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Human Development and Health

Tissue regeneration in osteoarthritis: the effects of inflammatory cytokines on bioengineering strategies to repair arthritic joints

Volume I of I

by

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ABSTRACT

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TISSUE REGENERATION IN OSTEOARTHRITIS: THE EFFECTS OF INFLAMMATORY CYTOKINES ON BIOENGINEERING STRATEGIES TO REPAIR ARTHRITIC JOINTS

Emma Louise Williams

Osteoarthritis (OA) is the most common form of arthritis worldwide. Historically, treatments for OA have been analgesics, physiotherapy, joint injections and eventually joint replacement surgery. With the advent of tissue engineering (TE), there has been considerable interest in exploiting these techniques to devise new treatments for OA. Despite the development of skeletal stem cell (SSC) constructs aimed at creating viable cartilage and bone, few studies have examined the effects of inflammatory cytokines present in OA synovial tissues, on such constructs. Here, both *in vitro* and *in vivo* approaches were used to examine the effects of cytokines on the integrity of such bone constructs.

This work confirms IL-1β is a key cytokine for enhancing osteogenic differentiation in OA, whereas TNFα is inhibitory. OA supernatants were obtained from synovial tissue of patients undergoing hip or knee arthroplasty. Most OA supernatants produced an inhibitory effect on osteogenic differentiation in co-cultures with HBMSCs; a minority had additive osteogenic effects, consistent with a more inflammatory subtype of OA. miRNA profiles were analysed for the first time in these co-cultures, producing valuable insights into the pathways through which IL-1β may mediate its effects on osteogenic differentiation. Finally, *ex vivo* and *in vivo* models of bone formation, not previously used in the OA field, corroborated the findings of these *in vitro* studies. These data support the utility of focusing on upregulating IL1-β and inhibiting TNFα to modify existing bone TE strategies being developed locally, for use in the future treatment of OA. Findings were similar with other cell types such as induced pluripotent stem cells, which show promise for future TE strategies. This work also validated the organotypic chick femur and subcutaneous implant models for the future testing of other bioactive factors or miRNAs for incorporation into bone TE approaches for the management of OA.

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List of publications, presentations and awards

The following publications, presentations and awards have resulted from work completed as part of this candidature for Doctor of Philosophy.

Awards:

I was awarded a Cawley Fellowship from the Southampton Rheumatology Trust in order to carry out my PhD studies.

Academic articles:

Williams EL, Edwards CJ, Cooper C, Oreffo ROC. The osteoarthritic niche and modulation of skeletal stem cell function for regenerative medicine. J Tissue Eng Regen Med. 2013; 7 (8):589-608.

Williams EL, Edwards CJ, Cooper C, Oreffo ROC. Tissue Engineering Strategies: can they help us repair osteoarthritic joints? BioTech International 2012 Nov/Dec [Epub ahead of print]

Williams EL, Edwards CJ, Cooper C, Oreffo ROC. Impact of inflammation on the osteoarthritic niche; implications for regenerative medicine. Regenerative Medicine 2012 Jul;7(4):551-70.

Conference/symposia presentations:

What influence do inflammatory cytokines have on the development of bioengineering strategies to repair arthritic joints? – Southampton University Clinical Academic Trainees' (SoCAT) Conference, March 2011.

Conference poster presentations and abstracts:

Williams EL, Cooper C, Edwards CJ, Oreffo ROC. Strontium and cytokine modulated human bone marrow stromal cells – a future treatment for osteoarthritis? Accepted as an abstract and poster for the BSR annual meeting 2017.

Williams EL, Cooper C, Oreffo ROC, Edwards CJ. Tissue regeneration in Osteoarthritis – the effects of inflammation on current reparative strategies. Accepted as an abstract and poster for the University of Southampton Medical School Postgraduate Conference 2012.

Williams EL, Cooper C, Oreffo ROC, Edwards CJ. Inflammatory cytokines and bioengineering strategies for arthritis – friend or foe? Accepted as an abstract and poster for BSR annual meeting 2012.

Williams EL, Cooper C, Oreffo ROC, Edwards CJ. What influence do inflammatory cytokines have on the development of bioengineering strategies to repair arthritic joints? Presented as a poster at the 3rd combined BRS/BORS meeting, June 2011.

Williams EL, Roach HI, Cooper C, Oreffo ROC and Edwards CJ. 'The Effects of Inflammatory Cytokines from Arthritic Synovium on Skeletal Stem Cell Function – Implications for Reparative Strategies'. Accepted as an abstract and poster for University of Southampton School of Medicine Academic Clinical Trainees Conference 2010. Final poster number: 28.

Williams EL, Roach HI, Cooper C, Oreffo ROC and Edwards CJ. 'The Effects of Inflammatory Cytokines from Arthritic Synovium on Skeletal Stem Cell Function – Implications for Reparative Strategies'. Accepted as an abstract and poster for BSR annual meeting 2009. Final abstract number: 38.

Williams EL, Dunlop D, MacDonald TT, Edwards CJ. Does differential expression of the TGFβ-superfamily signalling molecule Smad-7 explain differences in joint healing associated with osteoarthritis and rheumatoid arthritis? Accepted as an abstract and poster for the BSR annual meeting 2008.

Regional presentations:

What influence do inflammatory cytokines have on bioengineering strategies to repair arthritic joints? – Southampton Regional Training Afternoon, Sept 2013.

Research in Inflammation and Arthroplasty – Osteoarthritis Research Syndicate (OARS) Regional Meeting, March 2012.

Research Presentation - Regional Acute Medicine Day, Southampton General Hospital, March 2010.

Other associated publications:

Williams EL, White K, Oreffo ROC. 'Isolation and enrichment of Stro-1 immunoselected mesenchymal stem cells from adult human bone marrow' pg67-73. In: Stem Cell Niche: Methods and Protocols. Ed. K. Turksen. Springer, 2013.

Roach HI and Williams EL. Epigenetic clues to Arthritic Diseases. Chapter VI 'Epigenetic Clues to Arthritic Diseases' pg159-182. In 'Epigenetics: Mechanisms, Functions and Human Effects'. Eds: Balázs Pintér and Zsolt Mészáros. Nova Publishers, 2009.

Academic Thesis: Declaration Of Authorship

I, Emma Williams

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

'Tissue regeneration in osteoarthritis: the effects of inflammatory cytokines on bioengineering strategies to repair arthritic joints'

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

Williams EL, Edwards CJ, Cooper C, Oreffo ROC. The osteoarthritic niche and modulation of skeletal stem cell function for regenerative medicine. J Tissue Eng Regen Med. 2013; 7 (8):589-608.

Williams EL, Edwards CJ, Cooper C, Oreffo ROC. Tissue Engineering Strategies: can they help us repair osteoarthritic joints? BioTech International 2012 Nov/Dec [Epub ahead of print].

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Signed: .....

Date: 19th November 2018.....

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Finally, a huge thank you to my family, especially my husband Richard and sons Henry and Luke, for putting up with the studying and associated stresses, especially during the writing up stage.

Definitions and Abbreviations

ACI – autologous chondrocyte implantation

ACLT – anterior cruciate ligament transection model of OA

ACVR2B – activin receptor type 2B

ADAMTS – ADAM metalloproteinase with thrombospondin type 1 motif

ALP – alkaline phosphatase

αMEM – alpha modified Eagle's medium

AMPK/JNK – AMP-activated protein kinase/c-Jun N-terminal kinase pathway

AP-1- activator protein -1

AS – ankylosing spondylitis

Bcl-2 – B-cell CLL/lymphoma 2

BCP – basic calcium phosphate

BMP – bone morphogenetic protein

BSA – bovine serum albumin

BSP – bone sialoprotein

CASR – calcium sensing receptor

CbFα1 – core-binding factor subunit alpha-1

CCB - craniocleidodysostosis

CCL2 – C-C motif chemokine ligand -2

CCL7 – C-C motif chemokine ligand-7

CCL11- C-C motif chemokine ligand-11

CHRDL1 – chordin-like-1

CM-01 – Carolinas Molecule-01

COL1A1 – Collagen 1A1

COL1A2 – Collagen 1A2

COMP – cartilage oligomeric protein

COX-2 – cyclo-oxygenase-2

cPLA2 – calcium-dependent phospholipase A2

CRP – C-reactive protein

CTG – cell tracker green

CTGF/CCN2 – connective tissue growth factor

CXCL-8 – C-X-C motif ligand 8

DAS-28 – disease activity score -28 joint count

DEPC – diethyl pyrocarbonate

DDR2 – discoid domain receptor 2

DIP – distal interphalangeal joints

Dkk-1 – Dickkopf -1

DKK-1 AS – Dkk-1 antisense nucleotide

DMARD – Disease Modifying Anti-Rheumatic Drugs

DMM – Destabilisation of the medial meniscus model of OA

DMOAD – Disease Modifying Osteoarthritis Drugs

DMSO – dimethyl sulfoxide

DR6 – death receptor 6 (also known as TNF receptor superfamily member 21 TNFRSF21)

DVL3 – dishevelled segment polarity protein-3

ECM – extracellular matrix

EH-1 – ethidium homodimer

EMA – European Medicines Agency

EphB2 – ephrin B2

ERK 1/2 - extracellular signal-regulated kinases 1/2

ET-1 - endothelin -1

FCS – fetal calf serum

FGF – fibroblast-like growth factor

FLS – fibroblast-like synoviocytes

FRAT2 – FRAT2, Wnt signalling parthway

GADD45β – growth arrest and DNA damage 45 beta

GAG – glucosaminoglycan

GDF-5 - growth differentiation factor 5

GLUT – glucose transporter protein

GNP – Gross National Product

Gp130 – glycoprotein 130

GP – General Practitioner

hADSC – human adipose tissue-derived stromal cells

HBMSC – human bone marrow-derived stromal cells

HDAC – histone deacetylase

hESC – human embryonic stem cells

HIF – hypoxia-inducible-factor -1α

HMGB1 – high mobility group box protein1

IGF-1 – insulin-like growth factor -1

IGFR -1 – insulin-like growth factor receptor 1

IL – interleukin

IL-1RA – IL-1 receptor antagonist

iNOS – inducible nitric oxide synthase

iPSC – induced pluripotent stem cells

IRAK – IL-1 receptor associated kinase

IRAP – IL-1 receptor antagonist protein

JAKi – janus kinase inhibitor

JNK – c-Jun N-terminal kinase

KL – Kellgren Lawrence

KOOS – Knee injection and Osteoarthritis Outcome Score

LIF – leukaemia inhibitory factor

MAPK – mitogen-activated protein kinase

MAPKAPK – mitogen-activated protein kinase activated protein kinase

MCPIP-1 – monocyte chemotactic protein-induced protein-1

M-CSF – macrophage colony stimulating factor

miRNA – microRNA

MMP – matrix metalloproteinase

MSC – mesenchymal stem cells

NFκB – nuclear factor kappa B

NGF – nerve growth factor

NO – nitric oxide

OA - osteoarthritis

OCN - osteocalcin

OP - osteoporosis

OP-1 – osteogenic protein-1

OPG - osteoprotegerin

OPN - osteopontin

OSM – oncostatin M

PPARγ – peroxisome proliferator-activated receptor gamma

PBS – phosphate buffered saline

PCNA – proliferating cell nuclear antigen

PCT – polymerase chain reaction

PDGF – platelet-derived growth factor

PGE2 – prostaglandin E2

PGRN - progranulin

PI3K – phosphatidylinositol 3-kinase

PKA – protein kinase A

POMC – pro-opiomelanocortin

PRAC – Pharmacovigilance Risk Assessment Committee

PRP – platelet-rich plasma

PsA – psoriatic arthritis

PTH-R – parathyroid hormone receptor

PTHrP – parathyroid hormone related peptide

RA – rheumatoid arthritis

RANK – receptor activator of nuclear factor kappa B

RANKL – receptor activator of nuclear factor kappa B ligand

RAS – rat sarcoma viral oncogene homolog

Runx-2 – runt-related transcription factor 2

SAP – stress-activated protein

scAAV – self-complementary adeno-associated virus

SEKOIA – Strontium Ranelate in Knee Osteoarthritis Trial

SET1A – SET Domain containing 1A

siRNA – small interfering RNA

Sirt-1 – Sirtuin -1

SLE – Systemic Lupus Erythematosus

Smad – mothers against decapentaplegic homolog -1

SNP – single nucleotide polymorphism

SOCS-3 – suppressor of cytokine signalling 3

SOD-2 – superoxide dismutase 2

SOX-9 – sex determining region Y – box 9

SSC – skeletal stem cells

STAT – signal transducer and activator of transcription protein

TAZ – PDZ- binding motif

TGFβ – transforming growth factor β

TIMP – tissue inhibitor of metalloproteinases-1

TLR-4 – toll-like receptor 4

TNFα – tumour necrosis factor α

TNF-R – tumour necrosis factor receptor

TRAF-6 – tumour necrosis factor associated factor 6

TRAP – tartrate-resistant acid phosphatase

TSA – Trichostatin A

UK – United Kingdom

VEGF – vascular endothelial growth factor

Wnt – Wingless-related integration site

WOMAC – Western Ontario and McMaster University Osteoarthritis Index

WTCRF- Wellcome Trust Clinical Research Facility

ZIP8 – hepatic metal ion transporter - 8

Chapter 1 Introduction

Impact of inflammation on the osteoarthritic niche; implications for regenerative medicine

1.1 The challenge of treating OA

Osteoarthritis (OA) is the most common form of arthritis worldwide. The majority of people over the age of 65 have radiographic evidence of OA and this proportion rises to approximately 80% in those over 75 (Arden and Nevitt 2006). The hallmarks of OA on plain radiographs are joint space narrowing, subchondral sclerosis, cysts and osteophyte formation, although a more aggressive erosive subtype can occur (Figures 1.1-1.2). The latest figures from Public Health England indicate that 18.2% of those over 45 years of age in England have OA of the knee and 10.9% have OA of the hip (ARUK Musculoskeletal Calculator). OA is a significant cause of morbidity, ranking as the sixth leading cause of disability globally (Woolf and Pfleger 2003). It costs the UK economy an estimated 1% of GNP per annum (ARMA, 2004). Every year 2 million people visit their GP due to symptoms caused by OA (ARC, 2002). In 2016, a total of 87,733 hip replacements and 98,147 knee replacements were performed in England and Wales, the majority of which were for patients with OA (National Joint Registry, 2017). Across the UK as a whole, rates of knee replacement operations in women have increased from 43 per 100,000 person years in 1991 to 137 per 100,000 person years in 2006 (Nursing Times, 2012).

