al., 1996; Prestwich and Matthew, 2002; Wang et al., 2006; Meinel et al., 2004a; Li et al., 2006b; Shin et al., 2004; Williams et al., 2005; Hollister, 2005; Yoshimoto et al., 2003; Meinel et al., 2005; Meinel et al., 2004b; Farrell et al., 2006; Farrell et al., 2007; Hofmann et al.,

2007; Martin et al., 1998; Lyons et al., 2010; Middleton and Tipton, 2000; Agrawal and Ray, 2001; Hutmacher, 2000; Rezwan et al., 2006; Wei and Ma, 2004; Zhang and Ma, 1999; Li et al., 2006a; Ma et al., 2001; Lyons et al., 2014; Murphy et al., 2014; Gleeson et al., 2010; d'Aquino et al., 2009). However, recently researchers have sought to design constructs that replicate aspects of the bone formation process that arise during bone development, specifically the formation of the cartilage template during endochondral ossification (Scotti et al., 2010; Scotti et al., 2013; Farrell et al., 2011; Farrell et al., 2009; Harada et al., 2014; van der Stok et al., 2014; Gawlitta et al., 2015; Visser et al., 2015) and prevascularisation of the construct (Correia et al., 2011; Ghanaati et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Scherberich et al., 2007; Duffy et al., 2011; Saleh et al., 2011; Fuchs et al., 2007). Each of these studies has advanced the understanding of in vitro and in vivo factors that can govern bone regeneration, see Figure 7.1.

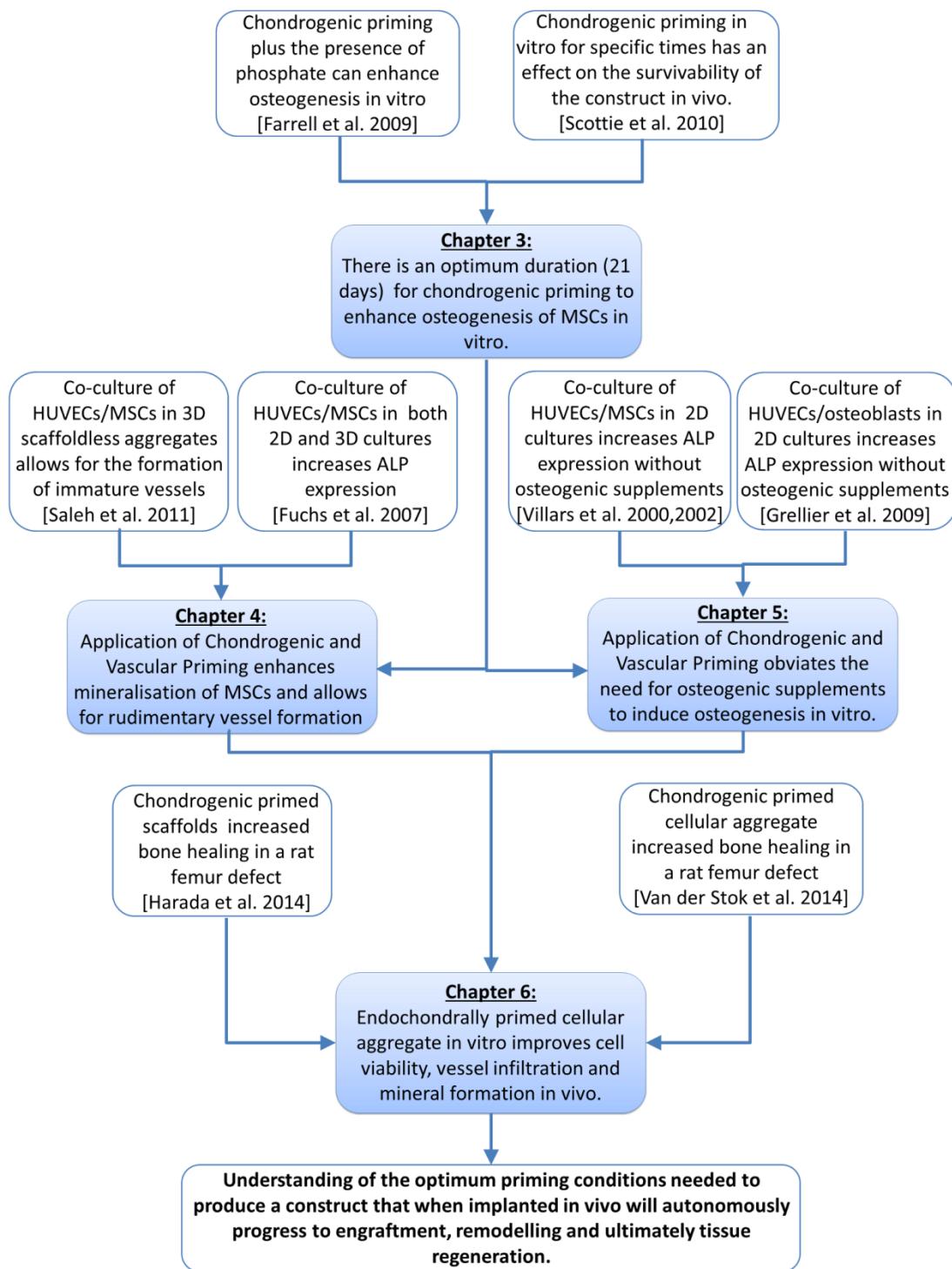


Figure 7.1: Graphical representation of the work conducted as part of this PhD Thesis in context with previous studies.

Farrell et al. was the first to investigate whether chondrogenic priming, for a period of 21 days, could enhance the osteogenic potential of MSCs in vitro. The results

of this study could not distinguish any significant differences in the expression/production of osteogenic markers (ALP, Collagen type II) between the chondrogenically primed group and the osteogenic group (Farrell et al., 2009). Scotti et al. reported that the duration of the chondrogenic priming period had a significant effect on the in vivo survivability (Scotti et al., 2010), but did not explore the effects of different time periods for osteogenic differentiation of MSCs in vitro. The results of Chapter 3 of this Thesis demonstrated the importance of the duration of chondrogenic priming for in vitro osteogenic differentiation potential of both human and murine MSCs, and also mineral deposition within the aggregate, see Figure 7.2. Future studies could implement these findings by chondrogenically priming aggregates for times (14-21 days) deemed appropriate from this Chapter to avoid a bone tissue engineered construct with uneven mineral deposition in vitro.

One of the most common methodologies used to overcome the issue of core degradation is to prevascularise constructs prior to implantation (Correia et al., 2011; Ghanaati et al., 2011; Pedersen et al., 2013; McFadden et al., 2013; Scherberich et al., 2007; Duffy et al., 2011). However, to date, no approach has sought to incorporate both chondrogenesis and vascularization simultaneously to produce a pre-vascularized cartilaginous construct in vitro, even though both stages are crucial for bone development by endochondral ossification and during fracture healing in vivo. Therefore, despite the novel understanding gained from Chapter 3 and other important studies (Scotti et al., 2010; Scotti et al., 2013; Farrell et al., 2011; Farrell et al., 2009; Harada et al., 2014; van der Stok et al., 2014; Gawlitta et al., 2015; Visser et al., 2015), a clear deficiency in knowledge regarding the ideal priming conditions still existed. The experimental studies from both Chapter 4 and 5 aimed to address this deficit of knowledge. Indeed these studies demonstrated for the first time that the application of

both chondrogenic and vascular priming improved osteogenesis and vasculogenesis, compared to chondrogenic priming alone, see Figure 7.2. Moreover, the results from Chapter 5 indicate that direct co-culture can even promote osteogenesis without the use of any supplementary osteogenic growth factors, and that the addition of these supplements actually inhibits both vasculogenesis and osteogenesis. These results indicate that the application of both chondrogenic priming and vascular priming (through the co-culture of both MSCs and HUVECs) can provide a bone tissue engineered aggregate that is mineralised throughout, complete with rudimentary vessels, all without the use of any external osteogenic supplements *in vitro*. A summary of all the results generated due to the application of chondrogenic and vascular priming can be seen in Figure 7.2. However, whether endochondrally priming MSCs would lead to enhanced mineralisation once implanted *in vivo* was still unknown.

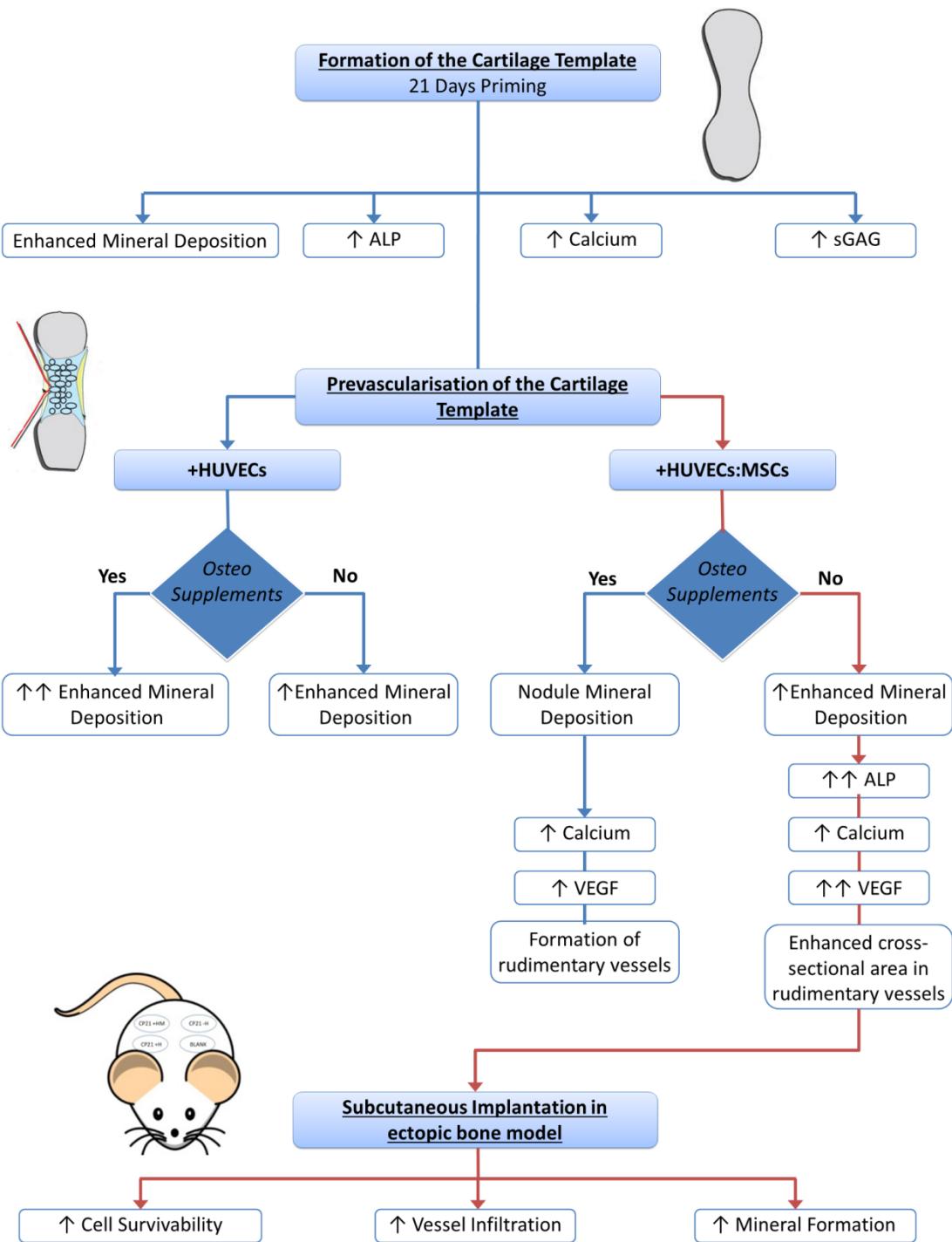


Figure 7.2: Graphical representation of cellular responses due to the application of chondrogenic and vascular priming in vitro as determined in Chapter 3, 4, 5 and 6.

Previous studies (Correia et al., 2011; Gerber and Ferrara, 2000; Freeman et al., 2015b) have postulated that, in order for bone to form in vivo vasculogenesis should

be induced prior to osteogenesis in vitro to obtain functional bone tissue when implanted in vivo. The results from Chapter 6 of this Thesis showed that the only aggregates to have mineralisation nodules present were the prevascularised cartilaginous aggregates. Moreover, the results from this Thesis show that they were also the only group to have viable human MSCs still present 21 days after implantation and mature vessels present within the aggregate. Taken together, the results from this Thesis found that endochondrally priming MSCs not only enhanced vasculogenesis and osteogenic differentiation potential of MSCs in vitro but also induced angiogenesis and thus mineralisation once implanted in vivo.

The provision of bone tissue constructs appropriate for clinical use are limited, due to issues with core degradation and an uneven distribution of bone mineral throughout the construct (Farrell et al., 2009; Scotti et al., 2010; Farrell et al., 2011). In particular no study has managed to design an in vitro bone tissue engineering construct that can ensure osteogenesis, angiogenesis and survivability once implanted in vivo. The individual studies reported in this Thesis built upon these previous studies and provided a novel understanding of potential endochondral priming conditions that might serve this purpose, as shown in Figure 7.1. When analysed together, the findings of these provide a novel insight into how future bone tissue engineered constructs might be designed.

7.4 Recommendations for Future Work

The studies described in this Thesis provide a novel insight on the optimum conditions needed to create a prevascularised mineralised aggregate that once implanted in vivo has enhanced survivability. Based on the findings of this Thesis the following recommendations are made for future research;

7.4.1 Experimental characterisation of the signalling pathways as a results of the co-culture of MSCs and HUVECs

The optimum conditions needed to enhance mineral deposition and vessel formation within a cellular aggregate both with (Chapter 4) and without (Chapter 5) the presence of osteogenic supplements were determined in this Thesis. Both studies showed that the addition of HUVECs and MSCs had a significant effect on the mineralisation and vascularisation potential of MSC aggregates. These studies also showed that this effect differed due to the inclusion or exclusion of osteogenic growth supplements. Future studies should investigate the exact signalling pathways that are activated due to the co-culture of HUVECs and MSCs both with and without the addition of osteogenic growth supplements to fully understand the direct cell-cell interaction between hMSCs and endothelial cells, and thus giving a deeper understanding of the interaction between these cells during the endochondral ossification process *in vivo* which is currently unknown. Specific pathways which have been shown to have an effect on the endochondral ossification process include Wnt/β-catenin (Day et al., 2005), FGF (Ornitz and Marie, 2002), Indian hedgehog hormone/parathyroid hormone related protein (St-Jacques et al., 1999), and TGF-β family (Sakou et al., 1999) signalling pathways (Kronenberg, 2003).

7.4.2 Understanding the effect of long term subcutaneous implantation of prevascularised cartilaginous cellular aggregates.

The results from Chapter 6 demonstrated that cell viability and mature vessel formation was enhanced after 4 weeks subcutaneous implantation within cellular aggregates that were first chondrogenic and vascular primed *in vitro*. However, micro computed tomography (μ CT) and Alizarin Red staining (mineralisation) showed no significant difference in mineralisation between any of the groups including the

control group other than the location of the mineralisation nodules. The choice of the 4 week time point allowed the author to see both early mineral formation and vessel infiltration to explore the in vivo potential of the prevascularised aggregates. However, the short duration of implantation (4 weeks) did not allow for significant mineralisation to occur. Future studies could expand on these results by implanting cellular aggregates subcutaneously for periods of 8 weeks or more.

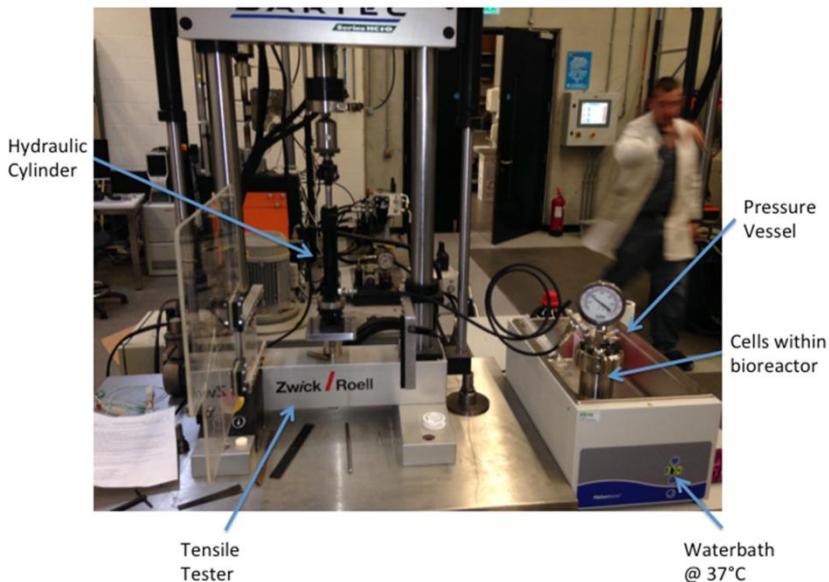
7.4.3 Understanding the effects the application of mechanical stimulation in vitro will have on the cellular aggregates when implanted in vivo.