With an ageing population, this cost will continue to rise. Unlike rheumatoid arthritis (RA) where disease modifying anti-rheumatic drugs (DMARDs) and biologics have revolutionised treatment over recent years, treatments for OA have been limited to painkillers, physiotherapy and joint injections. When these fail, patients are referred for joint replacement surgery. Although highly effective in reducing pain and improving joint function, joint replacement surgery is not without risk. With primary surgery being performed on ever younger patients, costly and complex revision surgery will become more prevalent. With the advent of tissue engineering strategies aimed at generating new bone and cartilage for repair of osteochondral defects, there has been considerable interest in exploiting these techniques to devise new treatments for OA.

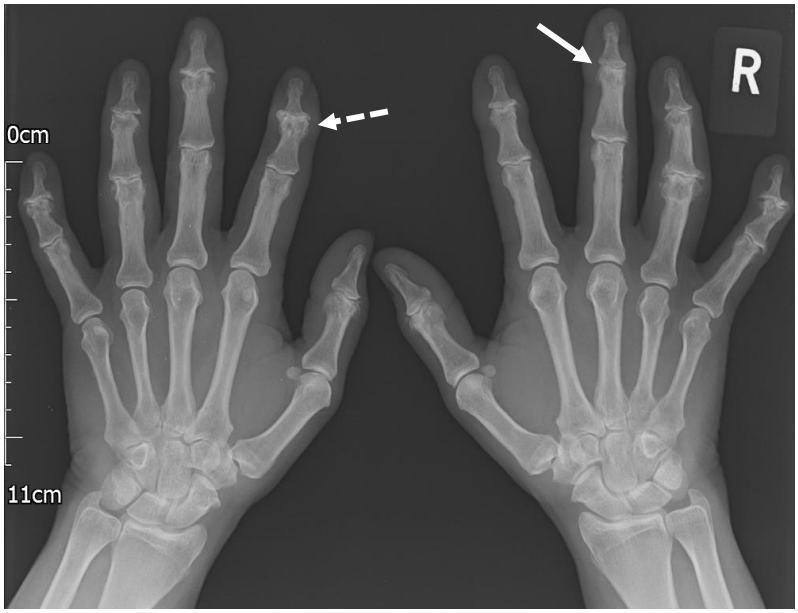


Figure 1.1 Plain radiographs from a patient with OA of the hands.

Plain arrow demonstrates typical distal interphalangeal joint (DIP) involvement with joint space narrowing and subchondral sclerosis; dashed arrow indicates DIP joint space narrowing and subchondral cysts.

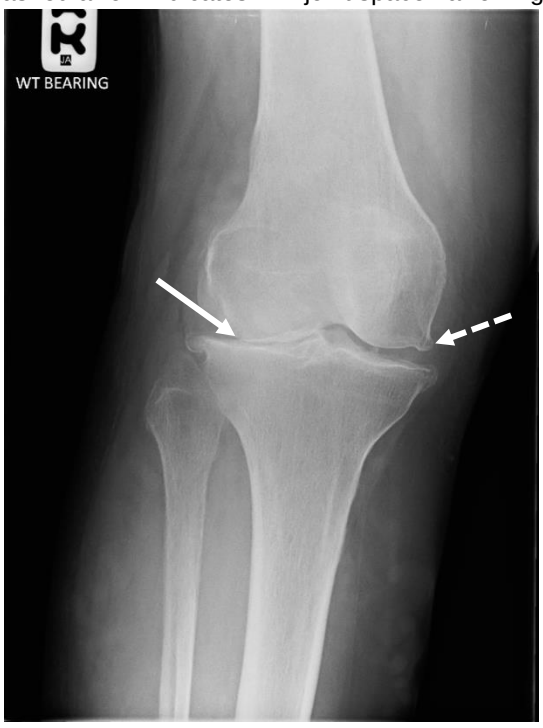


Figure 1.2 Plain radiographs from a patient with OA of the knee.

Plain arrow demonstrates typical knee joint involvement with joint space narrowing and subchondral sclerosis; dashed arrow indicates the presence of osteophytes.

However, to date, no consideration has been given to the OA niche and attendant inflammatory milieu for any regenerative strategy for the bone or cartilage in OA. This work aims to highlight the importance of understanding the osteoarthritic niche in order to modify existing tissue engineering strategies for the future treatment of OA. Initially, it will focus on the osteoarthritic niche and summarise tissue engineering principles, before moving on to consider how tissue engineering is already being used for musculoskeletal repair. Finally, approaches for modifying existing strategies to account for the inflammatory environment present within OA joints will be considered, together with suggestions for future progress.

1.2 Tissue engineering and OA

1.2.1 Principles of tissue engineering

Tissue engineering provides a useful approach to the repair of damaged tissues and has been used successfully in a variety of disease processes. This section will focus on how the tissue engineering paradigm can be used in musculoskeletal disease, particularly OA, initially as an adjunct but potentially in the future as an alternative to joint replacement. In order to discuss tissue engineering, it is first necessary to describe the key components of a tissue engineering strategy (Figure 1.3). This requires multipotent stem cells; in OA these will be mesenchymal stem cells (MSCs), more appropriately referred to as skeletal stem cells (SSCs).

SSCs have the potential to differentiate into bone, cartilage, adipose tissue, connective tissue and smooth muscle. Stem cells can be derived or generated (e.g. induced pluripotent stem cells) from multiple sources with varying degrees of success. The most commonly used sources remain bone marrow and peripheral blood. These SSCs are typically combined with a support or ‘scaffold’, ranging from a hydrogel through to a 3D synthetic scaffold (for a full review of scaffolds for skeletal tissue engineering see (Zippel, Schulze et al. 2010). The scaffold both supports the delivery of cells and aids cell survival and growth within the target tissue, as well as serving as a vehicle for growth factor delivery (Tare, Kanczler et al. 2010). Additional components pivotal to the tissue engineering paradigm are mechanical stimulation and the judicious selection of bioactive molecules including hormones, growth factors and cytokines, to maximise cell growth and integration with the target tissue (Dawson and Oreffo 2008).

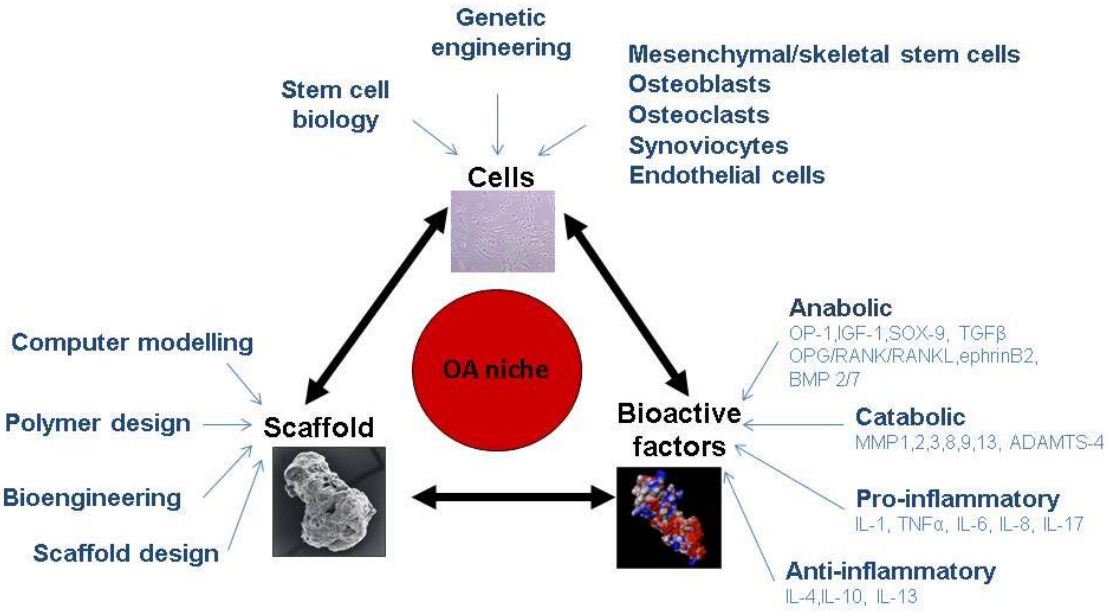


Figure 1.3 Schematic diagram of the tissue engineering paradigm highlighting the three main components required and the factors or processes that influence each of these
(adapted from (Williams, Edwards et al. 2012))
OP -1 – osteogenic protein -1; IGF-1 – insulin-like growth factor-1; SOX-9 - sex determining region Y – box 9; TGFβ – transforming growth factor beta; OPG – osteoprotegerin; RANK – receptor activator of nuclear factor-κβ; RANKL - receptor activator of nuclear factor-κβ ligand; BMP – bone morphogenetic protein; MMP – matrix metalloproteinase; ADAMTS-4 – ADAM metallopeptidase with thrombospondin type 1 motif, 4; IL – interleukin; TNFα – tumour necrosis factor alpha.

1.2.2 Current limitations of tissue engineering

Tissue engineering strategies have been successful in producing healing of long bone defects, fractures or cartilage damage in animal models (reviewed in (Arthur, Zannettino et al. 2009)). However, in terms of translation into clinical practice, only a few small-scale studies have shown equivalence or superiority to existing therapies. This has been most successful using autologous MSCs combined with a variety of different scaffolds to treat fracture non-union (Quarto, Mastrogiacomo et al. 2001), long bone defects (Quarto, Mastrogiacomo et al. 2001, Price, Connolly et al. 2003, Marcacci, Kon et al. 2007) and spinal fusion (Price, Connolly et al. 2003, Faundez, Taylor et al. 2006, Putzier, Strube et al. 2008). In contrast, results utilising this approach in craniofacial surgery proved mixed. Several groups have demonstrated that autologous mandibular periosteal cells cultured on polymer fleece (Schmelzeisen, Schimming et al. 2003), collagen scaffolds (Springer, Nocini et al. 2006), xenograft bone (Springer, Nocini et al. 2006, Beaumont, Schmidt et al. 2008) or

calcium phosphate ceramic scaffolds (Shayesteh, Khojasteh et al. 2008) can be used successfully for sinus augmentation surgery. However, other groups have failed to replicate these findings (Schimming and Schmelzeisen 2004). One of the best cited examples to date of cell-based tissue engineering remains the successful transplantation of a composite tissue-engineered airway using stem-cell derived chondrocytes. This included co-culture of viable chondrocytes and epithelial cells either side of a structural matrix, demonstrating the ability to create composite tissue constructs with implications for osteochondral repair (Macchiarini, Jungebluth et al. 2008). One of the particular challenges with utilising bioengineering strategies for an OA joint is the requirement for a composite scaffold like this, in the case of OA to bridge an osteochondral defect. The complex zonal structure of articular cartilage necessary for it to fulfil its biomechanical role is especially difficult to recreate, although new strategies are slowly beginning to address this (Sharma, Saxena et al. 2007, O'Shea and Miao 2008, Nguyen, Kudva et al. 2011). Not only do these difficulties exist in terms of managing to establish successful co-cultures and incorporation into the scaffold, they also impact on the implantation and behaviour of the construct within the target tissue. For example, autologous chondrocyte implantation (ACI) techniques, although successful in some patients with chondral damage to the knee, to date are limited by the occurrence of chondrocyte differentiation, which leads to healing of part of the defect by fibrosis. This fibrotic tissue is weaker than normal cartilage and therefore adversely affects the biomechanical properties of the OA joint as before. This phenomenon is termed a loss of phenotype or phenotypic conversion.

Tissue engineering strategies are currently being used locally to optimise existing impaction bone grafting strategies for revision hip surgery or treatment of substantial bone defects due to benign tumours or avascular necrosis(Tilley, Bolland et al. 2006). This builds on previous studies attempting to improve the success of impaction bone grafting strategies using recombinant human bone morphogenetic protein-2 (BMP-2) or 7, either alone or in combination with autologous bone graft. Results were disappointing compared with animal models (Gautschi, Frey et al. 2007). This raises the possibility that the unique environment, including the presence of inflammatory cytokines, may be inhibiting success. Therefore, current work is focused on identifying additional molecules such as other growth factors or inflammatory cytokines, which can either be applied to the cells prior to implantation or incorporated into the scaffold. In terms of cartilage repair, combining either chondrocytes or SSCs and overexpression of BMP-2, transforming growth factor beta1 (TGFβ1) or insulin-

like growth factor-1 (IGF-1) using viral vectors has proven effective in animal models but has yet to be fully tested in clinical trials (Nixon, Goodrich et al. 2007).

In all of the clinical situations described above, tissue engineering strategies have been used to repair bone or cartilage defects in otherwise normal joints. As mentioned above, the situation in OA is more complex as larger, diffuse osteochondral defects are present. Over recent years the use of SSCs, either by injection or implantation, to treat OA has received increasing interest. The results from *in vivo* SSC implantation in animal models of OA have been encouraging, which has resulted in multiple small case series reporting findings from using SSCs to treat patients with knee OA (Im 2018). The majority of studies used human bone marrow derived stromal cells (HBMSCs), although adipose-derived SSCs and human umbilical cord blood derived SSCs were used in a minority (Im 2018). Patient follow up varied from 6 to 60 months across the different studies and a variety of patient reported outcomes for pain and function were used. Some studies also reported structural outcomes including histological grading, arthroscopic findings or MRI appearances. Both clinical and histological, arthroscopic and MRI-based outcomes improved after injection of SSCs in the majority of studies, although the duration of improvement was variable (Im 2018). Several meta-analyses have evaluated these studies to date. Reviewing 18 studies including a total of 565 patients demonstrated improved outcomes at 12 and 24 months after SSC injections for knee OA (Cui, Wang et al. 2016). Factors associated with improved patient outcomes included having had arthroscopic debridement, addition of an activation agent (hyaluronic acid, platelet-rich plasma (PRP), collagen sheets, fibrin glue) or a lower Kellgren-Lawrence (KL) grade at presentation (Cui, Wang et al. 2016). Another meta-analysis yielded similarly positive results for SSC treatment at 12 and 24 months and confirmed the safety of this approach (Yubo, Yanyan et al. 2017). However, a systematic review of the data has suggested that the included trials were all at high risk of bias and that on the basis of the currently available evidence, it was not possible to recommend SSC treatment for knee OA (Pas HI 2017). A recent overview of all four systematic reviews of SSC treatment of knee OA concluded once again that most of the trials were at high risk of bias (Xing D 2017). Taking this into account, the authors concluded that the evidence to date gave moderate confidence in the safety of SSC treatment for knee OA but low confidence in the efficacy of this approach. Further high quality studies of SSC treatment for knee OA are therefore warranted.

What remains to be considered is how these strategies require modification to account for the *inflammatory milieu present in arthritic joints*. The normal development of bone and

cartilage will be reviewed briefly first, followed by an overview of normal bone and cartilage turnover and a summary of the pathophysiology of OA. Subsequent sections will then focus on the inflammatory environment present in the OA niche and how this will influence the development of future bioengineering strategies for the treatment of OA.

1.3 Normal skeletal development

The skeleton originates from embryonic mesenchymal tissue. The majority of bones in the body are derived from the lateral plate mesenchyme, with the exception of the craniofacial bones, which develop from neural crest cells (ectomesenchyme). Two main processes are involved; endochondral ossification and intramembranous ossification (Summerlee 2002, Lane 2003).

1.3.1 Endochondral ossification

The long bones of the skeleton develop predominantly by endochondral ossification. This begins with the mesenchymal cells clustering together to form a cartilage template or 'anlage', which acts as a scaffold on which the new bone forms. The transformation of this cartilage template to bone occurs initially in areas termed ossification centres and at the perichondrium. Longitudinal growth of the bones requires the development of the growth plate or physis, which has four histologically distinct zones (Figure 1.4). The uppermost of these is the resting zone, consisting of a layer of hyaline cartilage, produced by cartilage cells with some surrounding matrix, which are attached to the overlying epiphyseal bone. Next is the proliferating zone where the older cartilage cells begin to form vertical columns, separated by narrow bands of interstitial tissue. These cells then migrate to the hypertrophic zone, where they enlarge and incorporate stores of glycogen. Cells in this region then polarise, with those located closest to the underlying metaphysis starting to secrete alkaline phosphatase. If sufficient quantities of minerals are present, these cells then gradually mineralise, forming a layer of calcified cartilage adjacent to the metaphysis. Blood vessels then invade the connective tissue in this region and penetrate into the vertical columns. The bands of interstitial tissue are progressively removed, leaving vertical columns of calcified matrix known as the primary spongiosa. Bone matrix is then laid down on this primary spongiosa to form the secondary spongiosa, which eventually become the first trabeculae. Subsequent bone modelling by osteoblasts and osteoclasts convert these trabeculae into

adult trabeculae. This modelling is crucial in shaping the bone to its final adult proportions as both growth and modelling cease when the growth plates close after puberty.

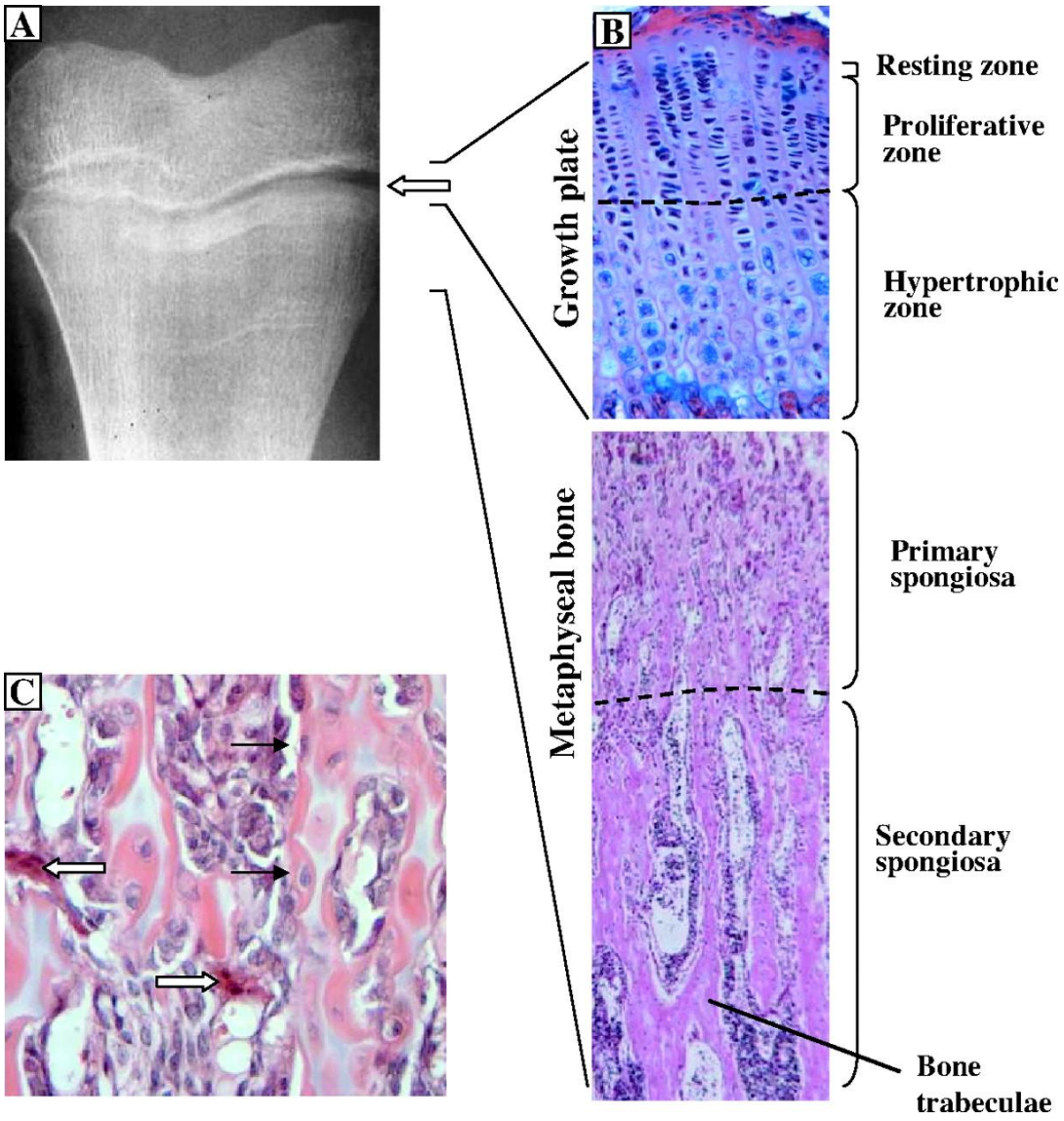


Figure 1.4 Structure and function of growth plate cartilage and metaphyseal bone in bone lengthening and bone mass accumulation
(Xian 2007)
Growth plate, situated at the ends of a long bone (A), is composed of the resting, proliferative, and hypertrophic zones (B). Through sequential activation, proliferation, maturation, hypertrophy, and apoptosis of chondrocytes, the growth plate produces a calcified template for new bone deposition in metaphysis (B). Calcified cartilage is first modeled to primary trabecular bone at the metaphysis, which is further modeled into more mature secondary spongiosa trabecular bone (B). This modeling is carried out by the coordinated action of these two cell types (C): osteoclasts (*block arrows*) and osteoblasts (*small arrows*) Images, taken from 8-wk-old male rats, were from the author's unpublished work. A, x-ray film; B, *upper panel*, alcin blue hematoxylin and eosin (H&E) staining (magnification, ×100); *lower panel*, H&E staining (magnification, ×25); C, H&E staining (magnification, ×400).

1.3.2 Intramembranous ossification

This is the process responsible for the growth of the majority of the axial skeleton, including the flat bones of the skull. It is also the mechanism through which structural remodelling occurs at the sub-periosteal surface of the long bones. Unlike in endochondral ossification, there is no cartilage template, instead bone is laid down by direct apposition within stromal connective tissue (Figure 1.5). Mesenchymal cells cluster together within ossification centres where they proliferate and then differentiate to form osteoblasts. The surrounding matrix changes from scattered, disorganised collagen fibrils to a denser and more homogeneous matrix. As the osteoblasts begin to secrete alkaline phosphatase, the matrix is progressively mineralised. Additional trabeculae are added by direct apposition. A proportion of trabeculae continue to expand, with blood vessels penetrating into their Haversian canals. Eventually these trabeculae will form cortical bone.

Growth in diameter of the shaft of the long bones also occurs by intramembranous ossification rather than endochondral ossification as mentioned above (Figure 1.5). This is due to continuous resorption by osteoclasts within the periosteum and results in the destruction of the metaphysis and formation of the diaphysis as the bone increases in length.

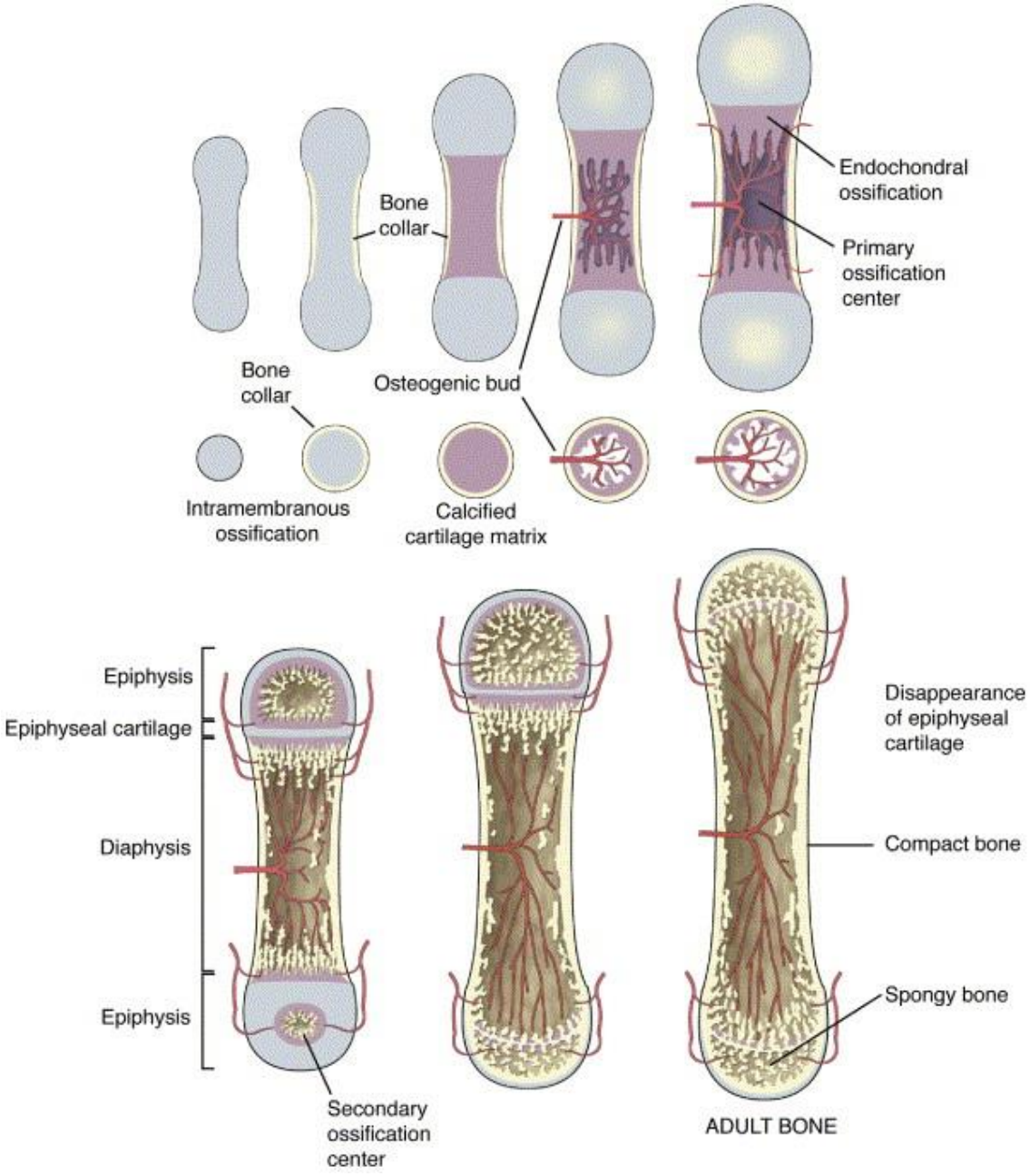


Figure 1.5 Schematic diagram of skeletal development demonstrating both endochondral and intramembranous ossification

(van Weeren 2006).

1.3.3 Alternative theories of bone development

Although skeletal development has traditionally been subdivided into two distinct processes as described above, subsequent evidence from studies in chicken tibiae suggests that this may not be the case (Caplan 1988, Abella, Scotece et al. 2016). This alternative

hypothesis of long bone formation proposes that it is not the cartilage template that arises first, instead it is a collar of bone-producing cells in the mid-diaphyseal region that originate first. This collar consists of 4 to 6 cells, termed the stacked cell layer, which surrounds a cartilaginous centre that develops later. This collar surrounds the developing bone and determines the dimensions of the cartilage rod (formerly believed to be the template for the adult bone), which is eventually eroded to leave the medullary cavity of the long bone. The developing vasculature is sandwiched between the rigid collar and the cartilage rod. The cells of the stacked layer, which lie adjacent to the vasculature, differentiate into osteogenic precursors and will eventually become osteoblasts. These then secrete osteoid and the rigid collar extends out towards the ends of the long bones. The mid-diaphyseal region is progressively mineralised and becomes bone. Further vascular invasion of the stacked cell layer occurs and these penetrating capillaries are then surrounded by further struts of osteoid and the two layers of bone are then interconnected by the formation of the supporting trabeculae. The cartilage rod is then progressively eroded and replaced by the marrow cavity.

1.4 Normal bone structure and function

1.4.1 Macroscopic structure

Bone has three main functions: to provide structural support for the trunk and limbs; to protect the delicate internal organs; and as a vital tissue in calcium homeostasis. Bone is extremely strong, making it very suitable for fulfilling the first two of these functions. The key to its strength lies in its unique combination of the fibrous protein collagen (good for resisting tensile strain) and apatite (good for withstanding compressive forces). There are two main types of bone, adapted according to the strain transmitted through the skeleton at different sites, termed cortical and trabecular bone.

Cortical or compact bone is located at the diaphysis of long bones, on the surface of flat bones and is also present as a thin shell around the epiphyses and metaphyses of long bones. 80-90% of cortical bone is calcified, consistent with its predominantly protective and mechanical functions. Cortical bone is dense and comprised of Haversian systems. These are cylindrical structures, also known as secondary osteons. Osteons refer to the individual amounts of bone formed by each Haversian system and they exhibit varying degrees of mineralisation, as mineralisation increases with the age of the osteon. Osteons in the

diaphysis of long bones are arranged along the long axis of the bone in order to confer maximal strength.