Mathematical and computational models have predicted that a variety of stimuli can both promote and inhibit cells to undergo chondrogenic and osteogenic differentiation during bone development (Carter et al., 1987; Carter and Wong, 1988; Wong and Carter, 1990a; Nowlan et al., 2008) or fracture healing (Blenman et al., 1989; Lacroix and Prendergast, 2002; Isaksson et al., 2006; Lacroix et al., 2002). Experimental studies have found that the application of cyclic hydrostatic pressure can influence the rate of endochondral ossification (Claes et al., 1998; Wong et al., 2003; Wong and Carter, 1990b), specifically, experimental studies have shown that the application of hydrostatic pressure can significantly enhance the chondrogenic differentiation process of stem cells and thus influence the rate of endochondral ossification (Miyanishi et al., 2006b; Miyanishi et al., 2006a; Finger et al., 2007; Ogawa et al., 2009; Vinardell et al., 2012; Luo and Seedhom, 2007; Angele et al., 2003; Wagner et al., 2008; Meyer et al., 2011). However, the ideal level of hydrostatic pressure and other physical parameters that will drive bone regeneration by endochondral ossification are still unknown. Endogenous cartilage within a joint is typically exposed to stresses between 3 and 10 MPa and can experience stresses as

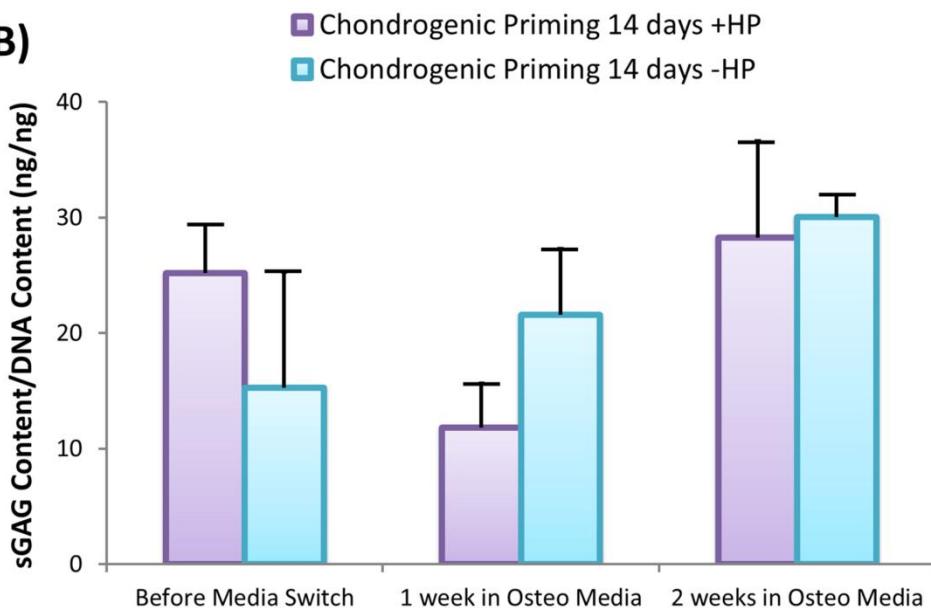
high as 18 MPa (Afroke et al., 1987; Hodge et al., 1986), while a typical human's cadence is generally up to 1 Hz (Waters et al., 1988). Recent studies have looked at applying pressures between 0.1 – 10 MPa to MSCs either in cellular aggregates or seeded on biomaterial scaffolds, these studies have suggested that chondrogenesis of MSCs exhibited the best response to applied loads of 10 MPa (Miyanishi et al., 2006b; Miyanishi et al., 2006a; Finger et al., 2007; Ogawa et al., 2009; Vinardell et al., 2012; Luo and Seedhom, 2007; Angele et al., 2003; Wagner et al., 2008; Meyer et al., 2011).

During the course of this Thesis a bioreactor was designed to apply hydrostatic pressure to the cellular aggregates developed in Chapters 3 – 5 of this Thesis, during the chondrogenic priming process, see Figure 7.3 (A)). A preliminary study was conducted in which 10 MPa of hydrostatic pressure was applied to human MSC cellular aggregates, for the 14 day chondrogenic priming period at 1 Hz for 1 hr/day. The results from this preliminary study show the positive potential of applying hydrostatic pressure, see Figure 7.3 (B) and (C). However, to date no study has sought to recreate the presence of multiple cell populations, their spatiotemporal differentiation and the extracellular mechanical environment that exists during endochondral ossification *in vivo*. Therefore, future studies should investigate an *in vitro* tissue regeneration strategy that not only mimics the cellular and biochemical environment, but also the mechanical environment applied during endochondral ossification *in vivo*.

(A)



(B)



(C)

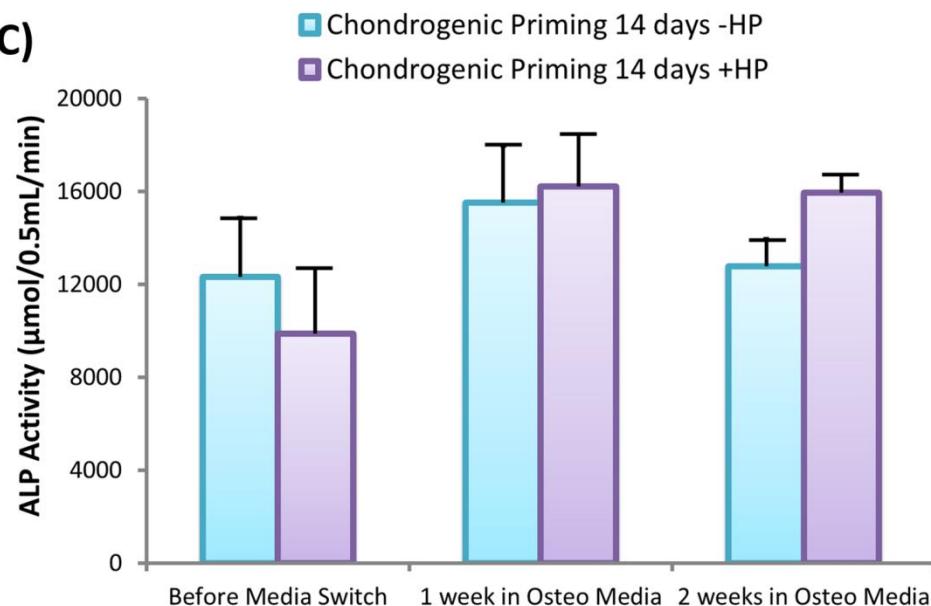


Figure 7.3: (A) Photograph of bioreactor setup. (B) sGAG/DNA content and (C) ALP Activity of aggregates chondrogenically primed for 14 days with (+HP) and without hydrostatic pressure (-HP) applied at both Pre-Medium Switch, 1 and 2 weeks Post Media Switch. Error bars denote standard deviation.

7.4.4 Applying the optimum priming conditions in vitro and investigating the regeneration potential of aggregates in a large bone defect model.

Once the optimum priming conditions, which mimic the cellular, biochemical and mechanical environment of endochondral ossification, have been established studies could investigate what effect these endochondrally primed aggregates would have at healing a large (5 – 15 mm) segmental bone defect. Using in vitro chondrogenic and vascular priming techniques, described in Chapters 3, 4, and 5, combined with the application of hydrostatic pressure and the techniques outlined in Chapter 6 of this Thesis, the viability, mineralisation capacity and overall bone tissue regeneration potential of these aggregates can be investigated. Taken together, the results from this Thesis and future studies on the application of mechanical stimuli can provide a novel insight into the design of future bone tissue engineered constructs.

7.5 Conclusions

In conclusion, this Thesis has presented experimental studies performed throughout the course of the author's PhD studies to investigate whether an in vitro tissue regeneration approach that mimicked endochondral ossification could serve as an effective approach to enhance osteogenic differentiation of MSCs in vitro and ultimately once implanted in vivo. In vitro cell culture methods were used to investigate the effect of chondrogenic and vascular priming on both the osteogenic

and vasculogenic potential of MSCs *in vitro*. The results from these studies demonstrated that allowing cells to first form a cartilage template, and sequentially prevascularising this template, through the co-culture with HUVECs and MSCs, actually enhanced the mineralisation potential of MSCs compared to allowing them to mineralise straightaway (following an intramembranous ossification-like pathway). Moreover, these results revealed that the optimum conditions needed to produce a fully mineralised prevascularised construct *in vitro*, which include chondrogenic priming for 21 days plus the co-culture with HUVECs and MSCs for 3 weeks without the addition of any osteogenic supplements. Finally, an *in vivo* animal model was developed to examine if applying these optimum conditions would produce a construct, when implanted *in vivo*, will mineralise down the endochondral ossification pathway. These results also gave a novel insight into the effect of direct interaction between hMSCs and endothelial cells, which naturally occurs during endochondral ossification *in vivo*. The results from the final study of the Thesis showed that the prevascularised cartilaginous aggregates had enhanced cellular viability, vessel infiltration and thus mineralisation when implanted in an ectopic bone formation model. The results from this Thesis provide a novel understanding of the optimum conditions needed to regenerate bone tissue for treatment of a large bone defects. Future bone tissue engineered constructs could be designed with these conditions in mind such that the constructs themselves can autonomously progress to engraftment, remodelling and ultimately lead to the regeneration of bone tissue after implantation.

References

- Abou-Samra AB, Jüppner H, Force T, Freeman MW, Kong XF, Schipani E, Urena P, Richards J, Bonventre JV & Potts JT (1992). Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proceedings of the National Academy of Sciences* 89 (7):2732-2736.
- Adachi T, Aonuma Y, Tanaka M, Hojo M, Takano-Yamamoto T & Kamioka H (2009). Calcium response in single osteocytes to locally applied mechanical stimulus: differences in cell process and cell body. *Journal of Biomechanics* 42 (12):1989-95. doi: 10.1016/j.jbiomech.2009.04.034.
- Afoke NY, Byers PD & Hutton WC (1987). Contact pressures in the human hip joint. *Journal of Bone and Joint (Br)* 69 (4):536-41.
- Agrawal C & Ray RB (2001). Biodegradable polymeric scaffolds for musculoskeletal tissue engineering. *Journal of Biomedical Materials Research* 55 (2):141-150.
- Ahlmann E, Patzakis M, Roidis N, Shepherd L & Holtom P (2002). Comparison of anterior and posterior iliac crest bone grafts in terms of harvest-site morbidity and functional outcomes. *Journal of Bone and Joint Surgery* 84 (5):716-20. doi: <http://dx.doi.org/>.
- Allen AB, Gazit Z, Su S, Stevens HY & Guldberg RE (2014). In Vivo Bioluminescent Tracking of Mesenchymal Stem Cells Within Large Hydrogel Constructs. *Tissue Engineering Part C: Methods* 20 (10):806-16. doi: 10.1089/ten.TEC.2013.0587.
- Allen MR, Hock JM & Burr DB (2004). Periosteum: biology, regulation, and response to osteoporosis therapies. *Bone* 35 (5):1003-1012. doi: <http://dx.doi.org/10.1016/j.bone.2004.07.014>.
- Americancancersociety. (2014). *What are the key statistics about osteosarcoma?* [Online]. Available: <http://www.cancer.org/cancer/osteosarcoma/detailedguide/osteosarcoma-key-statistics> [Accessed 23-03-2015 2015].
- Amini AR, Laurencin CT & Nukavarapu SP (2012). Bone Tissue Engineering: Recent Advances and Challenges. *Critical Reviews in Biomedical Engineering* 40 (5):363-408.
- Anderson HC (2003). Matrix vesicles and calcification. *Current Rheumatology Reports* 5 (3):222-6.
- 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. *Journal of Orthopaedic Research* 21 (3):451-7. doi: 10.1016/S0736-0266(02)00230-9.
- Arinze TL, Peter SJ, Archambault MP, Van Den Bos C, Gordon S, Kraus K, Smith A & Kadiyala S (2003). Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. *Journal of Bone and Joint Surgery* 85 (10):1927-35.
- Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS & Lian JB (1990). Factors That Promote Progressive Development of the Osteoblast Phenotype

- in Cultured Fetal-Rat Calvaria Cells. *Journal of Cellular Physiology* 143 (2):213-221. doi: 10.1002/jcp.1041430203.
- Athanasiou KA, Niederauer GG & Agrawal CM (1996). Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. *Biomaterials* 17 (2):93-102.
- Aubin JE (2001). Regulation of osteoblast formation and function. *Reviews in Endocrine and Metabolic Disorders* 2 (1):81-94. doi: 10.1023/A:1010011209064.
- Aubin JE, Turksen K & Heersche JNM (1993) Osteoblastic Cell Lineage. In: Noda M (ed) *Cellular and Molecular Biology*, Academic Press, Inc., pp. 2-29.
- Augello A, Kurth TB & De Bari C (2010). Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. *Eur Cell Mater* 20 121-33. doi: vol020a11 [pii].
- Baddoo M, Hill K, Wilkinson R, Gaupp D, Hughes C, Kopen GC & Phinney DG (2003). Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. *Journal of Cellular Biochemistry* 89 (6):1235-1249. doi: 10.1002/jcb.10594.
- Barry FP & Murphy JM (2004). Mesenchymal stem cells: clinical applications and biological characterization. *The International Journal of Biochemistry & Cell Biology* 36 (4):568-584. doi: <http://dx.doi.org/10.1016/j.biocel.2003.11.001>.
- Belmont PJJ, Mccriskin BJ, Sieg RN, Burks R & Schoenfeld AJ (2012). Combat wounds in Iraq and Afghanistan from 2005 to 2009. *Journal of Trauma and Acute Care Surgery* 73 (1):3-12. doi: 10.1097/TA.0b013e318250bfb4.
- Benders KE, Van Weeren PR, Badylak SF, Saris DB, Dhert WJ & Malda J (2013). Extracellular matrix scaffolds for cartilage and bone regeneration. *Trends Biotechnol* 31 (3):169-76. doi: 10.1016/j.tibtech.2012.12.004.
- Birmingham E, Niebur GL, Mchugh PE, Shaw G, Barry FP & Mcnamara LM (2012). Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. *European Cells & Materials* 23 13-27.
- Bittner K, Vischer P, Bartholmes P & Bruckner P (1998). Role of the Subchondral Vascular System in Endochondral Ossification: Endothelial Cells Specifically Derepress Late Differentiation in Resting Chondrocytes in Vitro. *Experimental Cell Research* 238 (2):491-497. doi: <http://dx.doi.org/10.1006/excr.1997.3849>.
- Blenman PR, Carter DR & Beaupre GS (1989). Role of mechanical loading in the progressive ossification of a fracture callus. *Journal of Orthopaedic Research* 7 (3):398-407. doi: 10.1002/jor.1100070312.
- Boden SD (1999). Bioactive factors for bone tissue engineering. *Clinical Orthopaedics and Related Research* 367 S84-S94.
- Boerckel JD, Kolambkar YM, Dupont KM, Uhrig BA, Phelps EA, Stevens HY, García AJ & Guldberg RE (2011). Effects of protein dose and delivery system on BMP-mediated bone regeneration. *Biomaterials* 32 (22):5241-5251. doi: <http://dx.doi.org/10.1016/j.biomaterials.2011.03.063>.
- Bolland BJRF, Kanczler JM, Ginty PJ, Howdle SM, Shakesheff KM, Dunlop DG & Oreffo ROC (2008). The application of human bone marrow stromal cells and poly(dl-lactic acid) as a biological bone graft extender in impaction bone grafting. *Biomaterials* 29 (22):3221-3227. doi: <http://dx.doi.org/10.1016/j.biomaterials.2008.04.017>.
- Bonewald LF (2002). Osteocytes: a proposed multifunctional bone cell. *Journal of Musculoskeletal & Neuronal Interactions* 2 (3):239-241.