Trabecular or cancellous bone forms the epiphyseal and metaphyseal regions of the long bones, together with the cortex of flat and shorter bones. Only 15-25% of trabecular bone is calcified and its functions are largely mechanical and metabolic. In contrast to cortical bone, trabecular bone consists of a looser three-dimensional lattice comprised of bone plates and columns arranged within a shell of cortical bone.

Osteogenic cells also line the periosteum (a layer between the cortical surface of the bone and the overlying soft tissues) and the endosteum (the corresponding layer between the metaphysis and the marrow cavity).

1.4.2 Microscopic structure

At a microscopic level, bone is a combination of collagen fibres (90% type I collagen) and ground substance. The latter includes both glycoproteins and proteoglycans. Hydroxyapatite crystals are also found within both the collagen fibrils and the ground substance. There are then four main cell types: osteocytes; osteoblasts; osteoclasts and mesenchymal stem cells. Osteocytes are derived from osteoblasts but once surrounded by calcified matrix they become trapped and differentiate further. They are embedded in small osteocytic lacunae but have multiple cell processes to allow communication with one another and with other cell types and form networks of canaliculi. The primary function of osteocytes is to detect and respond to changes in mechanical loading of the bone via these canaliculi. Osteocytes have been implicated in regulating osteoblast and osteoclast function (Bonewald 2017).

Osteoblasts are derived from local mesenchymal stem cell populations. In order for osteogenic differentiation of MSCs to occur, the nuclear factor CBFA1 is required. Further osteogenic cues result in the formation of preosteoblasts and finally mature osteoblasts. Osteoblasts are responsible for the production of bone matrix and its mineralisation. Osteoblasts occur in clusters along the bone surface, referred to as a bone-forming site. Osteoblasts typically survive between 15 days and 8 weeks before either undergoing apoptosis or differentiating into a bone lining cell or osteocyte.

Mesenchymal stem cells are the precursors to osteoblasts as mentioned above. They line both the internal and external endosteal surfaces of cortical bone and the external endosteal surface of trabecular bone. MSCs and fibroblasts together form a layer over the periosteal surface of all bones.

Osteoclasts originate from haematopoietic stem cells in the bone marrow. Macrophage colony stimulating factor (M-CSF) is needed to stimulate differentiation and proliferation of macrophages from these haematopoietic stem cells, then the presence of bone stromal cells or osteoblasts, together with other local factors, result in the development of osteoclast precursors. Receptor activator of natural killer (NK)-κB ligand (RANKL) produced by osteoblasts then interacts with the RANK receptor on these osteoclast precursors to stimulate osteoclastogenesis. Osteoclasts are the bone cells responsible for bone resorption. Osteoclasts are multinucleated giant cells found on calcified bone surfaces within resorption pits or Howship's lacunae. Osteoclasts have a ruffled border in contact with the resorption site, surrounded by sealing zones. They secrete multiple lysosomal enzymes to dissolve the bone including tartrate resistant acid phosphatase (TRAP).

1.5 Normal bone turnover

Normal bone turnover is a dynamic process (Figure 1.6). Osteoblasts form the building blocks for new bone (Quiescence). In response to stimulation by a combination of cytokines and growth factors as mentioned above, preosteoclasts become activated and undergo differentiation to mature osteoclasts (Activation). These osteoclasts attach to bone matrix proteins, secrete proteinases from their ruffled border resulting in acidic conditions, leading to the formation of resorption pits (Resorption). This resorption phase is followed by a reversal phase during which both osteoclasts and osteoblasts are absent. Next, osteoblasts appear within the resorption pit and produce matrix (Formation) until the new bone (osteon) is produced (Mineralisation). The repair process is then complete and the osteoblasts become resting bone lining cells on the surface of the newly formed bone (Quiescence). In a healthy individual, a careful balance is maintained between bone resorption by osteoclasts and the filling in of these resorption pits by osteoblasts, resulting in bone remodelling. Alterations of this delicate balance in either direction lead to changes in bone density, mineralisation and strength.

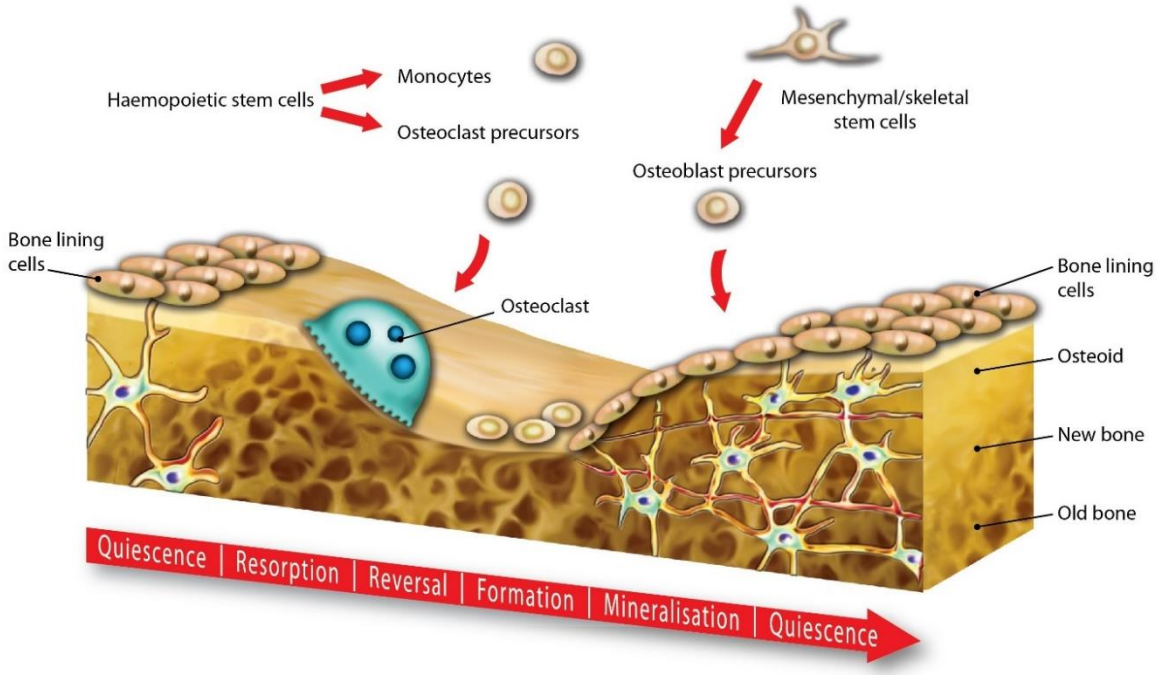


Figure 1.6 Schematic diagram of the 5 stages of normal bone turnover.

Figure prepared by Emma Williams with input from Studio Creative for a review article currently in preparation.

1.6 Normal cartilage metabolism

A functioning articular cartilage is crucial for the efficient functioning of a synovial joint. Cartilage dissipates the mechanical forces applied to the weight-bearing surfaces of the bones during movement and damage to it results in pain and loss of function. Adult articular cartilage is comprised of a cellular component, chondrocytes, embedded within a specialised extracellular matrix (ECM). The latter incorporates a range of complex molecules including collagens, proteoglycans and non-collagen proteins. Chondrocytes are crucial in maintaining this ECM, although the exact mechanisms through which chondrocytes exert control over the composition of the ECM is still only partially understood, particularly because chondrocytes lack direct access to the vascular network (Goldring and Marcu 2009). Instead, chondrocytes rely on active transport of nutrients via transporters such as the constitutive glucose transporter proteins GLUT3 and GLUT8 (Mobasheri, Richardson et al. 2005). Chondrocytes also have to survive at low oxygen tensions, down to as low as 1%, which is achieved through upregulation of so-called survival factors, including hypoxia-inducible factor-1-alpha (HIF-1α) and growth arrest and DNA damage 45 beta (GADD45β)

(Goldring and Marcu 2009), as well as angiogenic factors including vascular endothelial growth factor (VEGF) (Lin, Chen et al. 2004).

The homeostasis of cartilage metabolism relies on a careful balance between degradation and formation of the collagens (predominantly type II but also types VI and IX) and proteoglycans. Healthy cartilage has four zones; superficial, middle, deep and calcified cartilage zones, all with differently arranged collagen fibrils of varying thickness. The proportion of proteoglycan increases from superficial to deep zones. The major proteoglycan is aggrecan, which protects the collagen fibrils from compressive forces. The non-collagen proteins (biglycan, decorin, fibromodulin, matrillins and cartilage oligomeric protein (COMP)) act to catalyse fibril formation and cross-linkage (Goldring and Marcu 2009). In healthy cartilage, remodelling and repair is mediated through the actions of a large group of proteases directed against both the collagen and proteoglycan components of cartilage. These include the aggrecanases and matrix metalloproteinases (MMPs). The former include the a disintegrin and metalloproteinase (ADAM) family of proteinases, of which ADAM with thrombospondin-1 domains-4 and 5 (ADAMTS-4 and 5) are now recognised as the most important subtypes in cartilage degradation (Arner 2002, Plaas, Osborn et al. 2007). The MMPs can be subdivided into collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysin-1 (MMP-3) and membrane type 1 (MT1) MMP (MMP-14) (Cawston and Wilson 2006, Sandy 2006, Rengel, Ospelt et al. 2007, Murphy and Nagase 2008). Additional MMPs including MMP-10, are induced in response to mechanical strain or inflammatory cytokines. A common factor to all arthritic diseases is a shift towards cartilage catabolism, with upregulation of proteases either via mechanical strain, genetic factors or local damage, triggering production of inflammatory cytokines (IL-1, IL-6 and TNFα) mainly by chondrocytes in OA, or predominantly via synovial cell-mediated cytokine release in RA. This will be covered in more detail in subsequent sections.

1.7 Pathophysiology of OA

OA is a progressive condition occurring over years. Many patients have stable symptoms for long periods of time, followed by exacerbations with increased pain, early morning stiffness and joint effusions. Others have rapidly progressive OA with joint destruction and a poor prognosis. There is often a poor correlation between clinical symptoms and the radiographic appearance of affected joints. A number of risk factors for OA have been identified to date including: increasing age, ethnicity, obesity, hypercholesterolaemia, joint

deformity, ligamentous laxity, repetitive physical activity, occupational factors, previous injury to the joint, muscle weakness, peripheral neuropathy and genetic factors (Goldring and Goldring 2007). The profile of risk factors and their relative importance varies according to the joint involved.

OA is a condition that affects the whole synovial joint. As OA progresses, the surrounding muscles weaken, ligaments become lax and the joint capsule thickens. Not only tissue and cellular factors are important but also the local and systemic influences of a wide range of growth factors and inflammatory cytokines (Goldring 2000, Goldring and Otero 2011). Within the cartilage are mechanoreceptors that are sensitive to changes in pressure (Millward-Sadler and Salter 2004). Although dynamic stimulation has been shown in mechanical loading experiments to increase matrix synthesis, static compression reduces the matrix proteoglycan content, causes damage to the collagen network and reduces the synthesis of cartilage matrix proteins (Guilak, Fermor et al. 2004). This explains the adverse effects of abnormal loading (due to increased BMI or repetitive occupations or sporting activities) on a joint that was originally structurally normal, eventually leading to OA, as well as how joint deformity, previous injury or ligamentous laxity can result in accelerated OA under normal loading. Mechanical stress is detected by these mechanoreceptors, resulting in chondrocyte activation. This activation results in chondrocyte differentiation and hypertrophy. These activated receptors can stimulate the secretion of proteinases such as MMPs, chemokines and inflammatory cytokines that in turn perpetuate the differentiated chondrocyte phenotype, resulting in cartilage degradation (Pulai, Chen et al. 2005).

Firstly, there is oedema of the extracellular matrix with the formation of microcracks resulting in areas of focal cartilage loss. The formation of focal cartilage defects is exacerbated by the inability of cartilage repair to keep pace with damage due to reduced synthesis of tissue inhibitors of metalloproteinases (TIMPs), the natural inhibitors of MMPs. Fissuring and pitting of the cartilage follows, eventually leading to fragmentation of the cartilage with loose bodies released into the joint space. These same cartilage degradation products in turn increase synovial inflammation. *In vitro* studies have provided supportive evidence that it is the presence of this cartilage debris within the joint space that leads to increased production of pro-inflammatory cytokines such as TNFα, resulting in increased joint pain and progressive cartilage damage (Cameron-Donaldson, Holland et al. 2004). Synovitis, characterised by inflammatory cell infiltrates and increased production of IL-1β, TNFα and PGE2, has been demonstrated in synovial samples from patients with both early and late stage knee OA, although levels of IL-1β and TNFα were lower in late stage OA

(Benito, Veale et al. 2005). Inflamed synovial tissues also secrete catabolic and proinflammatory mediators, resulting in the overproduction of proteolytic enzymes within the joint, setting up a positive feedback loop. Areas of patchy inflammation are therefore found adjacent to areas of cartilage damage. Microcysts then form within exposed areas of subchondral bone.

Within the subchondral bone, osteoblasts are also stimulated by mechanical stress, resulting in the production of MMP-1 and 13, prostaglandin E2 (PGE2) and IL-6, together with inhibition of cartilage matrix components, leading to the degradation of subchondral bone. Alongside this, there is reduced blood flow to subchondral bone in OA, leading to venous stasis, hypertension, thrombosis and focal ischaemic bone necrosis. This occurs because both venous stasis and reductions in mechanical loading trigger osteocyte apoptosis and bone resorption by osteoclasts (Findlay 2007). Early signs of abnormal bone remodelling in an OA joint are detectable as bone marrow lesions (BMLs) on MRI and subchondral ischaemia may well be the mechanism that produces these changes. Production of both insulin-like growth factor (IGF) and tissue growth factor beta (TGFβ) by subchondral bone is responsible for new bone formation, producing the characteristic subchondral sclerosis and osteophyte formation at the joint margins, apparent on radiographs in patients with established OA, together with subchondral cyst formation (Figures 1.1 and 1.2)(Hill, Gale et al. 2001, Buckland-Wright 2004, Burr 2004). Subchondral osteoblasts in OA produce increased levels of alkaline phosphatase (ALP), osteocalcin (OCN), TGFβ1, IGF-1, urokinase, IL-6, IL-8, C-terminal type 1 procollagen propeptide and osteoprotegerin (OPN). These osteoblasts are also resistant to the effects of parathyroid hormone (PTH), which may be another factor contributing to abnormal bone remodelling in OA. This abnormal remodelling results in increased accumulation of osteoid and reduced mineralisation of the cartilage matrix, producing abnormal type 1 collagen with reduced capacity to withstand mechanical loading. Vascular invasion at the osteochondral junction, together with advancement of the calcified cartilage zone contribute to the further reduction of the thickness of the articular cartilage in OA (Lane, Villacin et al. 1977, Burr and Schaffler 1997). This angiogenesis at the osteochondral junction has been shown to be associated with cartilage changes and clinical disease activity (Walsh, Bonnet et al. 2007).

There is ongoing debate as to whether the primary defect in OA is in the cartilage, with the changes in subchondral bone being a secondary phenomenon, or vice versa. It is widely accepted that previous cartilage damage, be that due to trauma or previous surgery, increases the risk of developing hip and knee OA. A recent matched case-control study

using the Clinical Practice Research Datalink (CPRD) demonstrated that an anterior cruciate ligament injury resulted in a 7-fold increase in risk of TKR due to OA, whilst a previous meniscal injury resulted in a 15-fold increased risk (Khan T 2018). This supports the development of a cartilage or meniscal lesion being a primary event in the development and progression of OA. Following this theory, once cartilage damage occurs, it results in abnormal loading within the joint, leading to secondary changes in subchondral bone. These bone and cartilage changes in turn cause abnormal joint mechanics leading to progressive cartilage damage and new lesions within the subchondral bone.

However, subchondral bone marrow oedema, demonstrated as bone marrow lesions (BMLs) on MRI, has also been shown to be an independent risk factor for the progression and severity of knee OA (Felson, Chaisson et al. 2001). Similarly, changes in subchondral bone apparent on 99mTc HMDP scanning have been found to precede the development of radiographic joint damage in generalised nodal osteoarthritis of the hand (Hutton, Higgs et al. 1986). This evidence led to increased interest in the role of subchondral bone changes and synovial inflammation in the initiation and progression of OA (Radin and Rose 1986, Haywood, McWilliams et al. 2003). In some animal models of OA, changes occur within the subchondral bone first, with cartilage damage and focal cartilage loss as a secondary phenomenon (Pickarski, Hayami et al. 2011). An alternative theory on the development of OA therefore suggests that it is the repeated microfractures within subchondral bone in early OA that make it stiffer and less effective at transmitting load, leading to secondary cartilage damage. In addition to this mechanical damage leading to chondrocyte activation as described above, the subchondral osteoblasts have also been shown to produce growth factors and cytokines such as IL-6, MMPs and PGE2 in early OA, that lead to cartilage damage, with subsequent abnormal cartilage metabolism and excess cartilage degradation. Ongoing research is in progress to clarify which of these theories is closer to the exact pathophysiology of human OA.

Subsequent sections will focus on the microenvironment of the OA joint, termed the OA niche, including how this is affected by inflammation. Within this discussion, each individual component of the OA joint will be considered in more detail, together with key growth factors and inflammatory cytokines.

1.8 The OA niche

The understanding of any cell type requires an appreciation of all the factors that the cell is exposed or will interact with. These include cell-cell interactions, cell-matrix interactions, autocrine and paracrine hormonal influences, growth factors and cytokines. This can be encapsulated in the concept of a cell's 'niche', which defines the unique attributes of a cell's microenvironment that influence its development. The concept was first introduced for haematopoietic cells within the bone marrow over 30 years ago (Schofield 1978) but has been revived within the tissue engineering field as an aid to understanding stem cell development (Spradling, Drummond-Barbosa et al. 2001, Shi and Gronthos 2003). Similarly, each disease state will have a specific balance of influences required to perpetuate the course of the disease, creating its unique signature or niche. For musculoskeletal diseases such as OA, the principle cells of interest are SSCs, also termed MSCs (reviewed in (Tare, Babister et al. 2008)). A highly specific SSC niche is now well recognised, with functional and anatomical properties that permit these cells to switch between resting and activated states as required (Tare, Babister et al. 2008). To understand this unique microenvironment it is necessary to consider the individual components: progenitor cells; SSCs; skeletal progenitors; osteoblasts; osteoclasts; osteocytes; chondrocytes; synoviocytes; extracellular matrix (ECM) and bioactive factors including inflammatory cytokines and chemokines, growth factors and hormonal influences. For the purposes of this review the focus will be narrowed to considering how inflammation affects each of these compartments.

1.8.1 Impact of inflammation on the OA niche

Traditionally OA has been regarded primarily as a degenerative process, a consequence of ageing. Treatments have tended to focus on symptomatic management rather than halting disease progression. Following the success of DMARDs for treating RA over the last 30 years and biologic agents in the last 18 years, researchers have begun to revisit the role of inflammation in OA. It has even been suggested that the association of OA with obesity may be due to increased adipose tissue acting as another source of inflammatory cytokines driving the disease process, rather than a mechanical loading effect (Aspden 2011). Whilst OA has less of an inflammatory component than RA and other inflammatory arthropathies, both animal and human studies have reinforced the importance of inflammation in its pathogenesis (Pelletier, Martel-Pelletier et al. 2001, Goldring and Otero 2011). Women with early OA of the knees have been shown to have elevated levels of C-

reactive protein (CRP) and higher levels increase the likelihood of disease progression (Goldring 1999). The same inflammatory cytokines known to drive RA, namely interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα), are now known to be involved in this inflammatory component of OA (Pelletier, Roughley et al. 1991, Martel-Pelletier, Alaaeddine et al. 1999, Goldring 2000, Fernandes, Martel-Pelletier et al. 2002). Further recent studies have demonstrated the importance of cytokines in the pathophysiology of OA (reviewed by (Malemud 2010, Kapoor, Martel-Pelletier et al. 2011)). Evidence for IL-1β and TNFα occupying a pivotal role in the development of OA continues to accrue, with elevated levels of these cytokines or their gene expression reported in synovial tissues, synovial fluid and/or plasma in various animal models (Huebner, Seifer et al. 2007, Ley, Ekman et al. 2007, Maccoux, Salway et al. 2007). Studies of human OA synovial tissue or fluid have produced similar results (Kobayashi, Squires et al. 2005, Bondeson, Wainwright et al. 2006, Doss, Menard et al. 2007). As a result of these findings, there is now increasing interest in investigating whether biologics have a role in the future treatment of OA. The role of each of these cytokines, as well as others more recently implicated in OA, are summarised in Table 1.1 and will be considered in turn.

Cytokine/ growth factor	Tissue/cells of origin	Effects on target tissues
IL-1	Activated macrophages	Activates T +EC cells; causes acute inflammation; stimulates acute phase reactants
IL-2	T_h cells	T cell growth factor; promotes B cell growth; activates monocytes
IL-3	T_h cells	Promotes growth and differentiation of bone marrow progenitors; promotes B cell growth
IL-4	T_{h2} cells+ activated macrophages	Stimulates differentiation of T_{h0} to T_{h2} cells; stimulates activated B and T cells; reduced T_{h1}, macrophage, IL-12 and IFNγ
IL-5	T_{h2} cells+ Mast cells	Promotes growth+ differentiation of B cells+ eosinophils
IL-6	Activated macrophages, T cells, osteoblasts	Acute phase response; pro+ anti-inflammatory cytokine
IL-8	Platelets+ endothelial cells	Neutrophil activation/chemotaxis
IL-10	T_{reg}	Inhibits T_{h1}, T_{h2},T_{h17}, T_c
IL-12	Dendritic cells+ activated Macrophages	Promotes growth+ differentiation of bone marrow progenitors; promotes B cell growth
IL-17	T_{h17} cells	Chemokine production; destruction of extracellular pathogens
IL-18	Macrophages	Cell mediated immunity; stimulates NK and T cells to produce IFNγ
IL-23	T_{h17} cells	IL-23R activation leads to T_{h17} proliferation
TNFα	Activated macrophages	Acute inflammation; chemokine production, recruitment of neutrophils and macrophages
TGFβ	Most cell types	Proliferation + cellular differentiation
IFNγ	Natural killer cells + T_{h1} cells + T_c	Innate + adaptive immunity; ↑iNOS; suppresses osteoclast formation

Table 1.1 Summary of cytokine effects on different target tissues and their interactions

Adapted from (Williams, Edwards et al. 2012).

1.8.2 Cytokines

Cytokines are small (17-30 kDa) cell signalling proteins that occupy a crucial role in intercellular communication. A wide variety of cell types can produce cytokines (as listed in Table 1.1), which then exert autocrine, paracrine or endocrine effects. The main cytokines implicated in OA and RA are IL-1β, TNFα, IL-6 and, more recently, IL-17 and IL-18. As IL-1β and TNFα have been studied together previously in the literature and share many downstream pathways, they will be considered together first, followed by the IL-6 superfamily of cytokines as a whole, as the latter group all have a similar mechanism of action. Finally, the emerging data on IL-17 and IL-18 will be reviewed individually as these cytokines are thought to act through different pathways. TGFβ will be discussed in the subsection on anabolic factors due to its involvement in osteophyte formation rather than joint destruction.

1.8.2.1 IL-1 and TNFα

The chondrogenic niche

The articular cartilage is the principal site of damage in OA, due to an imbalance between anabolic and catabolic pathways, the latter being mediated by inflammatory cytokines. Catabolic pathways are driven primarily by IL-1β and TNFα, which trigger a molecular cascade involving matrix metalloproteinases (MMP1,2,3,8,9,13) and protein kinase C ζ. These are responsible for the ECM destruction seen in OA (Chikanza and Fernandes 2000, LaVallie, Chockalingam et al. 2006). These interactions also produce phenotypic changes in articular chondrocytes, which are therefore termed ‘degradative chondrocytes’. This appears to be mediated via epigenetic mechanisms. These can be subdivided into three main types: alterations in DNA methylation; histone modifications; changes in non-coding RNAs (reviewed in Reynard and Loughlin 2012). To date, in OA, such changes have mainly been studied in chondrocytes. Some of the changes first described include hypomethylation of CpG sites in the promoter regions of MMP-3, 9, 13 and ADAMTS-4 of these degradative chondrocytes (Roach, Yamada et al. 2005, Cheung, Hashimoto et al. 2009, da Silva M.A. 2009) . Histological findings confirm this, showing co-localisation of IL-1β, MMP1,3,8,9,13 in the superficial zone of human OA cartilage samples where features of cartilage damage such as fibrillation, chondrocyte clustering and ECM disruption are concentrated (Tetlow, Adlam et al. 2001). These changes progress through the zones of cartilage as OA progresses.

The progression of OA can potentially be explained, at least in part, by prolonged exposure of articular chondrocytes to IL-1β, triggering demethylation of CpG sites in the IL-1β promoter region (Hashimoto, Oreffo et al. 2009) . Consequently, degradative chondrocytes continue to express IL-1β long after the inflammatory stimulus has been removed. Work from Imagawa and colleagues suggests this demethylation is mediated through nuclear factor kappa B (NFκB) pathways (Imagawa, de Andres et al. 2011). Increased methylation of CpG sites within the superoxide dismutase 2 (SOD2) gene and bone morphogenetic protein-7 (BMP-7) have also been reported in OA chondrocytes with increasing age, suggesting that downregulation of these genes may have a role in OA progression (Loeser, Im et al. 2009, Scott, Gabrielides et al. 2010).

The actions of IL-1 on OA chondrocytes also appear to be modulated via the other two methods of epigenetic modulation; histone modification and non-coding RNAs. Studies have shown that the addition of IL-1 to chondrocytes in culture resulted in recruitment of the histone methyltransferase SET1A to the promoter region of the cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) genes (El Mansouri 2011). This correlated with increased transcription of these genes, which was proposed to occur via histone methylation of their promoter regions. Upregulation of COX-2 and iNOS leads to cartilage damage in OA through a combination of chondrocyte apoptosis, increased synthesis of matrix metalloproteinases and inhibition of collagen synthesis. As increased levels of SET1A have been found within OA cartilage, this suggests that inhibiting SET1A could have therapeutic benefits in OA due to its inhibitory effects on this IL-1 mediated upregulation of COX-2 and iNOS.

The histone deacetylases (HDACs) HDAC1,2 and 7, are all know to be upregulated in OA chondrocytes. HDAC1 and 2 overexpression results in repression of extracellular matrix (ECM) genes, whilst HDAC7 overexpression results in increased MMP-13 expression (Hong, Derfoul et al. 2009, Higashiyama, Miyaki et al. 2010). HDAC inhibitors have been shown to inhibit IL-1β induced nitric oxide (NO) and prostaglandin E2 (PGE2) production, making them a potential option for OA treatment in the future (Chabane, Zayed et al. 2008) . Specific HDAC inhibitors have also been shown in other studies to prevent ECM degradation in human OA chondrocytes (Im and Choi 2013, Lu, Sun et al. 2014, Carpio, Bradley et al. 2016). The non-selective HDAC inhibitor Trichostatin A (TSA) has already been shown to be effective in reducing cartilage damage, expression of collagenases and IL-1 in a rabbit anterior cruciate ligament transection (ACLT) model of OA. A series of studies with the orally bioavailable inhibitor of HDAC class I and II, vorinostat, have provided further insights into

the mechanisms of action of HDAC inhibitors. Vorinostat produces downregulation of NF-κβ signalling and suppresses IL-1β induced activation of p38 and ERK 1/2 (Zhong et al. 2013). Additional studies have demonstrated that vorinostat can suppress MMP-13 production by inhibiting IL-6 induced signalling in OA chondrocytes (Makki and Haqqi 2016). Vorinostat has also been shown to upregulate miR-46 in fibroblast-like synoviocytes (FLS) from OA joints (Wang, Shih et al. 2013).

Another of the HDACs, HDAC4, is known to be involved in the regulation of chondrocyte hypertrophy. HDAC4 is downregulated in OA chondrocytes, suggesting that this alteration in expression is involved in promoting the catabolic chondrocyte phenotype as part of the pathogenesis of OA (Lu, Sun et al. 2014). Further support for this hypothesis comes from work demonstrating that reduced expression of HDAC4 is linked to increased expression of Runx-2 and the MMPs (K. Cao 2014). Therefore, upregulation of HDAC4 represents an alternative means of modulating the HDACs and another option for the treatment of OA.

Several microRNAs (miRNAs), including miR-9, miR-27b, mi-R34a, miR-98, miR-140 and miR-146 have been found to show differential expression between normal and OA cartilage (reviewed in (Yu, Chen et al. 2011, Barter, Bui et al. 2012, Reynard and Loughlin 2012)). Overexpression of miR-9, miR-98 and miR-146 in OA cartilage, reduces IL-1-induced TNFα production (Jones, Watkins et al. 2009). Additionally, alterations in miR-9 expression modulate MMP-13 secretion. Overall, these miRNAs appear to have protective effects in OA.