- Bonewald LF (2011). The amazing osteocyte. *Journal of Bone Mineral Research* 26 (2):229-38. doi: 10.1002/jbmr.320.
- Boskey AL (2013). Bone composition: relationship to bone fragility and antiosteoporotic drug effects. *BoneKEy Reports* 2 doi: 10.1038/bonekey.2013.181.
- Bourne GH (1972) Phosphate and Calcification. In: Bourne GH (ed) *Physiology and Pathology*, Academic Press, Ltd., p. 89.
- Boyce T, Edwards J & Scarborough N (1999). Allograft bone: the influence of processing on safety and performance. *Orthopedic Clinics of North America* 30 (4):571-581.
- Boyle WJ, Simonet WS & Lacey DL (2003). Osteoclast differentiation and activation. *Nature* 423 (6937):337-342.
- Bronckers AL, Gay S, Finkelman RD & Butler WT (1987). Developmental appearance of Gla proteins (osteocalcin) and alkaline phosphatase in tooth germs and bones of the rat. *Bone and Mineral* 2 (5):361-73.
- Bruder SP, Kraus KH, Goldberg VM & Kadiyala S (1998a). The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *Journal of Bone and Joint (Am)* 80 (7):985-96.
- Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N & Kadiyala S (1998b). Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. *Journal of Orthopaedic Research* 16 (2):155-162. doi: DOI 10.1002/jor.1100160202.
- Brydone AS, Meek D & MacLaine S (2010). Bone grafting, orthopaedic biomaterials, and the clinical need for bone engineering. *Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine* 224 (12):1329-43.
- Buga A-M, Margaritescu C, Scholz C, Radu E, Zelenak C & Popa-Wagner A (2014). Transcriptomics of post-stroke angiogenesis in the aged brain. *Frontiers in Aging Neuroscience* 6 doi: 10.3389/fnagi.2014.00044.
- Burg KJ, Porter S & Kellam JF (2000). Biomaterial developments for bone tissue engineering. *Biomaterials* 21 (23):2347-2359.
- Burger EH & Klein-Nulend J (1999). Mechanotransduction in bone—role of the lacuno-canalicular network. *The Journal of the Federation of American Societies for Experimental Biology* 13 (9001):101-112.
- Cameron DA (1968). The Golgi apparatus in bone and cartilage cells. *Clinical Orthopaedics and Related Research* 58 191-211.
- Canalis E, Pash J & Varghese S (1993). Skeletal growth factors. *Critical Reviews in Eukaryotic Gene Expression* 3 (3):155-66.
- Cancedda R, Giannoni P & Mastrogiovanni M (2007). A tissue engineering approach to bone repair in large animal models and in clinical practice. *Biomaterials* 28 (29):4240-50. doi: 10.1016/j.biomaterials.2007.06.023.
- Caplan AI (1990). Cell delivery and tissue regeneration. *Journal of Controlled Release* 11 (1-3):157-165. doi: [http://dx.doi.org/10.1016/0168-3659\(90\)90129-H](http://dx.doi.org/10.1016/0168-3659(90)90129-H).
- Caplan AI (1991). Mesenchymal stem cells. *Journal of Orthopaedic Research* 9 (5):641-50. doi: 10.1002/jor.1100090504.
- Caplan AI (2005). Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Engineering* 11 (7-8):1198-211. doi: 10.1089/ten.2005.11.1198.
- Caplan AI (2008). All MSCs are pericytes? *Cell Stem Cell* 3 (3):229-230.

- Cardoso L, Herman BC, Verborgt O, Laudier D, Majeska RJ & Schaffler MB (2009). Osteocyte Apoptosis Controls Activation of Intracortical Resorption in Response to Bone Fatigue. *Journal of Bone and Mineral Research* 24 (4):597-605. doi: 10.1359/jbmr.081210.
- Carlevaro MF, Cermelli S, Cancedda R & Descalzi Cancedda F (2000). Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *Journal of Cell Science* 113 (Pt 1) 59-69.
- Carter DR, Orr TE, Fyhrie DP & Schurman DJ (1987). Influences of Mechanical Stress on Prenatal and Postnatal Skeletal Development. *Clinical Orthopaedics and Related Research* 219 237-250.
- Carter DR & Wong M (1988). The role of mechanical loading histories in the development of diarthrodial joints. *Journal of Orthopaedic Research* 6 (6):804-816. doi: 10.1002/jor.1100060604.
- Chan BP & Leong KW (2008). Scaffolding in tissue engineering: general approaches and tissue-specific considerations. *European Spine Journal* 17 Suppl 4 467-79. doi: 10.1007/s00586-008-0745-3.
- Chan CKF, Chen CC, Luppen CA, Kim JB, Deboer AT, Wei K, Helms JA, Kuo CJ, Kraft DL & Weissman IL (2009). Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature* 457 (7228):490-U9. doi: Doi 10.1038/Nature07547.
- Chapekar MS (2000). Tissue engineering: challenges and opportunities. *Journal of Biomedical Materials Research* 53 (6):617-20. doi: 10.1002/1097-4636(2000)53:6<617::AID-JBM1>3.0.CO;2-C.
- Chapman PG & Villar RN (1992). The bacteriology of bone allografts. *Journal of Bone and Joint (Br)* 74 (3):398-9.
- Cheng NC, Estes BT, Awad HA & Guilak F (2009). Chondrogenic differentiation of adipose-derived adult stem cells by a porous scaffold derived from native articular cartilage extracellular matrix. *Tissue Engineering Part A* 15 (2):231-41. doi: 10.1089/ten.tea.2008.0253.
- Cheng SL, Yang JW, Rifas L, Zhang SF & Avioli LV (1994). Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. *Endocrinology* 134 (1):277-86. doi: 10.1210/endo.134.1.8275945.
- Claes LE, Heigele CA, Neidlinger-Wilke C, Kaspar D, Seidl W, Margevicius KJ & Augat P (1998). Effects of Mechanical Factors on the Fracture Healing Process. *Clinical Orthopaedics and Related Research* 355 S132-S147.
- Clarke B (2008). Normal Bone Anatomy and Physiology. *Clinical Journal of the American Society of Nephrology* 3 (Suppl 3):S131-S139. doi: 10.2215/cjn.04151206.
- Coleman CM, Vaughan EE, Browe DC, Mooney E, Howard L & Barry F (2013). Growth Differentiation Factor-5 Enhances In Vitro Mesenchymal Stromal Cell Chondrogenesis and Hypertrophy. *Stem Cells and Development* doi: 10.1089/scd.2012.0282.
- Cormack DH, (2001). Essential Histology, Philadelphia, Pennsylvania, USA, Lippincott Williams and Wilkins.
- Correia C, Grayson WL, Park M, Hutton D, Zhou B, Guo XE, Niklason L, Sousa RA, Reis RL & Vunjak-Novakovic G (2011). In vitro model of vascularized bone: synergizing vascular development and osteogenesis. *PLoS One* 6 (12):28352. doi: 10.1371/journal.pone.0028352.

- Coventry MB & Tapper EM (1972). Pelvic instability: a consequence of removing iliac bone for grafting. *Journal of Bone and Joint (Am)* 54 (1):83-101.
- Cowin SC, Moss-Salentijn L & Moss ML (1991). Candidates for the mechanosensory system in bone. *Journal of Biomechanical Engineering* 113 (2):191-7.
- Cowin SC, Weinbaum S & Zeng Y (1995). A case for bone canaliculi as the anatomical site of strain generated potentials. *Journal of Biomechanics* 28 (11):1281-1297. doi: [http://dx.doi.org/10.1016/0021-9290\(95\)00058-P](http://dx.doi.org/10.1016/0021-9290(95)00058-P).
- Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J & Peault B (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 3 (3):301-13. doi: 10.1016/j.stem.2008.07.003.
- Crockett JC, Rogers MJ, Coxon FP, Hocking LJ & Helfrich MH (2011). Bone remodelling at a glance. *Journal of Cell Science* 124 (7):991-998.
- Currey JD & Brear K (1990). Hardness, Young's modulus and yield stress in mammalian mineralized tissues. *Journal of Materials Science: Materials in Medicine* 1 (1):14-20. doi: 10.1007/bf00705348.
- Curtin CM, Cunniffe GM, Lyons FG, Bessho K, Dickson GR, Duffy GP & O'brien FJ (2012). Innovative collagen nano-hydroxyapatite scaffolds offer a highly efficient non-viral gene delivery platform for stem cell-mediated bone formation. *Advanced Materials* 24 (6):749-54. doi: 10.1002/adma.201103828.
- Curtis-Prior PB, (1988). Prostaglandins: Biology and Chemistry of Prostaglandins and Related Eicosanoids, Edinburgh, Churchill Livingstone.
- D'aquino R, De Rosa A, Lanza V, Tirino V, Laino L, Graziano A, Desiderio V, Laino G & Papaccio G (2009). Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. *European Cells & Materials* 18 75-83.
- D'souza RN, Niederreither K & De Crombrugghe B (1993). Osteoblast-specific expression of the alpha 2(I) collagen promoter in transgenic mice: correlation with the distribution of TGF-beta 1. *Journal of Bone Mineral Research* 8 (9):1127-36. doi: 10.1002/jbmr.5650080914.
- Da Silva Meirelles L, Caplan AI & Nardi NB (2008). In search of the in vivo identity of mesenchymal stem cells. *Stem Cells* 26 (9):2287-99. doi: 10.1634/stemcells.2007-1122.
- Dawson JI & Oreffo RO (2008). Bridging the regeneration gap: stem cells, biomaterials and clinical translation in bone tissue engineering. *Archives of Biochemistry and Biophysics* 473 (2):124-31. doi: 10.1016/j.abb.2008.03.024.
- Dawson JI, Wahl DA, Lanham SA, Kanczler JM, Czernuszka JT & Oreffo ROC (2008). Development of specific collagen scaffolds to support the osteogenic and chondrogenic differentiation of human bone marrow stromal cells. *Biomaterials* 29 (21):3105-3116. doi: <http://dx.doi.org/10.1016/j.biomaterials.2008.03.040>.
- Day TF, Guo X, Garrett-Beal L & Yang Y (2005). Wnt/β-Catenin Signaling in Mesenchymal Progenitors Controls Osteoblast and Chondrocyte Differentiation during Vertebrate Skeletogenesis. *Developmental Cell* 8 (5):739-750. doi: <http://dx.doi.org/10.1016/j.devcel.2005.03.016>.
- Deb A, Wang S, Skelding KA, Miller D, Simper D & Caplice NM (2003). Bone marrow-derived cardiomyocytes are present in adult human heart: A study of gender-mismatched bone marrow transplantation patients. *Circulation* 107 (9):1247-9.

- Dick HM & Strauch RJ (1994). Infection of massive bone allografts. *Clinical Orthopaedics and Related Research* (306):46-53.
- Dickson G, Buchanan F, Marsh D, Harkin-Jones E, Little U & McCaigue M (2007). Orthopaedic tissue engineering and bone regeneration. *Technology and Health Care* 15 (1):57-67.
- Dietrich JW, Canalis EM, Maina DM & Raisz LG (1976). Hormonal control of bone collagen synthesis in vitro: effects of parathyroid hormone and calcitonin. *Endocrinology* 98 (4):943-9. doi: 10.1210/endo-98-4-943.
- Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R & Prockop DJ (1999). Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* 107 (2):275-81. doi: bjh1715 [pii].
- Dimitriou R, Jones E, Mcgonagle D & Giannoudis PV (2011). Bone regeneration: current concepts and future directions. *BMC Medicine* 9 66. doi: 10.1186/1741-7015-9-66.
- Dolan EB, Haugh MG, Tallon D, Casey C & McNamara LM (2012). Heat-shock-induced cellular responses to temperature elevations occurring during orthopaedic cutting. *Journal of The Royal Society Interface* 9 (77):3503-13. doi: 10.1098/rsif.2012.0520.
- Doll B, Towle H, Hollinger J, Reddi A & Mellonig J (1990). The osteogenic potential of two composite graft systems using osteogenin. *Journal of Periodontology* 61 (12):745-750.
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D & Horwitz E (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8 (4):315-317. doi: 10.1080/14653240600855905.
- Doty SB & Schofield BH (1976). Enzyme histochemistry of bone and cartilage cells. *Progress in Histochemistry Cytochemistry* 8 (1):1-38.
- Duffy GP, McFadden TM, Byrne EM, Gill SL, Farrell E & O'Brien FJ (2011). Towards in vitro vascularisation of collagen-GAG scaffolds. *European Cells & Materials* 21 15-30.
- Duvall CL, Taylor WR, Weiss D & Guldberg RE (2004). Quantitative microcomputed tomography analysis of collateral vessel development after ischemic injury. *American Journal of Physiology - Heart and Circulatory Physiology* 287 (1):H302-10. doi: 10.1152/ajpheart.00928.2003.
- Dziak R & Brand JS (1974). Calcium transport in isolated bone cells. I. Bone cell isolation procedures. *Journal of Cellular Physiology* 84 (1):75-83. doi: 10.1002/jcp.1040840109.
- Dziak R & Stern PH (1975). Calcium transport in isolated bone cells. III. Effects of parathyroid hormone and cyclic 3',5'-AMP. *Endocrinology* 97 (5):1281-7. doi: 10.1210/endo-97-5-1281.
- Ecarot-Charrier B, Glorieux FH, Van Der Rest M & Pereira G (1983). Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. *Journal of Cell Biology* 96 (3):639-643. doi: 10.1083/jcb.96.3.639.
- Einhorn TA (1998). The cell and molecular biology of fracture healing. *Clinical Orthopaedics and Related Research* (355):7-21.
- El-Serafi AT, Wilson DI, Roach HI & Oreffo RO (2011). Developmental plasticity of human foetal femur-derived cells in pellet culture: self assembly of an osteoid shell around a cartilaginous core. *European Cells & Materials* 21 558-67.

- Elder BD, Eleswarapu SV & Athanasiou KA (2009). Extraction techniques for the decellularization of tissue engineered articular cartilage constructs. *Biomaterials* 30 (22):3749-56. doi: 10.1016/j.biomaterials.2009.03.050.
- Elsinger EC & Leal L (1996). Coralline hydroxyapatite bone graft substitutes. *The Journal of foot and ankle surgery* 35 (5):396-399.
- Enneking WF, Eady JL & Burchardt H (1980). Autogenous cortical bone grafts in the reconstruction of segmental skeletal defects. *J Bone Joint Surg Am* 62 (7):1039-58.
- Enterprises K, (2001). *The Ortho Factbook*, Knowledge Enterprises Inc.
- Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H & Guilak F (2002). Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. *Biochemical and Biophysical Research Communications* 290 (2):763-9. doi: 10.1006/bbrc.2001.6270.
- Erickson IE, Huang AH, Chung C, Li RT, Burdick JA & Mauck RL (2009). Differential maturation and structure-function relationships in mesenchymal stem cell- and chondrocyte-seeded hydrogels. *Tissue Engineering Part A* 15 (5):1041-52. doi: 10.1089/ten.tea.2008.0099.
- Eriksson AB, Axelrod DW & Melsen F, (1994). *Bone Histomorphometry*, New York, Raven Press.
- Estes BT, Diekman BO, Gimble JM & Guilak F (2010). Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. *Nature Protocols* 5 (7):1294-1311.
- European_Commission. (2011). *Statistics - accidents data* [Online]. Available: http://ec.europa.eu/transport/road_safety/specialist/statistics/index_en.htm [Accessed 26/05/2015].
- Farrell E, Both SK, Odorfer KI, Koevoet W, Kops N, O'brien FJ, Baatenburg De Jong RJ, Verhaar JA, Cuijpers V, Jansen J, Erben RG & Van Osch GJ (2011). In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells. *BMC Musculoskeletal Disorders* 12 31. doi: 10.1186/1471-2474-12-31.
- Farrell E, Byrne EM, Fischer J, O'brien FJ, O'connell BC, Prendergast PJ & Campbell VA (2007). A comparison of the osteogenic potential of adult rat mesenchymal stem cells cultured in 2-D and on 3-D collagen glycosaminoglycan scaffolds. *Technol Health Care* 15 (1):19-31.
- Farrell E, O'brien FJ, Doyle P, Fischer J, Yannas I, Harley BA, O'Connell B, Prendergast PJ & Campbell VA (2006). A collagen-glycosaminoglycan scaffold supports adult rat mesenchymal stem cell differentiation along osteogenic and chondrogenic routes. *Tissue Engineeering* 12 (3):459-68. doi: 10.1089/ten.2006.12.459.
- Farrell E, Van Der Jagt OP, Koevoet W, Kops N, Van Manen CJ, Hellingman CA, Jahr H, O'brien FJ, Verhaar JA, Weinans H & Van Osch GJ (2009). Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? *Tissue Engineering Part C* 15 (2):285-95. doi: 10.1089/ten.tec.2008.0297.
- Fatherazi S, Matsa-Dunn D, Foster BL, Rutherford RB, Somerman MJ & Presland RB (2009). Phosphate regulates osteopontin gene transcription. *Journal of Dental Research* 88 (1):39-44. doi: 10.1177/0022034508328072.
- Favus MJ & Christakos S, (1996). *Primer on the metabolic bone diseases and disorders of mineral metabolism.*, Philadelphia, PA, USA, American Society of Bone and Mineral Research.