In contrast, IL-1 stimulation leads to a reduction in miR-27b expression in chondrocytes. Levels of miR-27b are known to be reduced in OA cartilage and demonstrate an inverse correlation with their target protein MMP-13 (Akhtar, Rasheed et al. 2010). Expression of miR-34a in chondrocytes is significantly upregulated by IL-1β and its inhibition leads to a reduction in chondrocyte apoptosis in a rat model of OA (Abouheif, Nakasa et al. 2010). This has led to the suggestion that inhibitors of this miRNA could represent novel therapies for OA. Similarly, IL-1β can reduce the expression of miRNA-140 in chondrocytes in vitro (Miyaki, Nakasa et al. 2009). miR-140 is expressed by differentiated human articular chondrocytes and acts to modulate the IL-1 response. When transfected into chondrocytes, ds-miR140 inhibits IL-1β induced ADAMTS-5 expression and restores IL-1β-induced suppression of aggrecan gene expression. IL-1β also downregulates miR-30a expression via activation of Activator protein 1 (AP-1) (Ji Q 2016). AP-1 and miR-30a are critical to IL-1β induced upregulation of ADAMTS-5 in OA (Ji Q 2016). IL-1β also induces expression of miR-146 in chondrocytes in vitro but can also suppress IL-1-induced expression of MMP-13

though its inhibitory effects on the IL-1 receptor associated kinase (IRAK-1) and TNF associated factor-6 (TRAF-6) genes (Yamasaki, Nakasa et al. 2009). miR-146 is upregulated in early OA cartilage and down-regulated in later disease, suggesting a role in the development of inflammatory OA (Jones, Watkins et al. 2009).

MiR-365 is up regulated in OA cartilage and its expression is increased by IL-1β (Yang X 2016). Mechanical loading also increases miR-365 levels in growth plate chondrocytes, leading to chondrocyte differentiation and increased expression of MMP-13 and Collagen type X (Col X) (Yang X 2016). miR-365 targets HDAC-4, which acts to regulate the effects of mechanical stress and proinflammatory cytokines on chondrocyte differentiation. Downregulation of miR-365 could therefore represent another future target for OA therapies, preventing or ameliorating OA by inhibiting the differentiation of chondrocytes to a catabolic phenotype. Tables 1.2 and 1.3 summarise the multiple other miRNAs involved in cartilage homeostasis with possible roles in the pathogenesis of OA.

OA chondrocytes express increased levels of IL-1β, TNFα and inducible nitric oxide synthase (iNOS), compared with chondrocytes from patients with RA, seronegative arthritis or trauma, placing them firmly at the centre of the inflammatory damage seen in OA (Melchiorri, Meliconi et al. 1998). At the receptor level, both IL-1β and TNFα can influence the expression of TNF receptor (TNF-R) isoforms p55 and p75 (Alsalameh, Mattka et al. 1999). The expression of the active TNF-R isoform p55 is increased in OA chondrocytes *ex vivo* but varies in the articular cartilage *in vivo*. OA synovial supernatants also usually have insufficient levels of soluble TNF-R to counteract the adverse effects of TNFα, confirming that TNFα is crucial to localised cartilage damage (Webb, Westacott et al. 1997, Westacott, Barakat et al. 2000). Both IL-1β and TNFα have been shown to occupy a key role in both proteoglycan breakdown and inhibition of new proteoglycan synthesis (Pratta, Dimeo et al. 1989, Shinmei, Masuda et al. 1989, Shinmei, Masuda et al. 1991, Malfait, Verbruggen et al. 1994).

IL-1α and β and interleukin-1 receptor 1 (IL-1R1) in particular are all upregulated in OA, overriding the beneficial effects that should occur with the upregulation of IGF-1 and insulin-like growth factor receptor 1 (IGFR1) seen in OA cartilage (Wang, Verdonk et al. 2003). IL-1 and TNFα also stimulate production of vascular endothelial-like growth factor (VEGF) by dedifferentiated chondrocytes *in vivo*, providing a putative mechanism for cartilage neovascularisation in OA (Honorati, Cattini et al. 2004). IL-1β, TNFα and interleukin-17 (IL-17) all increase NO and PGE2 production, which are already known to be produced spontaneously by menisci from OA patients (LeGrand, Fermor et al. 2001).

miRNAs	Alterations in OA	Target	Reference
miR-9	↑ in OA cartilage and bone	MCPIP-1, MMP-13	(Jones, Watkins et al. 2009, Song, Kim et al. 2013, Makki, Haseeb et al. 2015)
miR-21	↑ in OA cartilage	GDF-5	(Zhang, Beckel et al. 2014)
miR-22	↑ in OA	PPARγ, BMP-7	(Iliopoulos, Malizos et al. 2008)
miR-29	↑ in OA	ADAMTS-5,-6,-14,-17,-19; FZD3; DVL3; FRAT2; CK2A2; Col3α1; osteonectin	(Le, Swingler et al. 2016)
miR -33a	↑ in OA chondrocytes	MMP-13	(Kostopoulou, Malizos et al. 2015)
miR-34a	↑ in OA cartilage	EPhA5, Col2a1	(Abouheif, Nakasa et al. 2010, Wu, Tian et al. 2014)
miR-98	↑ in OA chondrocytes	Bcl-2, TNF	(Jones, Watkins et al. 2009, Wang, Chen et al. 2017)
miR-145	↑ in OA chondrocytes	Col 2, aggrecan, Smad 3, Sox-9, TNFRSFIIB	(Martinez-Sanchez, Dudek et al. 2012, Yang WH 2014)
miR-146a	↑ in early OA	Smad 4, IL-1β, TRAF 6, IRAK 1	(Taganov, Boldin et al. 2006, Jones, Watkins et al. 2009, Yamasaki, Nakasa et al. 2009, Nakasa, Nagata et al. 2011, Li J 2012, Okuhara, Nakasa et al. 2012, Wang, Shih et al. 2013, Si, Zeng et al. 2016, West and McDermott 2017)
miR-146b	↑ in OA cartilage	SOX-5	(Budd E 2017)
miR-155	↑ in PBMC from OA patients	MMP-1,-13	(Ceppi, Pereira et al. 2009) (Okuhara, Nakasa et al. 2012)
miR-181	↑ in blood or cartilage of OA patients	TNFα, MMP-13	(Okuhara, Nakasa et al. 2012, Song, Kim et al. 2013, Xia, Tian et al. 2017)
miR-194	↑ in OA	SOX-5	(Wu, Tian et al. 2014, Xu, Li et al. 2016)
miR-218	↑ in moderate to severe OA	PIK3C2A	(Lu, Ji et al. 2017)
miR-320a	↑in OA chondrocytes	ADAMTS-5	(Peng, Liang et al. 2017)
miR-335	↑ in OA	DKK-1	(Zhang, Chen et al. 2011, Kopanska, Szala et al. 2017)
miR-365	↑ in OA cartilage	HDAC-4	(Yang X 2016)
miR-455	↑ in OA cartilage	ACVR2B, Smad2, CHRDL1	(Swingler, Wheeler et al. 2012)
miR-483	↑ in OA cartilage	Matrillin 3 and TIMP-2, MMP-13	(Qi, Ma et al. 2013) (Wang, Zhang et al. 2017)
miR-675	↑ in OA cartilage	COL2A1	(Steck, Boeuf et al. 2012, Trzeciak and Czarny-Ratajczak 2014)

Table 1.2 Summary of the miRNAs upregulated in OA

miRNAs	Alterations in OA	Target	Reference
miR-16	↓ in OA synovial fluid	Smad-7	(Murata, Yoshitomi et al. 2010, Xu and Xu 2017)
miR-17	↓in murine model of OA	p62/sequestosome 1	(Li H 2018)
miR-24	↓ in OA chondrocytes	$p16^{ink4\alpha}$	(Philipot, Guerit et al. 2014)
miR-25	↓ in OA	α5 integrin	(Papanagnou, Stivarou et al. 2016)
miR-27a/b	↓ in OA	MMP-13	(Akhtar, Rasheed et al. 2010)
miR-33b	↓in serum from OA patients	lnsR, IGFR1	(Ntoumou, Tzetis et al. 2017)
miR-92a	↓ in OA cartilage	ADAMTS-4,-5	(Mao, Wu et al. 2017)
miR-101	↓ in OA cartilage	COX 2 gene	(Akhtar and Haqqi 2012, Dai, Zhang et al. 2012)
miR-125b	↓ in OA cartilage	ADAMTS-4, MMP-13	(Matsukawa, Sakai et al. 2013, Wu, Tian et al. 2014)
miR-127	↓ in OA cartilage	MMP-13	(Park, Cheon et al. 2013)
miR-130a	↓ in OA tissues	TNF	(Li YP 2015)
miR-132	↓ in OA synovial fluid	GDF-5	(Murata, Yoshitomi et al. 2010, Liu, Yang et al. 2017)
miR-138	↓ in OA cartilage	Forkhead Box C1 (FOCX1)	(Yuan, Zhang et al. 2016)
miR-140	↓ in OA	HDAC-4, CXCL12, Smad 3, ADAMTS-5, DNPEP, RALA, IGFBP5; FUT1	(Tuddenham, Wheeler et al. 2006, Miyaki, Nakasa et al. 2009, Tardif, Hum et al. 2009, Yamasaki, Nakasa et al. 2009, Miyaki, Sato et al. 2010, Pais, Nicolas et al. 2010, Nakamura, Inloes et al. 2011, Liang, Zhuang et al. 2012, Wang, Gu et al. 2018)
miR-140-3p	↓in serum from OA patients	lnsR, IGFR1	(Ntoumou, Tzetis et al. 2017)
miR-146a	↓ in late stage OA	Smad 4, IL-1β, TRAF 6, IRAK 1	(Taganov, Boldin et al. 2006, Jones, Watkins et al. 2009, Yamasaki, Nakasa et al. 2009, Nakasa, Nagata et al. 2011, Li J 2012, Okuhara, Nakasa et al. 2012, Wang, Shih et al. 2013, Si, Zeng et al. 2016, West and McDermott 2017)
miR-148a	↓ in OA cartilage	Col10A1, MMP-13, ADAMTS -5	(Vonk, Kragten et al. 2014)
miR-149	↓ in OA chondrocytes	TNFα, IL-1β, IL-6	(Santini, Politi et al. 2014)
miR-204	↓in OA cartilage	IL-1β	(Song, Zhu et al. 2017)
miR-210	↓ in rat model of OA	DR6	(Zhang, Cao et al. 2015)
miR-223	↓ in OA synovial fluid and OA PBMC	COMP	(Murata, Yoshitomi et al. 2010, Okuhara, Nakasa et al. 2012, Coustry, Posey et al. 2018)
miR-355	↓ in OA	Wnt	(Portal-Nunez, Esbrit et al. 2016)
miR-488	↓ in OA cartilage	SLC39A8/ZIP 8, MMP-13	(Song, Kim et al. 2013)
miR-558	↓ in OA chondrocytes	COX-2, MMP-1,-13	(Park, Cheon et al. 2013)
miR-608	↓ in OA chondrocytes in severe OA	MMP-13	(Lin, Liu et al. 2009)
miR-671	↓in serum from OA patients	lnsR, IGFR1	(Ntoumou, Tzetis et al. 2017)

Table 1.3 Summary of the miRNAs downregulated in OA

The osteogenic niche

Elevated levels of cytokines and MMPs occur not only in OA cartilage as described above, but also in serum and subchondral bone (Hulejova, Baresova et al. 2007). Evidence now suggests that OA *subchondral bone* mediates the phenotypic changes seen in chondrocytes in OA cartilage by stimulating the production of inflammatory cytokines and MMPs (Hulejova, Baresova et al. 2007). In co-culture systems, sclerotic zone osteoblasts have been shown to reduce the expression of sex determining region Y – box 9 (SOX-9), type II collagen, parathyroid hormone related peptide (PTHrP) and parathyroid hormone receptor (PTH-R) genes by human articular chondrocytes, producing hypertrophic differentiation of the chondrocytes (Sanchez, Deberg et al. 2008). This is thought to be mediated via activation of extracellular regulated kinases 1/2 (ERK1/2) phosphorylation together with deactivation of p38 phosphorylation (Prasadam, van Gennip et al. 2010).

Osteoblasts within OA subchondral bone, particularly in the sclerotic zone, undergo phenotypic changes themselves, including upregulation of MMP-13, type 1A1 collagen (COL1A1), type 1A2 collagen (COL1A2), osteocalcin, alkaline phosphatase (ALP), VEGF and transglutaminase gene expression and decreased matrix mineralisation (Sanchez, Deberg et al. 2008). These osteoblasts also display abnormal expression of osteoprotegerin (OPG) and receptor activator of NFκB (RANK) (Kwan Tat, Pelletier et al. 2008). Osteoblasts can therefore be sub classified depending on their levels of PGE_2 into low or high OA osteoblasts (Massicotte, Lajeunesse et al. 2002, Massicotte, Fernandes et al. 2006, Kwan Tat, Pelletier et al. 2008, Kwan Tat, Pelletier et al. 2008, Tat, Pelletier et al. 2008, Tat, Pelletier et al. 2009). OA subchondral osteoblasts from low OA patients show increased expression of ephrin-B4 (EphB4), which can be further increased in response to PGE_2 and IL-17 (Zhao, Irie et al. 2006). This results in impaired bone resorption via inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in osteoblasts, reducing levels of IL-1β, IL-6, MMP-1,9,13 and RANK ligand (RANKL). There is therefore, an important and yet poorly appreciated *bone component* in OA and a need to address both cartilage and bone regeneration and the interaction of bone cells and tissue in OA (Figure 1.7).

The synovial niche

Elevated levels of inflammatory cytokines have been reported in both synovium and synovial fluid from OA joints. Whether these are causing joint damage or occur as a result of pre-existing cartilage damage remains the subject of debate (Deng, Chai et al. 1998) . *In vitro* studies support the hypothesis that it is cartilage debris that leads to increased TNFα and worsening joint pain and cartilage damage (Cameron-Donaldson, Holland et al. 2004) . In

contrast, findings from an *in vivo* mouse model of OA suggest that synovial lining macrophages are integral to the formation of osteophytes (van Lent, Blom et al. 2004). Subsequent *in vitro* work using human OA and RA synovial cell cultures demonstrated that removal of these synovial lining macrophages produced significant decreases in IL-1β, TNFα, fibroblast-derived cytokines, MMPs and ADAMTS-4, all of which are implicated in OA joint damage (Bondeson, Blom et al. 2010) . IL-1β production in OA appears to be independent of both TNFα and NFκB pathways, although NFκB inhibitors have previously been shown to decrease levels of MMP-1,-3 and IL-6 in OA synoviocytes and synovial tissue explants (Lauder, Carty et al. 2007). Therefore, synovial lining macrophages appear to be a possible target for the treatment of OA but further research into the other factors involved in osteophyte formation is still needed.