- Felix R & Fleisch H (1979). Increase in alkaline phosphatase activity in calvaria cells cultured with diphosphonates. *Biochemical Journal* 183 (1):73-81.
- Fell HB & Robison R (1929). The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated in vitro. *Biochemical Journal* 23 (4):767-784 5.
- Fell HB & Robison R (1934). The development of the calcifying mechanism in avian cartilage and osteoid tissue. *Biochemical Journal* 28 (6):2243-53.
- Fiedler J, Leucht F, Waltenberger J, Dehio C & Brenner RE (2005). VEGF-A and PIGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. *Biochemical and Biophysical Research Communications* 334 (2):561-8. doi: 10.1016/j.bbrc.2005.06.116.
- Finger AR, Sargent CY, Dulaney KO, Bernacki SH & Loba EG (2007). Differential effects on messenger ribonucleic acid expression by bone marrow-derived human mesenchymal stem cells seeded in agarose constructs due to ramped and steady applications of cyclic hydrostatic pressure. *Tissue Engineering* 13 (6):1151-1158.
- Finkelman RD, Bell NH, Strong DD, Demers LM & Baylink DJ (1992). Ovariectomy Selectively Reduces the Concentration of Transforming Growth-Factor-Beta in Rat Bone - Implications for Estrogen Deficiency-Associated Bone Loss. *Proceedings of the National Academy of Sciences of the United States of America* 89 (24):12190-12193. doi: 10.1073/pnas.89.24.12190.
- Finkemeier CG (2002). Bone-grafting and bone-graft substitutes. *Journal of Bone and Joint (Am)* 84-A (3):454-64.
- Foster BL, Nociti FH, Jr., Swanson EC, Matsa-Dunn D, Berry JE, Cupp CJ, Zhang P & Somerman MJ (2006). Regulation of cementoblast gene expression by inorganic phosphate in vitro. *Calcified Tissue International* 78 (2):103-12. doi: 10.1007/s00223-005-0184-7.
- Franceschi RT & Iyer BS (1992). Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *Journal of Bone Mineral Research* 7 (2):235-46. doi: 10.1002/jbmr.5650070216.
- Franceschi RT, Iyer BS & Cui Y (1994). Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *Journal of Bone Mineral Research* 9 (6):843-54. doi: 10.1002/jbmr.5650090610.
- Fratzl P, Gupta HS, Paschalis EP & Roschger P (2004). Structure and mechanical quality of the collagen-mineral nano-composite in bone. *Journal of Materials Chemistry* 14 (14):2115-2123. doi: 10.1039/B402005g.
- Freeman F, Haugh M & Mcnamara L (2013). Investigation of the optimal timing for chondrogenic priming of MSCs to enhance osteogenic differentiation in vitro as a bone tissue engineering strategy. *Journal of tissue engineering and regenerative medicine* doi: 10.1002/term.1793.
- Freeman FE, Allen AB, Stevens HY, Guldberg RE & Mcnamara LM (2015a). Effects of in vitro endochondral priming and pre-vascularisation of human MSC cellular aggregates in vivo. *Stem Cell Res Ther* 6 218. doi: 10.1186/s13287-015-0210-2.
- Freeman FE, Haugh MG & Mcnamara L (2015b). An in vitro bone tissue regeneration strategy combining chondrogenic and vascular priming enhances the mineralisation potential of MSCs in vitro whilst also allowing for vessel formation. *Tissue Engineering Part A* 21 (7-8):1320-1332. doi: 10.1089/ten.tea.2014.0249.

- Friedenstein AJ, Chailakhjan RK & Lalykina KS (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell and Tissue Kinetics* 3 (4):393-403.
- Friedenstein AJ, Chailakhyan RK & Gerasimov UV (1987). Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell and Tissue Kinetics* 20 (3):263-72.
- Friedenstein AJ, Petrakova KV, Kurolesova AI & Frolova GP (1968). Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* 6 (2):230-47.
- Frohlich M, Grayson WL, Wan LQ, Marolt D, Drobnić M & Vunjak-Novakovic G (2008). Tissue Engineered Bone Grafts: Biological Requirements, Tissue Culture and Clinical Relevance. *Current Stem Cell Research & Therapy* 3 (4):254-264. doi: 10.2174/157488808786733962.
- Frost HM (1990). Skeletal structural adaptations to mechanical usage (SATMU): 1. Redefining Wolff's law: the bone modeling problem. *The Anatomical Record* 226 (4):403-13. doi: 10.1002/ar.1092260402.
- Fuchs E, Tumbar T & Guasch G (2004). Socializing with the Neighbors: Stem Cells and Their Niche. *Cell* 116 (6):769-778. doi: [http://dx.doi.org/10.1016/S0092-8674\(04\)00255-7](http://dx.doi.org/10.1016/S0092-8674(04)00255-7).
- Fuchs S, Hofmann A & Kirkpatrick CJ (2007). Microvessel-like structures from outgrowth endothelial cells from human peripheral blood in 2-dimensional and 3-dimensional co-cultures with osteoblastic lineage cells. *Tissue Engineering* 13 (10):2577-88. doi: 10.1089/ten.2007.0022.
- Gawlitta D, Benders KEM, Visser J, Van Der Sar AS, Kempen DHR, Theyse LFH, Malda J & Dhert WJA (2015). Decellularized Cartilage-Derived Matrix as Substrate for Endochondral Bone Regeneration. *Tissue Engineering Part A* 21 (3-4):694-703. doi: 10.1089/ten.tea.2014.0117.
- Gawlitta D, Fledderus JO, Van Rijen MH, Dokter I, Alblas J, Verhaar MC & Dhert WJ (2012). Hypoxia impedes vasculogenesis of in vitro engineered bone. *Tissue Engineering Part A* 18 (1-2):208-18. doi: 10.1089/ten.TEA.2010.0731.
- Ge Z, Baguenard S, Lim LY, Wee A & Khor E (2004). Hydroxyapatite–chitin materials as potential tissue engineered bone substitutes. *Biomaterials* 25 (6):1049-1058.
- Gerber HP & Ferrara N (2000). Angiogenesis and bone growth. *Trends in Cardiovascular Medicine* 10 (5):223-8. doi: 10.1016/S1050-1738(00)00074-8.
- Ghanaati S, Fuchs S, Webber MJ, Orth C, Barbeck M, Gomes ME, Reis RL & Kirkpatrick CJ (2011). Rapid vascularization of starch-poly(caprolactone) in vivo by outgrowth endothelial cells in co-culture with primary osteoblasts. *Journal of tissue engineering and regenerative medicine* 5 (6):136-43. doi: 10.1002/jterm.373.
- Giovannini S, Diaz-Romero J, Aigner T, Heini P, Mainil-Varlet P & Nesic D (2010). Micromass co-culture of human articular chondrocytes and human bone marrow mesenchymal stem cells to investigate stable neocartilage tissue formation in vitro. *European Cells & Materials* 20 245-59.
- Gleeson J, Plunkett N & O'Brien F (2010). Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration. *European Cell Materials* 20 218-230.

- Glowacki J, Rey C, Glimcher MJ, Cox KA & Lian J (1991). A role for osteocalcin in osteoclast differentiation. *Journal of Cellular Biochemistry* 45 (3):292-302. doi: 10.1002/jcb.240450312.
- Golub EE, Harrison G, Taylor AG, Camper S & Shapiro IM (1992). The role of alkaline phosphatase in cartilage mineralization. *Journal of Bone Mineral Research* 17 (2):273-8.
- Goshima J, Goldberg VM & Caplan AI (1991a). The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. *Clinical Orthopaedics and Related Research* (269):274-83.
- Goshima J, Goldberg VM & Caplan AI (1991b). Osteogenic potential of culture-expanded rat marrow cells as assayed in vivo with porous calcium phosphate ceramic. *Biomaterials* 12 (2):253-258. doi: [http://dx.doi.org/10.1016/0142-9612\(91\)90209-S](http://dx.doi.org/10.1016/0142-9612(91)90209-S).
- Goshima J, Goldberg VM & Caplan AI (1991c). The osteogenic potential of culture-expanded rat marrow mesenchymal cells assayed in vivo in calcium phosphate ceramic blocks. *Clinical Orthopaedics and Related Research* (262):298-311.
- Gray H & Standring S (2008) Musculoskeletal system. In: S S (ed) Gray's Anatomy: The Anatomical Basis of Clinical Practice., Elsevier/Churchill Livingstone, pp. 83-135.
- Grellier M, Ferreira-Tojais N, Bourget C, Bareille R, Guillemot F & Amédée J (2009a). Role of vascular endothelial growth factor in the communication between human osteoprogenitors and endothelial cells. *Journal of Cell Biomechanics* 106 (3):390-8. doi: 10.1002/jcb.22018.
- Grellier M, Ferreira-Tojais N, Bourget C, Bareille R, Guillemot F & Amédée J (2009b). Role of vascular endothelial growth factor in the communication between human osteoprogenitors and endothelial cells. *Journal of Cellular Biochemistry* 106 (3):390-398. doi: 10.1002/jcb.22018.
- Groot CG, Danes JK, Blok J, Hoogendojk A & Hauschka PV (1986). Light and electron microscopic demonstration of osteocalcin antigenicity in embryonic and adult rat bone. *Bone* 7 (5):379-85.
- Guillotin B, Bareille R, Bourget C, Bordenave L & Amédée J (2008). Interaction between human umbilical vein endothelial cells and human osteoprogenitors triggers pleiotropic effect that may support osteoblastic function. *Bone* 42 (6):1080-1091. doi: <http://dx.doi.org/10.1016/j.bone.2008.01.025>.
- Hall BK, (1941). Bones and Cartilage: developmental and evolutionary skeletal biology, San Diago, California, Elsevier Academic Press.
- Hall BK (1987). Earliest evidence of cartilage and bone development in embryonic life. *Clinical Orthopaedics and Related Research* (225):255-72.
- Hamidouche Z, Hay E, Vaudin P, Charbord P, Schule R, Marie PJ & Fromigue O (2008). FHL2 mediates dexamethasone-induced mesenchymal cell differentiation into osteoblasts by activating Wnt/beta-catenin signalling-dependent Runx2 expression. *The Journal of the Federation of American Societies for Experimental Biology* 22 (11):3813-22. doi: 10.1096/fj.08-106302.
- Hans-Peter G, Thiennu HV, Anne MR, Joe K, Zena W & Napoleone F (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nature Medicine* 5 (6):623-628. doi: 10.1038/9467.
- Harada N, Watanabe Y, Sato K, Abe S, Yamanaka K, Sakai Y, Kaneko T & Matsushita T (2014). Bone regeneration in a massive rat femur defect through

- endochondral ossification achieved with chondrogenically differentiated MSCs in a degradable scaffold. *Biomaterials* 35 (27):7800-7810. doi: <http://dx.doi.org/10.1016/j.biomaterials.2014.05.052>.
- Harvey W (1988) Source of prostaglandins and their influence on bone resorption and formation. In: Harvey W & Bennet A (eds) *Prostaglandins in Bone Resorption*, CRC Press, pp. 27-41.
- Haugh MG, Meyer EG, Thorpe SD, Vinardell T, Duffy GP & Kelly DJ (2011). Temporal and spatial changes in cartilage-matrix-specific gene expression in mesenchymal stem cells in response to dynamic compression. *Tissue Engineering Part A* 17 (23-24):3085-93. doi: 10.1089/ten.tea.2011.0198.
- Haynesworth SE, Goshima J, Goldberg VM & Caplan AI (1992). Characterization of cells with osteogenic potential from human marrow. *Bone* 13 (1):81-88. doi: [http://dx.doi.org/10.1016/8756-3282\(92\)90364-3](http://dx.doi.org/10.1016/8756-3282(92)90364-3).
- Heise U, Osborn J & Duwe F (1990). Hydroxyapatite ceramic as a bone substitute. *International Orthopaedics* 14 (3):329-338.
- Herbertson A & Aubin JE (1995). Dexamethasone alters the subpopulation make-up of rat bone marrow stromal cell cultures. *Journal of Bone Mineral Research* 10 (2):285-94. doi: 10.1002/jbmr.5650100216.
- Hibi H, Yamada Y, Ueda M & Endo Y (2006). Alveolar cleft osteoplasty using tissue-engineered osteogenic material. *International Journal of Oral and Maxillofacial Surgery* 35 (6):551-555. doi: <http://dx.doi.org/10.1016/j.ijom.2005.12.007>.
- Hoch BL, Klein MJ & Schiller AL (2009) Bone and Joints. In: Rubin E & Reisner HM (eds) *Essentials of Rubin's Pathology*, Lippincott Williams & Wilkins, a Wolters Kluwer business, pp. 546-548.
- Hodge WA, Fijan RS, Carlson KL, Burgess RG, Harris WH & Mann RW (1986). Contact pressures in the human hip joint measured in vivo. *Proceedings of the National Academy of Sciences* 83 (9):2879-83.
- Hoey DA, Kelly DJ & Jacobs CR (2011). A role for the primary cilium in paracrine signaling between mechanically stimulated osteocytes and mesenchymal stem cells. *Biochemical and Biophysical Research Communications* 412 (1):182-187. doi: <http://dx.doi.org/10.1016/j.bbrc.2011.07.072>.
- Hofmann A, Ritz U, Verrier S, Eglin D, Alini M, Fuchs S, Kirkpatrick CJ & Rommens PM (2008). The effect of human osteoblasts on proliferation and neo-vessel formation of human umbilical vein endothelial cells in a long-term 3D co-culture on polyurethane scaffolds. *Biomaterials* 29 (31):4217-4226. doi: <http://dx.doi.org/10.1016/j.biomaterials.2008.07.024>.
- Hofmann S, Hagenmüller H, Koch AM, Müller R, Vunjak-Novakovic G, Kaplan DL, Merkle HP & Meinel L (2007). Control of in vitro tissue-engineered bone-like structures using human mesenchymal stem cells and porous silk scaffolds. *Biomaterials* 28 (6):1152-1162. doi: <http://dx.doi.org/10.1016/j.biomaterials.2006.10.019>.
- Hollinger JO, Schmitt JM, Buck DC, Shannon R, Joh SP, Zegzula HD & Wozney J (1998). Recombinant human bone morphogenetic protein-2 and collagen for bone regeneration. *Journal of Biomedical Materials Research* 43 (4):356-364.
- Hollinger JO, Winn S & Bonadio J (2000). Options for tissue engineering to address challenges of the aging skeleton. *Tissue Engineering* 6 (4):341-50. doi: 10.1089/107632700418065.
- Hollister SJ (2005). Porous scaffold design for tissue engineering. *Nature Materials* 4 (7):518-524.