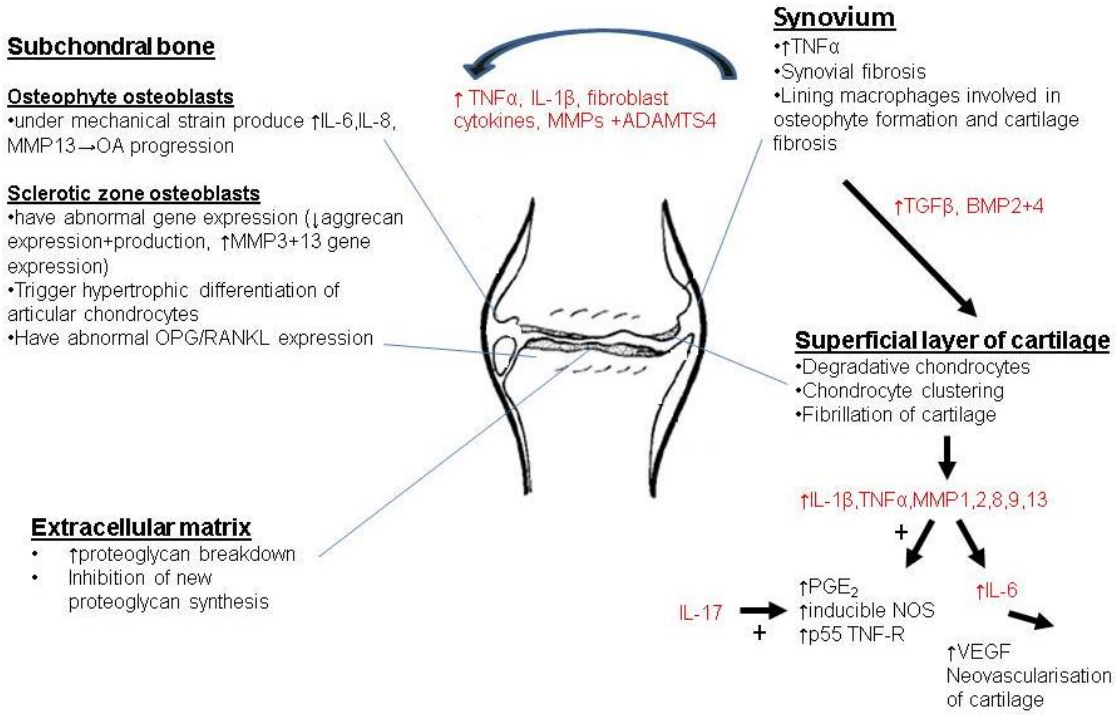


Figure 1.7 Summary of the pathophysiological changes of OA at each level of the synovial joint, focusing on key cytokines and growth factors
(adapted from (Williams, Edwards et al. 2012)).
TNFα – tumour necrosis factor alpha; IL – interleukin; MMP – matrix metalloproteinase; ADAMTS-4 – ADAM metallopeptidase with thrombospondin type 1 motif, 4; TGFβ – transforming growth factor beta; BMP – bone morphogenetic protein; PGE2 – prostaglandin E2; iNOS – inducible nitric oxide synthase; p55 TNF-R – p55 tumour necrosis factor receptor; VEGF – vascular endothelial growth factor.

Connective Tissue Growth Factor (CTGF /CCN2) is a key proinflammatory mediator in the IL-1β-mediated synovial inflammation seen in OA (Wang Z 2013). When human knee OA fibroblast-like synoviocytes (FLS) were incubated with CTGF, this led to increased production of IL-6, IL-8, C-C motif ligand 2 (CCL2), CCL20, MMP-1 and MMP-3, provided that IL-1β was also present. CTGF also produced increased phosphorylation of ERK 1/2 and p38 and increased activation of the NFκB pathway, demonstrating that the interaction of CTGF with IL-1β drives the inflammatory synovial response in OA synovium by initiating this inflammatory cascade (Wang Z 2013).

Increased levels of CCL11 (or eotaxin-1) and MMP-9 are found in OA synovial fluid compared to plasma (Chang, Shen et al. 2016). IL-1β and TNFα stimulation leads to the release of CCL11 from OA FLS via the C-C Chemokine Receptor 3 (CCR3) and ERK signalling pathways (Chang, Shen et al. 2016). Further research into the role of these proinflammatory chemokines is needed to clarify which might be the optimal target for future OA therapies.

The perivascular niche

Both venous stasis and reductions in mechanical loading can trigger osteocyte apoptosis and bone resorption by osteoclasts (Findlay 2007). Subchondral ischaemia may well be the mechanism that produces the bone marrow oedema seen on magnetic resonance imaging (MRI) in early OA, which if prolonged can lead to both bone damage and increased bone turnover, as well as degenerative change within the articular cartilage. Atherosclerosis may have a causal link with the progression of OA (Conaghan, Vanharanta et al. 2005), suggesting that improving perfusion may be more important than targeting inflammation for this facet of OA pathogenesis.

1.8.2.2 IL-6 and IL-6 protein superfamily members

IL-6

The chondrogenic niche

More recently, the importance of IL-6 in the pathogenesis of both OA and RA has been highlighted. This is supported by evidence demonstrating reduced serum IL-6 levels in patients with GC and CC single nucleotide polymorphisms (SNPs) in the promoter region of the IL-6 gene, compared to patients with the GG phenotype, indicating that the presence of 572 G/C IL-6 is a protective factor for the presence and severity of hip and knee OA in the elderly (Fernandes MT 2015). Increased expression of IL-6 and MMP-9 have been demonstrated in OA articular cartilage from patients with knee OA, compared to in normal

cartilage (Qu, Wang et al. 2015). Both IL-1 and to a lesser extent TNFα potentiate the production of IL-6 by human articular chondrocytes from both normal or OA subjects, which can be effectively blocked by specific antibodies to IL-6 (Guerne, Carson et al. 1990). In OA one of the main roles of IL-6 therefore appears to be rendering human articular chondrocytes more susceptible to the effects of other pro-inflammatory cytokines, particularly TNFα. Chondrocyte-mediated IL-6 and IL-1 production may also upregulated in response to the presence of fibronectin degradation fragments produced by the action of MMPs (Stanton, Ung et al. 2002). Similarly, IL-6 production by chondrocytes can also be driven by intact collagen 2 or discoid domain receptor 2 (DDR2), together with increases in IL-1 β, IL-8, MMP-1,-3,-13 and -14 (Klatt, Zech et al. 2009). This suggests that targeting IL-6 either specifically or via interrupting the type II collagen/DDR2 intracellular signalling pathway, may provide another means of reducing inflammation in OA.

IL-6 treatment of chondrocytes or cartilage explants has been shown to reduce proteoglycan content and increase production of MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 (Latourte, Cherifi et al. 2017). Functional studies indicate that IL-6 induced STAT 3 and ERK1/2 signalling. Inhibition of either IL-6 or Stat 3 reduced the formation of both cartilage lesions and osteophytes in the Destabilisation of the Medial Meniscus (DMM) murine model of OA, whilst inhibition of IL-6 also reduced synovitis (Latourte, Cherifi et al. 2017). These studies suggest that targeting either IL-6 or STAT-3 signalling could be an effective means of treating OA.

In vitro studies using primary murine chondrocytes and *in* vivo studies in a meniscectomy model of OA have demonstrated that basic calcium phosphate (BCP) and IL-6 form a positive feedback loop that leads to the development of OA (Nasi, So et al. 2016). Inhibition of either IL-6 or chondrocyte mineralisation therefore represent additional promising therapeutic strategies for the treatment of OA.

The osteogenic niche

As mentioned above for IL-1 and TNFα, subchondral bone osteoblasts from OA patients are also known to demonstrate increased expression of IL-6, as well as MMP-13 and RANKL (Sakao, Takahashi et al. 2008). The degree of upregulation of gene expression also correlated with the severity of cartilage damage amongst this group of patients. *In vitro* studies using primary human OA osteoblast cultures have shown that the maximal levels of IL-6 are expressed at Day 21 of culture, corresponding to the mineralisation phase of the osteoblast differentiation (Chenoufi, Diamant et al. 2001). *In vitro* work using both mature osteoblasts and bone marrow stromal cells from OA patients, showed that the cells

constitutively expressed IL-6, providing further support for the hypothesis that IL-6 is involved in bone remodelling in OA (Lisignoli, Piacentini et al. 2000). Pre-incubation of non-sclerotic zone osteoblasts with IL-6 was sufficient to induce a comparable degree of inhibition of aggrecan synthesis and increase in MMP-3 and -13 expression in co-cultures with OA chondrocytes, as that seen with sclerotic zone osteoblasts (Sanchez, Deberg et al. 2005). These data confirm that subchondral zone osteoblasts may well have an important role in cartilage degradation and OA progression. Additionally, mRNA expression and protein production of IL-6, as well as IL-8 and MMP-3, have also been shown to be upregulated in osteoblasts derived from osteophytes, suggesting that these cells also potentially influence the progression of OA (Sakao, Takahashi et al. 2008).

Given the increasing recognition of the importance of biomechanical factors in OA progression, several research groups have focused particularly on the role of mechanical loading on OA progression. *In vitro* studies applying a compressive force to murine calvarial osteoblasts within a 3D membrane demonstrated that compression increased expression of IL-6 and cyclo-oxygenase 2 (COX-2) mRNA within the osteoblasts themselves, as well as increasing the IL-6 and prostaglandin E2 (PGE2) levels within the culture supernatant (Sanchez, Gabay et al. 2009). Once again, similar findings were seen when mechanical loading was applied to cultures of OA osteoblasts isolated from osteophytes, with increased IL-6 (and IL-8) mRNA expression and protein production (Sakao, Takahashi et al. 2008). These data suggest that part of the influence of mechanical stress on OA progression may occur through stimulation of the production of pro-inflammatory cytokines including IL-6.

These findings led to interest in the effects of therapeutic agents such as hyaluronic acid, already used to treat OA, on local cytokine expression. Work in cultured human OA osteoblasts indicated that hyaluronic acid, particularly those of higher molecular weights, reduced levels of IL-6 and PGE2, suggesting that this may be one mechanism through which they exerted their therapeutic effect (Lajeunesse, Delalandre et al. 2003). Significant reductions in the levels of IL-6 found in the synovial fluid of patients with OA have also been reported in small numbers of patients (Sezgin, Demirel et al. 2005). However, further studies will be required to confirm this, particularly in humans where intra-articular hyaluronic acid has variable efficacy in clinical practice.

The synovial niche

Levels of MMP-1, IL-6, IL-8 and CCL5 are significantly higher in synovial fluid from OA patients compared to that from normal controls (Monibi, Roller et al. 2016). In both equine and human OA, the levels of IL-6 in synovial fluid appear to correlate with the degree of

synovitis (Nishimura, Segami et al. 2002, Ley, Ekman et al. 2007). Synovial fluid levels of IL-6 have also been demonstrated to be significantly associated with pain, WOMAC scores and Neuropathic Pain Scores in patients with end-stage knee OA (Radojčić MR 2017). Therapeutic agents that reduce IL-6, such as intra-articular injections of hyaluronic acid for patients with knee OA, correspondingly improve patients' symptoms and signs, including reducing the size of knee effusions, in some studies (Sezgin, Demirel et al. 2005). Levels of IL-6 in synovial fluid also show a strong positive correlation with radiographic OA scores (Monibi, Roller et al. 2016). This provides further evidence to suggest that inhibiting IL-6 may well have beneficial effects on the synovial inflammatory component of OA.

Adipocytokines

There are a number of proteins derived from adipose tissue, termed adipocytokines or adipokines. These include leptin, which has been the most widely studied, adiponectin, resistin, visfatin, progranulin (PGRN) and omentin-1. The role of these individual proteins in each cellular compartment will now be considered

The chondrogenic niche

Leptin has a wide range of effects within cartilage. Some of these such as inducing the expression of growth factors, stimulating proteoglycan and collagen synthesis may be beneficial; others such as its potentiation of the stimulatory effects of pro-inflammatory cytokines on NO production by chondrocytes, may not (Aspden, Scheven et al. 2001). High (10μg/ml) concentrations of leptin resulted in cartilage damage in porcine cartilage explants (Phitak, Boonmaleerat et al. 2017). Additional studies in human articular chondrocytes have shown that this cartilage destruction is mediated through activation of NFκB, ERK, JNK and p38, resulting in increased secretion of MMP-3, MMP-13 and ADAMTS -4 (Phitak, Boonmaleerat et al. 2017). IL-1β exerts an additive detrimental effect on cartilage through further upregulation of NFκB and JNK pathways (Phitak, Boonmaleerat et al. 2017). Leptin has also been shown to affect chondrogenic progenitor cells (CPCs) differentiation, resulting in cell cycle arrest and senescence due to activation of the p53/p21 pathway and inhibition of the Sirt-1 pathway (Zhao, Dong et al. 2016). Activation of this leptin pathway correlates with cartilage damage and tissue senescence in OA patients, suggesting that this leptin induced senescence has a role in the pathogenesis of OA (Zhao, Dong et al. 2016).

miR-27 has recently been demonstrated to target leptin (Zhou, Li et al. 2017). Transfection of a miR27 mimic into OA chondrocytes induced expression of collagen II, collagen X, glucosaminoglycan and aggrecan (Zhou, Li et al. 2017). Overexpression of miR-

27 in a rat model of OA led to downregulation of IL-6, IL-8, MMP-9 and MMP-13 via inhibition of the NFκB pathway, suggesting modulating miR-27 as a future therapeutic strategy for treating OA (Zhou, Li et al. 2017).

Leptin produced greater increases in the catabolic factors MMP-1, MMP-3, MMP-13, iNOS and COX-2 in cartilage samples from knee OA patients when the cartilage had lower background expression of suppressor of cytokine signalling-3 (SOCS-3) (Koskinen-Kolasa, Vuolteenaho et al. 2016). Therefore, upregulation of SOCS-3 could be a suitable target for the treatment or prevention of OA in the future.