- Horisaka Y, Okamoto Y, Matsumoto N, Yoshimura Y, Kawada J, Yamashita K & Takagi T (1991). Subperiosteal implantation of bone morphogenetic protein adsorbed to hydroxyapatite. Clinical Orthopaedics and Related Research 268 303-312.
- Horne WC (1995). Toward a more complete molecular description of the osteoclast. Bone 17 (2):107-109. doi: [http://dx.doi.org/10.1016/S8756-3282\(95\)00175-1](http://dx.doi.org/10.1016/S8756-3282(95)00175-1).
- Howe CC, Overton GC, Sawicki J, Solter D, Stein P & Strickland S (1988). Expression of SPARC/osteonectin transcript in murine embryos and gonads. Differentiation 37 (1):20-5.
- Hung SC, Pochampally RR, Chen SC, Hsu SC & Prockop DJ (2007). Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. Stem Cells 25 (9):2363-70. doi: 10.1634/stemcells.2006-0686.
- Hunziker EB & Schenk RK (1989). Physiological mechanisms adopted by chondrocytes in regulating longitudinal bone growth in rats. Journal of Physiology 414 55-71.
- Hutmacher DW (2000). Scaffolds in tissue engineering bone and cartilage. Biomaterials 21 (24):2529-2543.
- Imai S, Heino TJ, Hienola A, Kurata K, Büki K, Matsusue Y, Väänänen HK & Rauvala H (2009). Osteocyte-derived HB-GAM (pleiotrophin) is associated with bone formation and mechanical loading. Bone 44 (5):785-794. doi: <http://dx.doi.org/10.1016/j.bone.2009.01.004>.
- Isaksson H, Wilson W, Van Donkelaar CC, Huiskes R & Ito K (2006). Comparison of biophysical stimuli for mechano-regulation of tissue differentiation during fracture healing. Journal of Biomechanics 39 (8):1507-1516. doi: <http://dx.doi.org/10.1016/j.jbiomech.2005.01.037>.
- Izquierdo E, Cañete JD, Celis R, Santiago B, Usategui A, Sanmartí R, Del Rey MJ & Pablos JL (2009). Immature Blood Vessels in Rheumatoid Synovium Are Selectively Depleted in Response to Anti-TNF Therapy. PLoS One 4 (12):e8131. doi: 10.1371/journal.pone.0008131.
- Jaiswal N, Haynesworth SE, Caplan AI & Bruder SP (1997a). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. Journal of Cellular Biochemistry 64 (2):295-312. doi: 10.1002/(SICI)1097-4644(199702)64:2<295::AID-JCB12>3.0.CO;2-I.
- Jaiswal N, Haynesworth SE, Caplan AI & Bruder SP (1997b). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. Journal of Cell Biochemistry 64 (2):295-312. doi: 10.1002/(SICI)1097-4644(199702)64:2<295::AID-JCB12>3.0.CO;2-I.
- Jee W (2001) Integrated Bone Tissue Physiology: Anatomy and Physiology. In: Cowin SC (ed) Bone Mechanics Handbook, Taylor and Francis.
- Jiang J, Nicoll SB & Lu HH (2005). Co-culture of osteoblasts and chondrocytes modulates cellular differentiation in vitro. Biochemical and Biophysical Research Communications 338 (2):762-70. doi: 10.1016/j.bbrc.2005.10.025.
- Jones E & Mcgonagle D (2008). Human bone marrow mesenchymal stem cells in vivo. Rheumatology (Oxford) 47 (2):126-31. doi: 10.1093/rheumatology/kem206.
- Jones SJ, Gray C, Sakamaki H, Arora M, Boyde A, Gourdie R & Green C (1993). The incidence and size of gap junctions between the bone cells in rat calvaria. Anatomy and Embryology 187 (4):343-352. doi: 10.1007/bf00185892.

- Jukes JM, Both SK, Leusink A, Sterk LMT, Van Blitterswijk CA & De Boer J (2008). Endochondral bone tissue engineering using embryonic stem cells. *Proceedings of the National Academy of Sciences* 105 (19):6840-6845. doi: 10.1073/pnas.0711662105.
- Kadiyala S, Jaiswal N & Bruder SP (1997a). Culture-expanded, bone marrow-derived mesenchymal stem cells can regenerate a critical-sized segmental bone defect. *Tissue Engineering* 3 (2):173-185. doi: 10.1089/ten.1997.3.173.
- Kadiyala S, Young RG, Thiede MA & Bruder SP (1997b). Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro. *Cell Transplantation* 6 (2):125-134. doi: [http://dx.doi.org/10.1016/S0963-6897\(96\)00279-5](http://dx.doi.org/10.1016/S0963-6897(96)00279-5).
- Kaigler D, Krebsbach PH, West ER, Horger K, Huang YC & Mooney DJ (2005). Endothelial cell modulation of bone marrow stromal cell osteogenic potential. *FASEB J* 19 (6):665-7. doi: 10.1096/fj.04-2529fje.
- Kamalia N, Mcculloch CA, Tenebaum HC & Limeback H (1992). Dexamethasone recruitment of self-renewing osteoprogenitor cells in chick bone marrow stromal cell cultures. *Blood* 79 (2):320-6.
- Kanczler JM, Ginty PJ, Barry JJA, Clarke NMP, Howdle SM, Shakesheff KM & Oreffo ROC (2008). The effect of mesenchymal populations and vascular endothelial growth factor delivered from biodegradable polymer scaffolds on bone formation. *Biomaterials* 29 (12):1892-1900. doi: <http://dx.doi.org/10.1016/j.biomaterials.2007.12.031>.
- Kanczler JM, Ginty PJ, White L, Clarke NMP, Howdle SM, Shakesheff KM & Oreffo ROC (2010). The effect of the delivery of vascular endothelial growth factor and bone morphogenic protein-2 to osteoprogenitor cell populations on bone formation. *Biomaterials* 31 (6):1242-1250. doi: <http://dx.doi.org/10.1016/j.biomaterials.2009.10.059>.
- Kanczler JM & Oreffo RO (2008). Osteogenesis and angiogenesis: the potential for engineering bone. *European Cells & Materials* 15 100-14. doi: vol015a08 [pii].
- Kato Y, Windle JJ, Koop BA, Mundy GR & Bonewald LF (1997). Establishment of an osteocyte-like cell line, MLO-Y4. *Journal of Bone Mineral Research* 12 (12):2014-23. doi: 10.1359/jbmr.1997.12.12.2014.
- Kawakami Y, Ishikawa T, Shimabara M, Tanda N, Enomoto-Iwamoto M, Iwamoto M, Kuwana T, Ueki A, Noji S & Nohno T (1996). BMP signaling during bone pattern determination in the developing limb. *Development* 122 (11):3557-3566.
- Kennedy OD, Herman BC, Laudier DM, Majeska RJ, Sun HB & Schaffler MB (2012). Activation of resorption in fatigue-loaded bone involves both apoptosis and active pro-osteoclastogenic signaling by distinct osteocyte populations. *Bone* 50 (5):1115-1122. doi: <http://dx.doi.org/10.1016/j.bone.2012.01.025>.
- Kim Y-J, Sah RLY, Doong J-YH & Grodzinsky AJ (1988). Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Analytical Biochemistry* 174 (1):168-176. doi: 10.1016/0003-2697(88)90532-5.
- Klein-Nulend J, Bacabac RG & Bakker AD (2012). Mechanical Loading and How It Affects Bone Cells: The Role of the Osteocyte Cytoskeleton in Maintaining Our Skeleton. *European Cells & Materials* 24 278-291.
- 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. *The Journal of the Federation of American Societies for Experimental Biology* 9 (5):441-5.

- Kneser U, Schaefer DJ, Polykandriotis E & Horch RE (2006). Tissue engineering of bone: the reconstructive surgeon's point of view. *Journal of Cellular and Molecular Medicine* 10 (1):7-19.
- Ko HC, Milthorpe BK & Mcfarland CD (2007). Engineering thick tissues--the vascularisation problem. *European Cells & Materials* 14 1-18; discussion 18-9.
- Kolambkar YM, Dupont KM, Boerckel JD, Huebsch N, Mooney DJ, Hutmacher DW & Guldberg RE (2011). An alginate-based hybrid system for growth factor delivery in the functional repair of large bone defects. *Biomaterials* 32 (1):65-74. doi: <http://dx.doi.org/10.1016/j.biomaterials.2010.08.074>.
- Komori T (2010). Regulation of osteoblast differentiation by Runx2. *Advances in Experimental Medicine and Biology* 658 43-9. doi: 10.1007/978-1-4419-1050-9_5.
- Korff T & Augustin HG (1998). Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *Journal of Cell Biology* 143 (5):1341-52.
- Kotobuki N, Ioku K, Kawagoe D, Fujimori H, Goto S & Ohgushi H (2005). Observation of osteogenic differentiation cascade of living mesenchymal stem cells on transparent hydroxyapatite ceramics. *Biomaterials* 26 (7):779-785. doi: <http://dx.doi.org/10.1016/j.biomaterials.2004.03.020>.
- Kotobuki N, Kawagoe D, Nomura D, Katou Y, Muraki K, Fujimori H, Goto S, Ioku K & Ohgushi H (2006). Observation and quantitative analysis of rat bone marrow stromal cells cultured in vitro on newly formed transparent β -tricalcium phosphate. *Journal of Materials Science: Materials in Medicine* 17 (1):33-41. doi: 10.1007/s10856-006-6327-1.
- Koyama E, Shibukawa Y, Nagayama M, Sugito H, Young B, Yuasa T, Okabe T, Ochiai T, Kamiya N, Rountree RB, Kingsley DM, Iwamoto M, Enomoto-Iwamoto M & Pacifici M (2008). A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. *Developmental Biology* 316 (1):62-73. doi: <http://dx.doi.org/10.1016/j.ydbio.2008.01.012>.
- Krebsbach PH, Mankani MH, Satomura K, Kuznetsov SA & Robey PG (1998). Repair of craniotomy defects using bone marrow stromal cells. *Transplantation* 66 (10):1272-1278. doi: Doi 10.1097/00007890-199811270-00002.
- Krecic-Stres H, Krkovic M, Koder J, Malicev E, Drobnic M, Marolt D & Kregar-Velikonja N (2007). Mesenchymal Stem Cells: a Modern Approach to Treat Long Bones Defects. 11th Mediterranean Conference on Medical and Biological Engineering and Computing 2007, Vols 1 and 2 16 (1-2):253-256.
- Krishnan L, Willett N & Guldberg R (2014). Vascularization Strategies for Bone Regeneration. *Annals of Biomedical Engineering* 42 (2):432-444. doi: 10.1007/s10439-014-0969-9.
- Kronenberg HM (2003). Developmental regulation of the growth plate. *Nature* 423 (6937):332-6. doi: 10.1038/nature01657.
- Kruyt MC, Dhert WJ, Oner C, Van Blitterswijk CA, Verbout AJ & De Bruijn JD (2004). Optimization of bone-tissue engineering in goats. *Journal of Biomedical Materials Research* 69 (2):113-120.
- Kuhn NZ & Tuan RS (2010). Regulation of stemness and stem cell niche of mesenchymal stem cells: implications in tumorigenesis and metastasis. *Journal of Cellular Physiology* 222 (2):268-77. doi: 10.1002/jcp.21940.

- Kusumbe AP, Ramasamy SK & Adams RH (2014). Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature* 507 (7492):323-8. doi: 10.1038/nature13145.
- Kyriakidou K, Lucarini G, Zizzi A, Salvolini E, Belmonte MM, Mollica F, Gloria A & Ambrosio L (2008). Dynamic co-seeding of osteoblast and endothelial cells on 3D polycaprolactone scaffolds for enhanced bone tissue engineering. *Journal of Bioactive and Compatible Polymers* 23 (3):227-243.
- Lacroix D & Prendergast PJ (2002). A mechano-regulation model for tissue differentiation during fracture healing: analysis of gap size and loading. *Journal of Biomechanics* 35 (9):1163-1171. doi: [http://dx.doi.org/10.1016/S0021-9290\(02\)00086-6](http://dx.doi.org/10.1016/S0021-9290(02)00086-6).
- Lacroix D, Prendergast PJ, Li G & Marsh D (2002). Biomechanical model to simulate tissue differentiation and bone regeneration: Application to fracture healing. *Medical and Biological Engineering and Computing* 40 (1):14-21. doi: 10.1007/bf02347690.
- Langenbach F & Handschel J (2013). Effects of dexamethasone, ascorbic acid and beta-glycerophosphate on the osteogenic differentiation of stem cells in vitro. *Stem Cell Research and Therapy* 4 (5):117. doi: 10.1186/scrt328.
- Lanyon LE (1993). Osteocytes, strain detection, bone modeling and remodeling. *Calcified Tissue International* 53 102-7.
- Leboy PS, Beresford JN, Devlin C & Owen ME (1991). Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. *Journal of Cellular Physiology* 146 (3):370-8. doi: 10.1002/jcp.1041460306.
- Leboy PS, Vaias L, Uschmann B, Golub E, Adams SL & Pacifici M (1989). Ascorbic acid induces alkaline phosphatase, type X collagen, and calcium deposition in cultured chick chondrocytes. *Journal of Biological Chemistry* 264 (29):17281-6.
- Lee J, Sung H-M, Jang J-D, Park Y-W, Min S-K & Kim E-C (2010). Successful Reconstruction of 15-cm Segmental Defects by Bone Marrow Stem Cells and Resected Autogenous Bone Graft in Central Hemangioma. *Journal of Oral and Maxillofacial Surgery* 68 (1):188-194. doi: <http://dx.doi.org/10.1016/j.joms.2009.08.031>.
- Lemaire V, Tobin FL, Greller LD, Cho CR & Suva LJ (2004). Modeling the interactions between osteoblast and osteoclast activities in bone remodeling. *Journal of Theoretical Biology* 229 (3):293-309. doi: 10.1016/j.jtbi.2004.03.023.
- Lenas P, Moos Jr M & Luyten FP (2009). Developmental engineering: a new paradigm for the design and manufacturing of cell-based products. Part II. From genes to networks: tissue engineering from the viewpoint of systems biology and network science. *Tissue Engineering Part B: Reviews* 15 (4):395-422.
- Li C, Vepari C, Jin H-J, Kim HJ & Kaplan DL (2006a). Electrospun silk-BMP-2 scaffolds for bone tissue engineering. *Biomaterials* 27 (16):3115-3124. doi: <http://dx.doi.org/10.1016/j.biomaterials.2006.01.022>.
- Li M, Wang X, Meintzer MK, Laessig T, Birnbaum MJ & Heidenreich KA (2000). Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3beta. *Molecular and Cellular Biology* 20 (24):9356-63.
- Li X, Feng Q, Liu X, Dong W & Cui F (2006b). Collagen-based implants reinforced by chitin fibres in a goat shank bone defect model. *Biomaterials* 27 (9):1917-23. doi: 10.1016/j.biomaterials.2005.11.013.

- Li XF, Zhang YZ, Kang HS, Liu WZ, Liu P, Zhang JG, Harris SE & Wu DQ (2005). Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *Journal of Biological Chemistry* 280 (20):19883-19887. doi: 10.1074/jbc.M413274200.
- Lian JB, Tassinari M & Glowacki J (1984). Resorption of implanted bone prepared from normal and warfarin-treated rats. *Journal of Clinical Investigation* 73 (4):1223-6. doi: 10.1172/JCI111308.
- Liao J, Hammerick KE, Challen GA, Goodell MA, Kasper FK & Mikos AG (2011). Investigating the role of hematopoietic stem and progenitor cells in regulating the osteogenic differentiation of mesenchymal stem cells in vitro. *Journal of Orthopaedic Research* 29 (10):1544-53. doi: 10.1002/jor.21436.
- Linkhart TA, Mohan S & Baylink DJ (1996a). Growth factors for bone growth and repair: IGF, TGF beta and BMP. *Bone* 19 (1):1-12.
- Linkhart TA, Mohan S & Baylink DJ (1996b). Growth factors for bone growth and repair: IGF, TGF β and BMP. *Bone* 19 (1, Supplement 1):S1-S12. doi: [http://dx.doi.org/10.1016/S8756-3282\(96\)00138-X](http://dx.doi.org/10.1016/S8756-3282(96)00138-X).
- Liu C, Zhao Y, Cheung WY, Gandhi R, Wang L & You L (2010). Effects of cyclic hydraulic pressure on osteocytes. *Bone* 46 (5):1449-56. doi: 10.1016/j.bone.2010.02.006.
- Liu X & Ma P (2004). Polymeric Scaffolds for Bone Tissue Engineering. *Annals of Biomedical Engineering* 32 (3):477-486. doi: 10.1023/B:ABME.0000017544.36001.8e.
- Logeart-Avramoglou D, Anagnostou F, Bizios R & Petite H (2005). Engineering bone: challenges and obstacles. *Journal of Cellular and Molecular Medicine* 9 (1):72-84.
- Luben RA, Wong GL & Cohn DV (1976). Biochemical characterization with parathormone and calcitonin of isolated bone cells: provisional identification of osteoclasts and osteoblasts. *Endocrinology* 99 (2):526-34. doi: 10.1210/endo-99-2-526.
- Luo Z-J & Seedhom BB (2007). Light and low-frequency pulsatile hydrostatic pressure enhances extracellular matrix formation by bone marrow mesenchymal cells: An in-vitro study with special reference to cartilage repair. *Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine* 221 (5):499-507. doi: 10.1243/09544119jeim199.
- Lyons F, Gleeson J, Partap S, Coghlan K & O'brien F (2014). Novel Microhydroxyapatite Particles in a Collagen Scaffold: A Bioactive Bone Void Filler? *Clinical Orthopaedics and Related Research®* 472 (4):1318-1328. doi: 10.1007/s11999-013-3438-0.
- Lyons FG, Al-Munajjed AA, Kieran SM, Toner ME, Murphy CM, Duffy GP & O'brien FJ (2010). The healing of bony defects by cell-free collagen-based scaffolds compared to stem cell-seeded tissue engineered constructs. *Biomaterials* 31 (35):9232-9243. doi: 10.1016/j.biomaterials.2010.08.056.
- Ma J, Van Den Beucken JJ, Yang F, Both SK, Cui FZ, Pan J & Jansen JA (2011). Coculture of osteoblasts and endothelial cells: optimization of culture medium and cell ratio. *Tissue Eng Part C Methods* 17 (3):349-57. doi: 10.1089/ten.TEC.2010.0215.
- Ma JL, Both SK, Yang F, Cui FZ, Pan JL, Meijer G, Jansen JA & Van Den Beucken JJJP (2014). Concise Review: Cell-Based Strategies in Bone Tissue Engineering and Regenerative Medicine. *Stem Cells Translational Medicine* 3 (1):98-107. doi: DOI 10.5966/sctm.2013-0126.

- Ma PX, Zhang R, Xiao G & Franceschi R (2001). Engineering new bone tissue in vitro on highly porous poly(α -hydroxyl acids)/hydroxyapatite composite scaffolds. *Journal of Biomedical Materials Research* 54 (2):284-293. doi: 10.1002/1097-4636(200102)54:2<284::aid-jbm16>3.0.co;2-w.
- Macintyre I, Zaidi M, Alam AS, Datta HK, Moonga BS, Lidbury PS, Hecker M & Vane JR (1991). Osteoclastic inhibition: an action of nitric oxide not mediated by cyclic GMP. *Proceedings of the National Academy of Sciences of the United States of America* 88 (7):2936-2940.
- Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO & Pittenger MF (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Engineering* 4 (4):415-28.
- Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS & Mirams M (2008). Endochondral ossification: How cartilage is converted into bone in the developing skeleton. *The International Journal of Biochemistry & Cell Biology* 40 (1):46-62. doi: 10.1016/j.biocel.2007.06.009.
- Maniatopoulos C, Sodek J & Melcher AH (1988). Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell and Tissue Research* 254 (2):317-330. doi: 10.1007/bf00225804.
- Marcacci M, Kon E, Moukhachev V, Lavroukov A, Kutepov S, Quarto R, Mastrogiacomo M & Cancedda R (2007). Stem cells associated with macroporous bioceramics for long bone repair: 6-to 7-year outcome of a pilot clinical study. *Tissue Engineering* 13 (5):947-955. doi: DOI 10.1089/ten.2006.0271.
- Marcacci M, Kon E, Zaffagnini S, Giardino R, Rocca M, Corsi A, Benvenuti A, Bianco P, Quarto R & Martin I (1999). Reconstruction of extensive long-bone defects in sheep using porous hydroxyapatite sponges. *Calcified Tissue International* 64 (1):83-90.
- Marieb EN & Hoen K, (2006). *Human Anatomy and Physiology*, Benjamin Cummings.
- Marks Jr SC & Seifert MF (1985). The lifespan of osteoclasts: Experimental studies using the giant granule cytoplasmic marker characteristic of beige mice. *Bone* 6 (6):451-455. doi: [http://dx.doi.org/10.1016/8756-3282\(85\)90223-6](http://dx.doi.org/10.1016/8756-3282(85)90223-6).
- Martin I (2014). Engineered tissues as customized organ germs. *Tissue Engineering Part A* 20 (7-8):1132-1133.
- 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. *Journal of Orthopaedic Research* 16 (2):181-189. doi: 10.1002/jor.1100160205.
- Mastrogiacomo M, Scaglione S, Martinetti R, Dolcini L, Beltrame F, Cancedda R & Quarto R (2006). Role of scaffold internal structure on in vivo bone formation in macroporous calcium phosphate bioceramics. *Biomaterials* 27 (17):3230-3237.
- Mauck RL, Nicoll SB, Seyhan SL, Ateshian GA & Hung CT (2003). Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. *Tissue Engineering* 9 (4):597-611. doi: 10.1089/107632703768247304.
- Mauck RL, Yuan X & Tuan RS (2006). Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. *Osteoarthritis Cartilage* 14 (2):179-89. doi: 10.1016/j.joca.2005.09.002.

- Mauney JR, Volloch V & Kaplan DL (2005). Role of adult mesenchymal stem cells in bone tissue engineering applications: current status and future prospects. *Tissue Engineering* 11 (5-6):787-802. doi: 10.1089/ten.2005.11.787.
- Mayr-Wohlfart U, Waltenberger J, Hausser H, Kessler S, Gunther KP, Dehio C, Puhl W & Brenner RE (2002). Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts. *Bone* 30 (3):472-7.
- Mcfadden TM, Duffy GP, Allen AB, Stevens HY, Schwarzmaier SM, Plesnila N, Murphy JM, Barry FP, Guldberg RE & O'brien FJ (2013). The delayed addition of human mesenchymal stem cells to pre-formed endothelial cell networks results in functional vascularization of a collagen-glycosaminoglycan scaffold in vivo. *Acta biomaterialia* 9 (12):9303-9316. doi: <http://dx.doi.org/10.1016/j.actbio.2013.08.014>.
- Mckibbin B (1978). The biology of fracture healing in long bones. *Journal of Bone and Joint (Br)* 60-B (2):150-62.
- Mcnamara LM (2011) Bone as a Material. In: Ducheyne P (ed) *Comprehensive Biomaterials*, vol 2. Elsevier Ltd., pp. 169-186.
- Mcnamara LM, Majeska RJ, Weinbaum S, Friedrich V & Schaffler MB (2009). Attachment of Osteocyte Cell Processes to the Bone Matrix. *The Anatomical Record* 292 (3):355-363. doi: Doi 10.1002/Ar.20869.
- Meijer GJ, De Bruijn JD, Koole R & Van Blitterswijk CA (2007). Cell-based bone tissue engineering. *PLoS Med* 4 (2):9. doi: 10.1371/journal.pmed.0040009.
- Meijer GJ, De Bruijn JD, Koole R & Van Blitterswijk CA (2008). Cell based bone tissue engineering in jaw defects. *Biomaterials* 29 (21):3053-3061. doi: 10.1016/j.biomaterials.2008.03.012.
- Meinel L, Fajardo R, Hofmann S, Langer R, Chen J, Snyder B, Vunjak-Novakovic G & Kaplan D (2005). Silk implants for the healing of critical size bone defects. *Bone* 37 (5):688-698. doi: <http://dx.doi.org/10.1016/j.bone.2005.06.010>.
- Meinel L, Karageorgiou V, Fajardo R, Snyder B, Shinde-Patil V, Zichner L, Kaplan D, Langer R & Vunjak-Novakovic G (2004a). Bone Tissue Engineering Using Human Mesenchymal Stem Cells: Effects of Scaffold Material and Medium Flow. *Annals of Biomedical Engineering* 32 (1):112-122. doi: 10.1023/B:ABME.0000007796.48329.b4.
- Meinel L, Karageorgiou V, Hofmann S, Fajardo R, Snyder B, Li C, Zichner L, Langer R, Vunjak-Novakovic G & Kaplan DL (2004b). Engineering bone-like tissue in vitro using human bone marrow stem cells and silk scaffolds. *Journal of Biomedical Materials Research Part A* 71A (1):25-34. doi: 10.1002/jbm.a.30117.
- Melero-Martin JM, De Obaldia ME, Kang SY, Khan ZA, Yuan L, Oettgen P & Bischoff J (2008). Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. *Circulation Research* 103 (2):194-202. doi: 10.1161/CIRCRESAHA.108.178590.
- Merz E & Bahlmann F, (2004). Ultrasound in Obstetrics and Gynecology, Thieme.
- Meyer EG, Buckley CT, Steward AJ & Kelly DJ (2011). The effect of cyclic hydrostatic pressure on the functional development of cartilaginous tissues engineered using bone marrow derived mesenchymal stem cells. *Journal of the Mechanical Behavior of Biomedical Materials* 4 (7):1257-1265. doi: 10.1016/j.jmbbm.2011.04.012.
- Meyer Von GH (1867). Die Architektur der Spongiosa. *Archiv für Anatomie, Physiologie und Wissenschaftliche Medicin.* (34):615-628.

- Middleton JC & Tipton AJ (2000). Synthetic biodegradable polymers as orthopedic devices. *Biomaterials* 21 (23):2335-2346.
- Miller A (1984). Collagen: the organic matrix of bone. *Philos Trans R Soc Lond B Biol Sci* 304 (1121):455-77.
- Miller EJ (1969). Biochemical studies on the structure of chick bone collagen. *Federation Proceedings* 28 (6):1839-45.
- Miller SC, Bowman BM, Smith JM & Jee WSS (1980). Characterization of endosteal bone-lining cells from fatty marrow bone sites in adult beagles. *The Anatomical Record* 198 (2):163-173. doi: 10.1002/ar.1091980204.
- Miller SC, De Saint-Georges L, Bowman BM & Jee WS (1989). Bone lining cells: structure and function. *Scanning Microsc* 3 (3):953-60; discussion 960-1.
- Miller SC & Jee WS (1987). The bone lining cell: a distinct phenotype? *Calcified Tissue International* 41 (1):1-5.
- Miot S, Brehm W, Dickinson S, Sims T, Wixmerten A, Longinotti C, Hollander AP, Mainil-Varlet P & Martin I (2012). Influence of in vitro maturation of engineered cartilage on the outcome of osteochondral repair in a goat model. *European Cells & Materials* 23 222-36.
- Mirjam Fröhlich WLG, Leo Q. Wan, Darja Marolt, Matej Drobnič, and Gordana Vunjak-Novaković (2008). Tissue Engineered Bone Grafts: Biological Requirements, Tissue Culture and Clinical Relevance. *Current Stem Cell Research & Therapy* 3 (4):254-264.
- Miyanishi K, Trindade MC, Lindsey DP, Beaupre GS, Carter DR, Goodman SB, Schurman DJ & Smith RL (2006a). Dose- and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor-beta3-induced chondrogenesis by adult human mesenchymal stem cells in vitro. *Tissue Engineering* 12 (8):2253-62. doi: 10.1089/ten.2006.12.2253.
- Miyanishi K, Trindade MC, Lindsey DP, Beaupre GS, Carter DR, Goodman SB, Schurman DJ & Smith RL (2006b). Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis in vitro. *Tissue Engineering* 12 (6):1419-28. doi: 10.1089/ten.2006.12.1419.
- Mohan S, Nakao Y, Honda Y, Landale E, Leser U, Dony C, Lang K & Baylink DJ (1995). Studies on the Mechanisms by Which Insulin-Like Growth-Factor (Igf) Binding Protein-4 (Igfbp-4) and Igfbp-5 Modulate Igf Actions in Bone-Cells. *Journal of Biological Chemistry* 270 (35):20424-20431.
- Morgan SM, Tilley S, Perera S, Ellis MJ, Kanczler J, Chaudhuri JB & Oreffo ROC (2007). Expansion of human bone marrow stromal cells on poly-(dl-lactide-co-glycolide) (PDLLGA) hollow fibres designed for use in skeletal tissue engineering. *Biomaterials* 28 (35):5332-5343. doi: <http://dx.doi.org/10.1016/j.biomaterials.2007.08.029>.
- Morrison SJ & Scadden DT (2014). The bone marrow niche for haematopoietic stem cells. *Nature* 505 (7483):327-34. doi: 10.1038/nature12984.
- Morrison SJ & Spradling AC (2008). Stem Cells and Niches: Mechanisms That Promote Stem Cell Maintenance throughout Life. *Cell* 132 (4):598-611. doi: <http://dx.doi.org/10.1016/j.cell.2008.01.038>.
- Mueller MB & Tuan RS (2008). Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. *Arthritis & Rheumatology* 58 (5):1377-88. doi: 10.1002/art.23370.
- Mundy GR (1993). Factors which stimulate bone growth in vivo. *Growth Regulation* 3 (2):124-8.

- Mundy GR & Poser JW (1983). Chemotactic activity of the gamma-carboxyglutamic acid containing protein in bone. *Calcified Tissue International* 35 (2):164-8.
- Murphy CM, Schindeler A, Gleeson JP, Yu NYC, Cantrill LC, Mikulec K, Peacock L, O'Brien FJ & Little DG (2014). A collagen-hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates. *Acta biomaterialia* 10 (5):2250-2258. doi: <http://dx.doi.org/10.1016/j.actbio.2014.01.016>.
- Muschler GF, Nitto H, Matsukura Y, Boehm C, Valdevit A, Kambic H, Davros W, Powell K & Easley K (2003). Spine fusion using cell matrix composites enriched in bone marrow-derived cells. *Clinical orthopaedics and related research* (407):102-118. doi: DOI 10.1097/01.blo.0000030504.43495.46.
- Nakagawa M, Kaneda T, Arakawa T, Morita S, Sato T, Yomada T, Hanada K, Kumegawa M & Hakeda Y (2000). Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. *The Journal of the Federation of American Societies for Experimental Biology* 473 (2):161-4. doi: S0014-5793(00)01520-9 [pii].
- Nakaoka R, Hsiong SX & Mooney DJ (2006). Regulation of chondrocyte differentiation level via co-culture with osteoblasts. *Tissue Engineering* 12 (9):2425-33. doi: 10.1089/ten.2006.12.2425.
- Nielsen GP & Rosenberg AE (2010) Bone-Forming Tumours. In: Goldblum JR (ed) *Bone and Soft Tissue Pathology*, Churchill Livingstone/Elsevier, p. 309.
- Noble BS (2008). The osteocyte lineage. *Archives of Biochemistry and Biophysics* 473 (2):106-11. doi: 10.1016/j.abb.2008.04.009.
- Nomura S, Wills AJ, Edwards DR, Heath JK & Hogan BL (1988). Developmental expression of 2ar (osteopontin) and SPARC (osteonectin) RNA as revealed by in situ hybridization. *Journal of Cell Biology* 106 (2):441-50.
- Noonan KJ, Stevens JW, Tammi R, Tammi M, Hernandez JA & Midura RJ (1996). Spatial distribution of CD44 and hyaluronan in the proximal tibia of the growing rat. *Journal of Orthopaedic Research* 14 (4):573-81. doi: 10.1002/jor.1100140411.
- Nowlan NC, Murphy P & Prendergast PJ (2007). Mechanobiology of embryonic limb development. *Annals of the New York Academy Sciences* 1101 389-411. doi: 10.1196/annals.1389.003.
- Nowlan NC, Murphy P & Prendergast PJ (2008). A dynamic pattern of mechanical stimulation promotes ossification in avian embryonic long bones. *Journal of Biomechanics* 41 (2):249-258. doi: <http://dx.doi.org/10.1016/j.jbiomech.2007.09.031>.
- O'Brien FJ (2011). Biomaterials & scaffolds for tissue engineering. *Materials Today* 14 (3):88-95. doi: [http://dx.doi.org/10.1016/S1369-7021\(11\)70058-X](http://dx.doi.org/10.1016/S1369-7021(11)70058-X).
- Oest ME, Dupont KM, Kong HJ, Mooney DJ & Guldberg RE (2007). Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects. *Journal of Orthopaedic Research* 25 (7):941-950.
- Ogawa R, Mizuno S, Murphy GF & Orgill DP (2009). The effect of hydrostatic pressure on three-dimensional chondroinduction of human adipose-derived stem cells. *Tissue Engineering Part A* 15 (10):2937-45. doi: 10.1089/ten.TEA.2008.0672.
- Ohgushi H, Dohi Y, Katuda T, Tamai S, Tabata S & Suwa Y (1996). In vitro bone formation by rat marrow cell culture. *Journal of Biomedical Materials Research* 32 (3):333-40. doi: 10.1002/(SICI)1097-4636(199611)32:3<333::AID-JBM5>;3.0.CO;2-T.

- Ohgushi H, Dohi Y, Tamai S & Tabata S (1993). Osteogenic differentiation of marrow stromal stem cells in porous hydroxyapatite ceramics. *Journal of Biomedical Materials Research* 27 (11):1401-1407. doi: 10.1002/jbm.820271107.
- Okazaki R, Inoue D, Shibata M, Saika M, Kido S, Ooka H, Tomiyama H, Sakamoto Y & Matsumoto T (2002). Estrogen Promotes Early Osteoblast Differentiation and Inhibits Adipocyte Differentiation in Mouse Bone Marrow Stromal Cell Lines that Express Estrogen Receptor (ER) α or β . *Endocrinology* 143 (6):2349-2356. doi: doi:10.1210/endo.143.6.8854.
- Oliveira SM, Amaral IF, Barbosa MA & Teixeira CC (2009a). Engineering endochondral bone: in vitro studies. *Tissue Eng Part A* 15 (3):625-34. doi: 10.1089/ten.tea.2008.0051.
- Oliveira SM, Mijares DQ, Turner G, Amaral IF, Barbosa MA & Teixeira CC (2009b). Engineering endochondral bone: in vivo studies. *Tissue Engineering Part A* 15 (3):635-43. doi: 10.1089/ten.tea.2008.0052.
- Oreffo R & Triffitt J (1999). Future potentials for using osteogenic stem cells and biomaterials in orthopedics. *Bone* 25 (2):5-9.
- Oreffo ROC, Driessens FCM, Planell JA & Triffitt JT (1998). Growth and differentiation of human bone marrow osteoprogenitors on novel calcium phosphate cements. *Biomaterials* 19 (20):1845-1854. doi: [http://dx.doi.org/10.1016/S0142-9612\(98\)00084-2](http://dx.doi.org/10.1016/S0142-9612(98)00084-2).
- Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, Mckay R, Nadal-Ginard B, Bodine DM, Leri A & Anversa P (2001). Bone marrow cells regenerate infarcted myocardium. *Nature* 410 (6829):701-5. doi: 10.1038/35070587.
- Ornitz DM & Marie PJ (2002). FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev* 16 (12):1446-65. doi: 10.1101/gad.990702.
- Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N & Phinney DG (2003). Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proceedings of the National Academy of Sciences of the United States of America* 100 (14):8407-8411. doi: DOI 10.1073/pnas.1432929100.
- Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB & Stein GS (1990). Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *Journal of Cellular Physiology* 143 (3):420-30. doi: 10.1002/jcp.1041430304.
- Pedersen TO, Blois AL, Xing Z, Xue Y, Sun Y, Finne-Wistrand A, Akslen LA, Lorens JB, Leknes KN, Fristad I & Mustafa K (2013). Endothelial microvascular networks affect gene-expression profiles and osteogenic potential of tissue-engineered constructs. *Stem Cell Research and Therapy* 4 (3):52. doi: 10.1186/scrt202.
- Peister A, Mellad JA, Larson BL, Hall BM, Gibson LF & Prockop DJ (2004). Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood* 103 (5):1662-8. doi: 10.1182/blood-2003-09-3070.
- Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, Aigner T & Richter W (2006). Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular

- invasion after ectopic transplantation in SCID mice. *Arthritis Rheum* 54 (10):3254-66. doi: 10.1002/art.22136.
- Perry CR (1999). Bone repair techniques, bone graft, and bone graft substitutes. *Clinical Orthopaedics and Related Research* (360):71-86.
- Peter SJ, Liang CR, Kim DJ, Widmer MS & Mikos AG (1998). Osteoblastic phenotype of rat marrow stromal cells cultured in the presence of dexamethasone, beta-glycerophosphate, and L-ascorbic acid. *Journal of Cellular Biochemistry* 71 (1):55-62. doi: 10.1002/(SICI)1097-4644(19981001)71:1<55::AID-JCB6>3.0.CO;2-0 [pii].
- Phelps EA & Garcia AJ (2009). Update on therapeutic vascularization strategies. *Regen Med* 4 (1):65-80. doi: 10.2217/17460751.4.1.65.
- 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 (5411):143-7.
- Poole KES, Van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, Lowik CW & Reeve J (2005). Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *The Journal of the Federation of American Societies for Experimental Biology* 19 (10):1842-+. doi: DOI 10.1096/fj.05-4221fje.
- Prestwich GD & Matthew H (2002). Hybrid, composite, and complex biomaterials. *Annals of the New York Academy Sciences* 961 (1):106-108.
- Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, Kon E & Marcacci M (2001). Repair of large bone defects with the use of autologous bone marrow stromal cells. *New England Journal of Medicine* 344 (5):385-386. doi: Doi 10.1056/Nejm200102013440516.
- Ramay HRR & Zhang M (2004). Biphasic calcium phosphate nanocomposite porous scaffolds for load-bearing bone tissue engineering. *Biomaterials* 25 (21):5171-5180. doi: <http://dx.doi.org/10.1016/j.biomaterials.2003.12.023>.
- Reddi AH (1992). Regulation of cartilage and bone differentiation by bone morphogenetic proteins. *Current opinion in cell biology* 4 (5):850-855.
- Reddi AH (1998). Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat Biotechnol* 16 (3):247-52. doi: 10.1038/nbt0398-247.
- Reddi AH (2007) Morphogenesis and Tissue Engineering. In: Lanza R, Langer R & Vacanti JP (eds) *Principles of Tissue Engineering*, Elsevier Academic Press, pp. 117-118.
- Reid RL (1968). Hernia through an Iliac Bone-Graft Donor Site. *Journal of Bone and Joint (Am)* 50 (4):757-760.
- Rezwan K, Chen QZ, Blaker JJ & Boccaccini AR (2006). Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials* 27 (18):3413-3431. doi: <http://dx.doi.org/10.1016/j.biomaterials.2006.01.039>.
- Rivron NC, Liu JJ, Rouwema J, De Boer J & Van Blitterswijk CA (2008). Engineering vascularised tissues in vitro. *European Cells & Materials* 15 27-40.
- Rizzo M & Moran SL (2008). Vascularized Bone Grafts and Their Applications in the Treatment of Carpal Pathology. *Seminars in Plastic Surgery* 22 (3):213-227. doi: 10.1055/s-2008-1081404.
- Roach HI (1992). Trans-differentiation of hypertrophic chondrocytes into cells capable of producing a mineralized bone matrix. *Bone and Mineral* 19 (1):1-20. doi: 10.1016/0169-6009(92)90840-a.

- Roach HI (1994). Why does bone matrix contain non-collagenous proteins? The possible roles of osteocalcin, osteonectin, osteopontin, and bone sialoprotein in bone mineralisation and resorption. *Cell Biology International* 18 (6):617-628. doi: 10.1006/cbir.1994.1088.
- Roach HI (1997). New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. *Journal of Bone Mineral Research* 12 (5):795-805. doi: 10.1359/jbmr.1997.12.5.795.
- Robison R (1923). The Possible Significance of Hexosephosphoric Esters in Ossification. *Journal of Biochemistry* 17 (2):286-93.
- Robling AG, Bellido T & Turner CH (2006). Mechanical stimulation in vivo reduces osteocyte expression of sclerostin. *Journal of Musculoskeletal & Neuronal Interactions* 6 (4):354.
- Rodan GA & Martin TJ (1981). Role of osteoblasts in hormonal control of bone resorption--a hypothesis. *Calcified Tissue International* 33 (4):349-51.
- Roodman GD (1996). Advances in bone biology: the osteoclast. *Endocrine Reviews* 17 (4):308-32. doi: 10.1210/edrv-17-4-308.
- Rose FR & Oreffo RO (2002). Bone tissue engineering: hope vs hype. *Biochemical and Biophysical Research Communications* 292 (1):1-7. doi: 10.1006/bbrc.2002.6519.
- Ross FP & Christiano AM (2006). Nothing but skin and bone. *Journal of Clinical Investigation* 116 1140-1149.
- Rouwkema J, De Boer J & Van Blitterswijk CA (2006). Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct. *Tissue Engineering* 12 (9):2685-93. doi: 10.1089/ten.2006.12.2685.
- Rucci N (2008). Molecular biology of bone remodelling. *Clinical Cases Mineral Bone Metabolism* 5 (1):49-56.
- Sage H, Vernon RB, Decker J, Funk S & Iruela-Arispe ML (1989). Distribution of the calcium-binding protein SPARC in tissues of embryonic and adult mice. *Journal of Histochemistry and Cytochemistry* 37 (6):819-29.
- Saito T, Kuang JQ, Bittira B, Al-Khalidi A & Chiu RC (2002). Xenotransplant cardiac chimera: immune tolerance of adult stem cells. *Ann Thorac Surg* 74 (1):19-24; discussion 24.
- Sakou T, Onishi T, Yamamoto T, Nagamine T, Sampath TK & Ten Dijke P (1999). Localization of Smads, the TGF- β Family Intracellular Signaling Components During Endochondral Ossification. *Journal of Bone and Mineral Research* 14 (7):1145-1152. doi: 10.1359/jbmr.1999.14.7.1145.
- Saleh FA, Whyte M & Genever PG (2011). Effects of endothelial cells on human mesenchymal stem cell activity in a three-dimensional in vitro model. *European Cells & Materials* 22 242-57; discussion 257.
- Samuel SP, Baran GR, Wei G & Davis BL (2009) Biomechanics - Part II. In: Khurana JS (ed) *Bone Pathology*, Humana Press, pp. 69-70. doi: 10.1007/978-1-59745-347-9.
- Sartoris D, Holmes R & Resnick D (1991). Coralline hydroxyapatite bone graft substitutes: radiographic evaluation. *The Journal of foot and ankle surgery* 31 (3):301-313.
- Schaffler M & Kennedy O (2012). Osteocyte Signaling in Bone. *Current Osteoporosis Reports* 10 (2):118-125. doi: 10.1007/s11914-012-0105-4.

- Schaffler MB, Cheung WY, Majeska R & Kennedy O (2014). Osteocytes: master orchestrators of bone. *Calcified Tissue International* 94 (1):5-24. doi: 10.1007/s00223-013-9790-y.
- Scherberich A, Galli R, Jaquiere C, Farhadi J & Martin I (2007). Three-dimensional perfusion culture of human adipose tissue-derived endothelial and osteoblastic progenitors generates osteogenic constructs with intrinsic vascularization capacity. *Stem Cells* 25 (7):1823-9. doi: 10.1634/stemcells.2007-0124.
- Schmitt JM, Hwang K, Winn SR & Hollinger JO (1999). Bone morphogenetic proteins: an update on basic biology and clinical relevance. *Journal of Orthopaedic Research* 17 (2):269-278.
- Schofield R (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood cells* 4 (1-2):7-25.
- Schwartz Z, Hancock RH, Dean DD, Brooks BP, Gomez R, Boskey AL, Balian G & Boyan BD (1995). Dexamethasone promotes von kossa-positive nodule formation and increased alkaline phosphatase activity in costochondral chondrocyte cultures. *Endocrine* 3 (5):351-60. doi: 10.1007/BF03021418.
- Schwarz S, Elsaesser AF, Koerber L, Goldberg-Bockhorn E, Seitz AM, Bermueller C, Dürselen L, Ignatius A, Breiter R & Rotter N (2012a). Processed xenogenic cartilage as innovative biomatrix for cartilage tissue engineering: effects on chondrocyte differentiation and function. *Journal of tissue engineering and regenerative medicine* n/a-n/a. doi: 10.1002/term.1650.
- Schwarz S, Koerber L, Elsaesser AF, Goldberg-Bockhorn E, Seitz AM, Durselen L, Ignatius A, Walther P, Breiter R & Rotter N (2012b). Decellularized Cartilage Matrix as a Novel Biomatrix for Cartilage Tissue-Engineering Applications. *Tissue Engineering Part A* 18 (21-22):2195-2209. doi: DOI 10.1089/ten.tea.2011.0705.
- Scotti C, Piccinini E, Takizawa H, Todorov A, Bourgine P, Papadimitropoulos A, Barbero A, Manz MG & Martin I (2013). Engineering of a functional bone organ through endochondral ossification. *Proceedings of the National Academy of Sciences* 110 (10):3997-4002.
- Scotti C, Tonnarelli B, Papadimitropoulos A, Scherberich A, Schaeren S, Schauerte A, Lopez-Rios J, Zeller R, Barbero A & Martin I (2010). Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. *Proceedings of the National Academy of Sciences* 107 (16):7251-7256. doi: 10.1073/pnas.1000302107.
- Shayesteh YS, Khojasteh A, Soleimani M, Alikhasi M, Khoshzaban A & Ahmadbeigi N (2008). Sinus augmentation using human mesenchymal stem cells loaded into a β -tricalcium phosphate/hydroxyapatite scaffold. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 106 (2):203-209. doi: <http://dx.doi.org/10.1016/j.tripleo.2007.12.001>.
- Sheehy EJ, Vinardell T, Buckley CT & Kelly DJ (2013). Engineering osteochondral constructs through spatial regulation of endochondral ossification. *Acta biomaterialia* 9 (3):5484-5492.
- Sheyn D, Kallai I, Tawackoli W, Cohn Yakubovich D, Oh A, Su S, Da X, Lavi A, Kimelman-Bleich N, Zilberman Y, Li N, Bae H, Gazit Z, Pelled G & Gazit D (2011). Gene-modified adult stem cells regenerate vertebral bone defect in a rat model. *Molecular Pharmaceutics* 8 (5):1592-601. doi: 10.1021/mp200226c.
- Shin M, Yoshimoto H & Vacanti JP (2004). In vivo bone tissue engineering using mesenchymal stem cells on a novel electrospun nanofibrous scaffold. *Tissue Engineering* 10 (1-2):33-41. doi: Doi 10.1089/107632704322791673.

- Smith A, (1968). The body, Walker.
- Song IH, Caplan AI & Dennis JE (2009). In vitro dexamethasone pretreatment enhances bone formation of human mesenchymal stem cells in vivo. *Journal of Orthopaedic Research* 27 (7):916-21. doi: 10.1002/jor.20838.
- Sowa DT & Weiland AJ (1987). Clinical applications of vascularized bone autografts. *Orthop Clin North Am* 18 (2):257-73.
- St-Jacques B, Hammerschmidt M & McMahon AP (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation (vol 13, pg 2072, 1999). *Genes & Development* 13 (19):2617-2617.
- St John TA, Vaccaro AR, Sah AP, Schaefer M, Berta SC, Albert T & Hilibrand A (2003). Physical and monetary costs associated with autogenous bone graft harvesting. *The American Journal of Orthopedics* 32 (1):18-23.
- Sun H, Qu Z, Guo Y, Zang G & Yang B (2007). In vitro and in vivo effects of rat kidney vascular endothelial cells on osteogenesis of rat bone marrow mesenchymal stem cells growing on polylactide-glycolic acid (PLGA) scaffolds. *Biomedical Engineering OnLine* 6 41. doi: 10.1186/1475-925X-6-41.
- Sun N, Lee A & Wu JC (2009). Long term non-invasive imaging of embryonic stem cells using reporter genes. *Nature Protocols* 4 (8):1192-1201.
- Tada H, Nemoto E, Foster BL, Somerman MJ & Shimauchi H (2011). Phosphate increases bone morphogenetic protein-2 expression through cAMP-dependent protein kinase and ERK1/2 pathways in human dental pulp cells. *Bone* 48 (6):1409-16. doi: 10.1016/j.bone.2011.03.675.
- Takai E, Mauck RL, Hung CT & Guo XE (2004). Osteocyte viability and regulation of osteoblast function in a 3D trabecular bone explant under dynamic hydrostatic pressure. *Journal of Bone Mineral Research* 19 (9):1403-10. doi: 10.1359/JBMR.040516.
- Tan SD, De Vries TJ, Kuijpers-Jagtman AM, Semeins CM, Everts V & Klein-Nulend J (2007). Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. *Bone* 41 (5):745-751. doi: <http://dx.doi.org/10.1016/j.bone.2007.07.019>.
- Tan SD, Kuijpers-Jagtman AM, Semeins CM, Bronckers ALJJ, Maltha JC, Von Den Hoff JW, Everts V & Klein-Nulend J (2006). Fluid shear stress inhibits TNF alpha-induced osteocyte apoptosis. *Journal of Dental Research* 85 (10):905-909.
- Tare RS, Howard D, Pound JC, Roach HI & Oreffo ROC (2005). Tissue engineering strategies for cartilage generation—Micromass and three dimensional cultures using human chondrocytes and a continuous cell line. *Biochemical and Biophysical Research Communications* 333 (2):609-621. doi: <http://dx.doi.org/10.1016/j.bbrc.2005.05.117>.
- Taylor AF, Saunders MM, Shingle DL, Cimbala JM, Zhou Z & Donahue HJ (2007). Mechanically stimulated osteocytes regulate osteoblastic activity via gap junctions. *American Journal of Physiology - Cell Physiology* 292 (1):C545-52. doi: 10.1152/ajpcell.00611.2005.
- Taylor GI (1983). The current status of free vascularized bone grafts. *Clin Plast Surg* 10 (1):185-209.
- Teitelbaum SL & Ross FP (2003). Genetic regulation of osteoclast development and function. *Nature Reviews Genetics* 4 (8):638-49. doi: 10.1038/nrg1122.

- Tenenbaum HC (1981). Role of organic phosphate in mineralization of bone in vitro. *Journal of Dental Research* 60 Spec No C 1586-9.
- Tenenbaum HC & Heersche JN (1985). Dexamethasone stimulates osteogenesis in chick periosteum in vitro. *Endocrinology* 117 (5):2211-7. doi: 10.1210/endo-117-5-2211.
- Tenenbaum HC & Heersche JNM (1982). Differentiation of osteoblasts and formation of mineralized bone in vitro. *Calcified Tissue International* 34 (1):76-79. doi: 10.1007/bf02411212.
- Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, Langer R & Snyder EY (2002). Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. *Proc Natl Acad Sci U S A* 99 (5):3024-9. doi: 10.1073/pnas.052678899.
- Thompson WR, Modla S, Grindel BJ, Czymbek KJ, Kirn-Safran CB, Wang L, Duncan RL & Farach-Carson MC (2011). Perlecan/Hspg2 deficiency alters the pericellular space of the lacunocanicular system surrounding osteocytic processes in cortical bone. *Journal of Bone Mineral Research* 26 (3):618-29. doi: 10.1002/jbmr.236.
- Thorpe SD, Buckley CT, Vinardell T, O'brien FJ, Campbell VA & Kelly DJ (2010). The response of bone marrow-derived mesenchymal stem cells to dynamic compression following TGF-beta3 induced chondrogenic differentiation. *Annals of Biomedical Engineering* 38 (9):2896-909. doi: 10.1007/s10439-010-0059-6.
- Thurner PJ, Lam S, Weaver JC, Morse DE & Hansma PK (2009). Localization of Phosphorylated Serine, Osteopontin, and Bone Sialoprotein on Bone Fracture Surfaces. *Journal of Adhesion* 85 (8):526-545. doi: 10.1080/00218460902996424.
- Toma C, Pittenger MF, Cahill KS, Byrne BJ & Kessler PD (2002). Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 105 (1):93-8.
- Tropel P, Noel D, Platet N, Legrand P, Benabid AL & Berger F (2004). Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow. *Experimental Cell Research* 295 (2):395-406. doi: 10.1016/j.yexcr.2003.12.030.
- Tsai TL, Wang BW, Squire MW, Guo LW & Li WJ (2015). Endothelial cells direct human mesenchymal stem cells for osteo- and chondro-lineage differentiation through endothelin-1 and AKT signaling. *Stem Cell Research & Therapy* 6 doi: 10.1186/S13287-015-0065-6.
- Tsiridis E, Upadhyay N & Giannoudis P (2007). Molecular aspects of fracture healing:Which are the important molecules? *Injury* 38 (1, Supplement):S11-S25. doi: <http://dx.doi.org/10.1016/j.injury.2007.02.006>.
- Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, Koga T, Martin TJ & Suda T (1990). Origin of Osteoclasts - Mature Monocytes and Macrophages Are Capable of Differentiating into Osteoclasts under a Suitable Microenvironment Prepared by Bone Marrow-Derived Stromal Cells. *Proceedings of the National Academy of Sciences of the United States of America* 87 (18):7260-7264. doi: 10.1073/pnas.87.18.7260.
- Ulrich-Merzenich G, Metzner C, Bhonde RR, Malsch G, Schiermeyer B & Vetter H (2002a). Simultaneous isolation of endothelial and smooth muscle cells from human umbilical artery or vein and their growth response to low-density

- lipoproteins. In Vitro Cellular & Developmental Biology-Animal 38 (5):265-272.
- Ulrich-Merzenich G, Metzner C, Schiermeyer B & Vetter H (2002b). Vitamin C and vitamin E antagonistically modulate human vascular endothelial and smooth muscle cell DNA synthesis and proliferation. European Journal of Nutrition 41 (1):27-34.
- Ulrich-Merzenich G, Zeitler H, Panek D, Bokemeyer D & Vetter H (2007). Vitamin C promotes human endothelial cell growth via the ERK-signaling pathway. European Journal of Nutrition 46 (2):87-94. doi: 10.1007/s00394-006-0636-5.
- Usdepartmentofdefence. (2014). *Casualty Status* [Online]. Available: <Http://www.defense.gov/news/casualty.pdf> [Accessed 23/03/2015 2015].
- Van Der Stok J, Koolen MK, Jahr H, Kops N, Waarsing JH, Weinans H & Van Der Jagt OP (2014). Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. European Cells & Materials 27 137-48; discussion 148.
- Vater C, Kasten P & Stiehler M (2011). Culture media for the differentiation of mesenchymal stromal cells. Acta biomaterialia 7 (2):463-77. doi: 10.1016/j.actbio.2010.07.037.
- Vaughan TJ, McCarthy CT & McNamara LM (2012). A three-scale finite element investigation into the effects of tissue mineralisation and lamellar organisation in human cortical and trabecular bone. Journal of the Mechanical Behavior of Biomedical Materials 12 (0):50-62. doi: <http://dx.doi.org/10.1016/j.jmbbm.2012.03.003>.
- Verbruggen Stefaan w, McCarthy Myles j, Haugh Matthew g, Voisin Muriel c & McNamara Laoise m (2015). Altered Mechanical Environment of Bone Cells in an Animal Model of Short- and Long-Term Osteoporosis. Biophysical Journal 108 (7):1587-1598. doi: <http://dx.doi.org/10.1016/j.bpj.2015.02.031>.
- Vermeulen AH, Vermeer C & Bosman FT (1989). Histochemical detection of osteocalcin in normal and pathological human bone. Journal of Histochemistry and Cytochemistry 37 (10):1503-8.
- Verseijden F, Posthumus-Van Sluijs SJ, Pavljasevic P, Hofer SO, Van Osch GJ & Farrell E (2010). Adult human bone marrow- and adipose tissue-derived stromal cells support the formation of prevascular-like structures from endothelial cells in vitro. Tissue Engineering Part A 16 (1):101-14. doi: 10.1089/ten.TEA.2009.0106.
- Villars F, Bordenave L, Bareille R & Amedee J (2000). Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF? Journal of Cell Biochemistry 79 (4):672-85.
- Villars F, Guillotin B, Amedee T, Dutoya S, Bordenave L, Bareille R & Amedee J (2002). Effect of HUVEC on human osteoprogenitor cell differentiation needs heterotypic gap junction communication. American Journal of Physiology - Cell Physiology 282 (4):C775-85. doi: 10.1152/ajpcell.00310.2001.
- Vinardell T, Rolfe RA, Buckley CT, Meyer EG, Ahearne M, Murphy P & Kelly DJ (2012). Hydrostatic pressure acts to stabilise a chondrogenic phenotype in porcine joint tissue derived stem cells. European Cells & Materials 23 121-32; discussion 133-4.
- Vinardell T, Thorpe SD, Buckley CT & Kelly DJ (2009). Chondrogenesis and integration of mesenchymal stem cells within an in vitro cartilage defect repair model. Annals of Biomedical Engineering 37 (12):2556-65. doi: 10.1007/s10439-009-9791-1.

- Visser J, Gawlitza D, Benders KEM, Toma SMH, Pouran B, Van Weeren PR, Dhert WJA & Malda J (2015). Endochondral bone formation in gelatin methacrylamide hydrogel with embedded cartilage-derived matrix particles. *Biomaterials* 37 (0):174-182. doi: <http://dx.doi.org/10.1016/j.biomaterials.2014.10.020>.
- Wagner DR, Lindsey DP, Li KW, Tummala P, Chandran SE, Smith RL, Longaker MT, Carter DR & Beaupre GS (2008). Hydrostatic pressure enhances chondrogenic differentiation of human bone marrow stromal cells in osteochondrogenic medium. *Annals of Biomedical Engineering* 36 (5):813-20. doi: 10.1007/s10439-008-9448-5.
- Wang HY, Pang B, Li Y, Zhu DL, Pang TX & Liu YJ (2012a). Dexamethasone has variable effects on mesenchymal stromal cells. *Cytotherapy* 14 (4):423-430. doi: Doi 10.3109/14653249.2011.652735.
- Wang X, Schröder HC, Wiens M, Ushijima H & Müller WEG (2012b). Bio-silica and bio-polyphosphate: applications in biomedicine (bone formation). *Current Opinion in Biotechnology* 23 (4):570-578. doi: <http://dx.doi.org/10.1016/j.copbio.2012.01.018>.
- Wang Y, Kim H-J, Vunjak-Novakovic G & Kaplan DL (2006). Stem cell-based tissue engineering with silk biomaterials. *Biomaterials* 27 (36):6064-6082. doi: <http://dx.doi.org/10.1016/j.biomaterials.2006.07.008>.
- Wang Y, McNamara LM, Schaffler MB & Weinbaum S (2007). A model for the role of integrins in flow induced mechanotransduction in osteocytes. *Proceedings of the National Academy of Sciences of the United States of America* 104 (40):15941-15946. doi: 10.1073/pnas.0707246104.
- Watanabe H, Yanagisawa T & Sasaki J (1995). Cytoskeletal architecture of rat calvarial osteoclasts: Microfilaments, intermediate filaments, and nuclear matrix as demonstrated by detergent perfusion. *The Anatomical Record* 243 (2):165-174. doi: 10.1002/ar.1092430203.
- Waters RL, Lunsford BR, Perry J & Byrd R (1988). Energy-speed relationship of walking: standard tables. *Journal of Orthopaedic Research* 6 (2):215-22. doi: 10.1002/jor.1100060208.
- Webster S, (2012). *Embryology at a Glance*, John Wiley & Sons.
- Wei G & Ma PX (2004). Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering. *Biomaterials* 25 (19):4749-4757. doi: <http://dx.doi.org/10.1016/j.biomaterials.2003.12.005>.
- Weiland AJ, Moore JR & Daniel RK (1983). Vascularized bone autografts. Experience with 41 cases. *Clin Orthop Relat Res* (174):87-95.
- Wensel TM, Iranikhah MM & Wilborn TW (2011). Effects of Denosumab on Bone Mineral Density and Bone Turnover in Postmenopausal Women. *Pharmacotherapy* 31 (5):510-523.
- Williams JM, Adewunmi A, Schek RM, Flanagan CL, Krebsbach PH, Feinberg SE, Hollister SJ & Das S (2005). Bone tissue engineering using polycaprolactone scaffolds fabricated via selective laser sintering. *Biomaterials* 26 (23):4817-4827. doi: DOI 10.1016/j.biomaterials.2004.11.057.
- Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, Skonier JE, Shpektor D, Jonas M, Kovacevich BR, Staehling-Hampton K, Appleby M, Brunkow ME & Latham JA (2003). Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *The EMBO Journal* 22 (23):6267-76. doi: 10.1093/emboj/cdg599.

- Winn SR, Uludag H & Hollinger JO (1999). Carrier systems for bone morphogenetic proteins. *Clinical Orthopaedics and Related Research* 367 S95-S106.
- Wolff J, (1986). *The Law of Bone Remodelling*, Berlin, Springer-Verlag Berlin Heidelberg.
- Wong M & Carter D (1990a). A theoretical model of endochondral ossification and bone architectural construction in long bone ontogeny. *Anatomy and Embryology* 181 (6):523-532. doi: 10.1007/bf00174625.
- Wong M & Carter DR (1990b). Theoretical stress analysis of organ culture osteogenesis. *Bone* 11 (2):127-131. doi: [http://dx.doi.org/10.1016/8756-3282\(90\)90060-C](http://dx.doi.org/10.1016/8756-3282(90)90060-C).
- Wong M, Siegrist M & Goodwin K (2003). Cyclic tensile strain and cyclic hydrostatic pressure differentially regulate expression of hypertrophic markers in primary chondrocytes. *Bone* 33 (4):685-693. doi: [http://dx.doi.org/10.1016/S8756-3282\(03\)00242-4](http://dx.doi.org/10.1016/S8756-3282(03)00242-4).
- Xaymardan M, Cimini M, Weisel RD & Li R (2010) Bone Marrow Stem Cells: Properties and Pluripotency. In: Carlson BM (ed) *Stem Cell Anthology*, Elsevier, p. 160.
- Xia X, Batra N, Shi Q, Bonewald LF, Sprague E & Jiang JX (2010). Prostaglandin Promotion of Osteocyte Gap Junction Function through Transcriptional Regulation of Connexin 43 by Glycogen Synthase Kinase 3/β-Catenin Signaling. *Molecular and Cellular Biology* 30 (1):206-219. doi: 10.1128/mcb.01844-08.
- Yang XB, Whitaker MJ, Sebald W, Clarke N, Howdle SM, Shakesheff KM & Oreffo RO (2004). Human osteoprogenitor bone formation using encapsulated bone morphogenetic protein 2 in porous polymer scaffolds. *Tissue Engineering* 10 (7-8):1037-1045.
- Ye K, Felimban R, Moulton SE, Wallace GG, Di Bella C, Traianedes K, Choong PF & Myers DE (2013). Bioengineering of articular cartilage: past, present and future. *Regen Med* 8 (3):333-49. doi: 10.2217/rme.13.28.
- Yoshikawa T, Ohgushi H, Dohi Y & Davies JE (1997). Viable bone formation in porous hydroxyapatite: Marrow cell-derived in vitro bone on the surface of ceramics. *Bio-Medical Materials and Engineering* 7 (1):49-58.
- Yoshimoto H, Shin YM, Terai H & Vacanti JP (2003). A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. *Biomaterials* 24 (12):2077-2082. doi: [http://dx.doi.org/10.1016/S0142-9612\(02\)00635-X](http://dx.doi.org/10.1016/S0142-9612(02)00635-X).
- Younger EM & Chapman MW (1989). Morbidity at bone graft donor sites. *Journal of Orthopaedic Trauma* 3 (3):192-195.
- Zamiri B, Shahidi S, Eslaminejad MB, Khoshzaban A, Gholami M, Bahramnejad E, Moghadasali R, Mardpour S & Aghdami N (2013). Reconstruction of human mandibular continuity defects with allogenic scaffold and autologous marrow mesenchymal stem cells. *Journal of Craniofacial Surgery* 24 (4):1292-7. doi: 10.1097/SCS.0b013e318294288a.
- Zhang R & Ma PX (1999). Poly (α -hydroxyl acids)/hydroxyapatite porous composites for bone-tissue engineering. I. Preparation and morphology. *Journal of Biomedical Materials Research* 44 (4):446-455. doi: [http://dx.doi.org/10.1002/\(SICI\)1097-4636\(19990315\)44:4<446::AID-JBM11>3.0.CO;2-F](http://dx.doi.org/10.1002/(SICI)1097-4636(19990315)44:4<446::AID-JBM11>3.0.CO;2-F).

- Zhao S, Zhang YK, Harris S, Ahuja SS & Bonewald LF (2002). MLO-Y4 osteocyte-like cells support osteoclast formation and activation. *Journal of Bone Mineral Research* 17 (11):2068-79. doi: 10.1359/jbmr.2002.17.11.2068.
- Zhao X, Liu L, Wang F-K, Zhao D-P, Dai X-M & Han X-S (2012). Coculture of vascular endothelial cells and adipose-derived stem cells as a source for bone engineering. *Annals of Plastic Surgery* 69 (1):91-98.
- Zomorodian E & Baghaban Eslaminejad M (2012). Mesenchymal Stem Cells as a Potent Cell Source for Bone Regeneration. *Stem Cells International* 2012 doi: 10.1155/2012/980353.
- Zuniga R. (2015). *Why Are Our Arms the Same Length* [Online]. 2014 Arizona Board of Regents. Available: <http://askabiologist.asu.edu/plosable/why-are-our-arms-same-length> [Accessed 30/04/2015].