Similarly, adiponectin causes degradation of OA cartilage by increasing expression of MMP-1,-3,-13 and inducible nitric oxide synthase (iNOS), mediated through the AMP-activated protein kinase and c-Jun N-terminal kinase (AMPK/JNK) pathway (Kang, Lee et al. 2010).

Visfatin is also expressed in OA cartilage and stimulates PGE2 synthesis, contributing to the pro-inflammatory environment present within OA cartilage and thereby cartilage damage (Gosset, Berenbaum et al. 2008).

Initially, although resistin appeared to have a role in the pathogenesis of RA, its role in OA remained unclear. However, recently resistin has been found to stimulate the release of sulphated glucosaminoglycans from meniscal tissue implants, associated with increased expression of MMP-2 and MMP-3 in meniscal tissue, similar to the effects of IL-1 (Nishimuta and Levenston 2017). Serum resistin levels have also been shown to be associated with cartilage defects and bone marrow lesions (BMLs) on MRI in patients with knee OA, providing further evidence of the importance of resistin in the pathogenesis of OA (Wang, Xu et al. 2017).

Two anti-inflammatory adipokines have recently been identified. Increased levels of progranulin are found in cartilage, synovium and infrapatellar fat pad tissue from OA patients (Abella, Scotece et al. 2016). Progranulin mRNA is increased in response to TNFα and IL-1β stimulation of human OA chondrocytes (Abella, Scotece et al. 2016). Progranulin counteracts the IL-1β induced expression of NOS2, COX-2, MMP-13 and VCAM-1 and functional studies suggest that at least some of these effects are mediated via the TNFR-1 (Abella, Scotece et al. 2016). Omentin-1 is the other anti-inflammatory adipokine (Li, Liu et al. 2017). In studies in human chondrocytes, omentin-1 reduced expression of MMP-1, MMP-3 and MMP-13 in response to stimulation with IL-1β and ameliorated the IL-1β induced downregulation of collagen II and aggrecan (Li, Liu et al. 2017). These protective effects on cartilage were mediated through blockade of the JAK-2/STAT3 pathway (Li, Liu et al. 2017).

Other groups have shown that levels of omentin-1 in synovial fluid are inversely correlated with symptoms and radiographic severity of OA, further supporting that upregulation of anti-inflammatory adipokines would be an alternative therapeutic option for the treatment of OA (Li, Zhao et al. 2012, Xu, Zhu et al. 2012).

The osteogenic niche

Leptin has now been recognised as an inhibitor of bone formation and bone mass, mediated through its effects on the sympathetic nervous system (Takeda, Elefteriou et al. 2003). Adiponectin acts as an inhibitor of osteoclast formation, whilst stimulating osteoblast formation (Oshima, Nampei et al. 2005, Tu, Zhang et al. 2011). Overall, this results in an increase in bone mineral density. To date, no information is available on the contribution of either resistin or visfatin to the osteogenic component of OA.

Serum visfatin concentrations have been shown to be higher in OA patients compared to healthy controls (Chen, Bao et al. 2010). Although significant levels of visfatin were present in synovial fluid, synovial tissue and adipose tissue, the highest levels were released from osteophytes, suggesting visfatin has an important role in the progression of OA (Chen, Bao et al. 2010).

The synovial niche

Leptin, adiponectin and resistin are all present in OA synovial fluid, but the significance of this finding is unclear (Gabay, Hall et al. 2008). Levels of leptin in both serum and synovial fluid from OA and RA patients are higher than in normal subjects (Yan M 2018). Increased adipokine concentrations (leptin, adiponectin, resistin and visfatin) in serum compared to synovial fluid have been reported in patients with hip and knee OA, whilst levels of IL-6 were higher in synovial fluid compared to serum (Bas, Finckh et al. 2014). Increased concentrations of IL-6, visfatin and leptin in synovial fluid samples correlated with worse pain in patients with hip OA, whereas increased synovial fluid levels of leptin and lower concentrations of adiponectin were associated with more severe pain in patients with knee OA (Bas, Finckh et al. 2014). This suggests that IL-6 and the adipokines are involved in the regulation of OA pain and exert differential effects depending on which joints are involved. Further research is required to elucidate the exact mechanisms involved and how they can best be modulated to reduce pain in OA patients.

Both articular adipose tissue and synovium taken from RA or OA patients are significant sources of adiponectin, which upregulates IL-6 and MMP-1 production (Ehling, Schaffler et al. 2006). This further emphasises the importance of IL-6 in the development of arthritis, as discussed above. However, only additional studies in this area will clarify whether targeting

adipocytokines or the synoviocytes themselves will be the more effective method of reducing this source of pro-inflammatory mediators.

Oncostatin M (OSM)

The chondrogenic niche

OSM appears to occupy a key role not only in bone remodelling, but also in regulating the chemokines and MMPs that drive cartilage degradation in OA (Lisignoli, Piacentini et al. 2000). Combinations of IL-1β and OSM added to cartilage explant cultures produce a synergistic effect on cartilage collagen resorption by stimulating both synthesis and activation of pro-MMPs (Cawston, Curry et al. 1998). Similarly, transfecting the genes for both IL-1 and OSM into a mouse model using an adenoviral vector stimulated production of MMPs and joint damage (Rowan, Hui et al. 2003). OSM produces additive effects in terms of bone and cartilage damage in an *in vivo* model of OA, compared with TNFα alone (Malemud, Islam et al. 2003, Malemud and Schulte 2008). The stimulatory effects of OSM on ADAMTS-4 and MMP-13 production by chondrocytes are thought to be mediated through Janus kinase (JAK)-3/signal transducers and activators of transcription (STAT)-13 signalling pathway, as well as by interactions between this pathway and the ERK1/2 and PI3K/AkT pathways (Malemud and Pearlman 2009). Studies done by our group have shown that expression of the suppressor of cytokine signalling (SOCS) proteins SOCS-2 and cytokine-inducible SH2-domain (CIS-1), inhibitors of cytokine signalling that act on the JAK/STAT pathway, are both reduced in OA chondrocytes compared to control (de Andres, Imagawa et al. 2011). Long-term treatment of these chondrocyte cultures with IL-1β, OSM or TNFα attenuated expression of SOCS-2 and CIS-1, suggesting that a positive feedback loop may exist under these conditions (de Andres, Imagawa et al. 2011, Sun 2013). These pathways may therefore provide suitable targets for OA treatment in the future.

Dickkopf-3 (Dkk-3), one of the Dkk family of Wnt antagonists has inhibitory effects on both IL-1β and OSM mediated proteoglycan loss from cartilage explants through its activation of TGFβ signalling, combined with inhibition of Wnt3a/activin signalling (Snelling, Davidson et al. 2016). This dual action results in protective effects on cartilage and could represent an alternative strategy for treating OA.

The osteogenic niche

OSM is expressed in OA osteoblasts (Wu R 2016). Endothelin -1 induces expression of OSM in human OA osteoblasts via transactivation of the OSM gene promoter (Wu R 2016). Leptin also increases OSM expression in human osteoblasts but its effects are mediated via increased phosphorylation of Akt, which results in downregulation of miR-93 and

transactivation of OSM production (Yang, Tsai et al. 2014). OSM itself stimulates production of IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) when applied to human endothelial cell lines (Bruce, Linsley et al. 1992, Brown, Liu et al. 1993). OSM activates osteoblasts and inhibits bone resorption, thereby occupying a key role in bone remodelling (Jay, Centrella et al. 1996). OSM also stimulates release of collagen fragments in the presence of inflammatory cytokines (Cawston, Curry et al. 1998) and increases expression of collagenase-3 by mature osteoblasts (Varghese, Yu et al. 1999).

The synovial niche

OSM is found at higher concentrations in synovial fluid from OA patients compared to that from healthy controls (Beekhuizen M 2013). Inhibition of OSM in cartilage explant cultures resulted in increased glucosaminoglycan production, suggesting that inhibition of OSM could have beneficial effects on cartilage repair in OA (Beekhuizen M 2013). Although synovial fluid levels of OSM have been found to correlate with markers of cartilage degradation in RA, this does not appear to be the case in OA (Manicourt, Poilvache et al. 2000). Increased levels of OSM expression have also been found in the synovial tissue of patients with knee OA compared to controls (Ni, Yuan et al. 2015). *In vitro* studies show that OSM increases osteoblast cell proliferation and differentiation, stimulating bone formation (Ni, Yuan et al. 2015). Overall, OSM has comparable effects to IL-6 in OA and comparative studies of OA and RA patients suggest that its role in OA may be more of a synergistic one, acting together with and potentiating the effects of IL-1β, TNFα and IL-6 rather than occupying a unique role as in RA (Manicourt, Poilvache et al. 2000).

Leukaemia inhibitory factor

The chondrogenic niche

The expression of LIF mRNA is increased in OA cartilage compared to that from normal controls (Jiang Y 2014). LIF expression is highest in severely degraded cartilage and in moderately degraded subchondral bone, demonstrating that levels of LIF in articular tissues vary, dependent on the clinical and radiographic severity of OA (Jiang Y 2014). LIF upregulation by IL-1β has been shown both in cultured chondrocytes and in cartilage explants, to be dependent on the activation of ERK 1/2, p38 kinase and NFκB (Fan, Bau et al. 2004). This effect appears to be ameliorated, at least partially, by TGFβ1, which raises further therapeutic possibilities (Malemud and Schulte 2008).

The osteogenic niche

LIF contributes to the regulation of bone formation and resorption (Taupin, Pitard et al. 1998). It is an osteoclast-stimulating factor whose effects are mediated through LIF receptors (LIF-R) expressed on mature osteoblasts (Cornish, Callon et al. 1993). When LIF binds to its specific receptor, it forms a heterodimer complex which interacts with the same glycoprotein (gp)130 signal transduction unit that the IL-6/IL-6 receptor complex interacts with to produce its downstream effects (Malemud and Pearlman 2009). This LIF/LIFR/gp130 interaction, after several intermediate steps, activates the JAK/STAT pathway discussed above, as well as the stress-activated protein (SAP)/mitogen-activated protein kinase (MAPK) signalling cascades (Malemud and Pearlman 2009). This highlights the suitability of these pathways as future therapeutic targets. LIF also stimulates osteoblast differentiation and proliferation (Cornish, Callon et al. 1993) and if over-expressed can cause over-production of osteoblasts by the bone marrow and result in new bone formation (Reid, Lowe et al. 1990). LIF actions are dose-dependent: at lower concentrations it causes an increase in osteoclast numbers, favouring bone resorption (Reid, Lowe et al. 1990); in higher concentrations it promotes DNA and protein synthesis (Bruce, Linsley et al. 1992, Cornish, Callon et al. 1993). Production of LIF by mature osteoblasts and bone marrow stem cells is significantly upregulated by IL-1β and TNFα (Lisignoli, Piacentini et al. 2000).

The synovial niche

LIF itself has also been shown to potentiate TNFα-induced PGE_2 release from human OA synovial fibroblasts, mainly by increasing levels of cytoplasmic phospholipase A2 (cPLA2) (Alaaeddine, Di Battista et al. 1999). Therefore, the therapeutic use of LIF in OA would require careful dose titration and tissue specific application in order to harness its beneficial effects on osteoblasts whilst minimising its influence on the chondrogenic and synovial compartments.

IL-17

The chondrogenic niche

IL-17 is a pro-inflammatory cytokine that received initial interest in terms of its involvement in RA, but its significance in OA was unclear. In recent years, IL-17 has been demonstrated to be a key cytokine, together with IL-23, in the pathogenesis of psoriasis, psoriatic arthritis (PsA) and ankylosing spondylitis (AS) (Raychaudhuri and Raychaudhuri 2017, Frieder, Kivelevitch et al. 2018, Wu, Yue et al. 2018). Inhibition of IL-17 with specific monoclonal antibodies such as secukinumab and ixekizumab leads to dramatic improvements in the clinical signs and symptoms of these conditions (Frieder, Kivelevitch et

al. 2018, Wu, Yue et al. 2018). Whereas RA is characterised by erosive damage to the joints, both erosive and sclerotic changes can be seen in PsA and AS at different stages of the disease. In OA, the predominant pattern of joint damage is subchondral sclerosis but a more erosive subtype can occur. Therefore, there has been renewed interest in whether IL-17 has a similarly significant role in the development of OA. In human OA chondrocytes, IL-17 increases NO production by up-regulating iNOS levels via pathways independent from those utilised by other pro-inflammatory cytokines such as IL-1β or TNFα (Martel-Pelletier, Mineau et al. 1999). The effects on NO production are predominantly controlled by activation of protein kinase A (PKA) and tyrosine kinase. IL-17 stimulated iNOS production in contrast appears to be regulated via NFκB signalling, with the p44/42 and p38 mitogen-activated protein kinases (MAPK) occupying a crucial role in ensuring full iNOS expression. It has been postulated that MAPK exerts its effects on NFκB via transactivation of NFκB by mitogen-activated protein-kinase activated protein kinase (MAPKAPK). In addition, IL-17 upregulates production of collagenase 3 by human OA chondrocytes through activating protein-1 (AP-1) mediated transcriptional activity, similarly to IL-1 but with differential protein complexes (Benderdour, Tardif et al. 2002). IL-17 also exerts synergistic effects with TNFα on cartilage degradation *in vitro* (Van Bezooijen, Van Der Wee-Pals et al. 2002) and can stimulate the release of chemokines, as well as PGE2 (Honorati, Bovara et al. 2002).

The osteogenic niche

Early microarray profiling on the role of IL-17 in arthritis in pre-osteoblast cell lines, suggested that the primary function of IL-17 in bone was to act synergistically with other cytokines to potentiate inflammation (Shen, Ruddy et al. 2005). In particular, IL-17 was found to enhance TNFα stimulated IL-6 synthesis via a p38 mitogen-activated protein kinase in osteoblasts (Tokuda, Kanno et al. 2004). Additionally, IL-17 appeared to enhance IL-6 synthesis stimulated by prostaglandin F(2α)(PGF(2α)) as well as endothelin-1 (ET-1) in osteoblasts (Tokuda, Kozawa et al. 2002). Further studies in bovine cartilage explant cultures confirmed that IL-17, either alone or acting synergistically with other proinflammatory cytokines, was able to stimulate the expression of MMP-1, MMP-3 and MMP-13, resulting in chondrocyte-mediated, MMP dependent, release of collagen II from cartilage (Koshy, Henderson et al. 2002). IL-17 has been demonstrated to stimulate the development of osteoclasts, in the presence of osteoblasts. The precise mechanism of action on osteoclasts was unknown until recent work showed that IL-17A was able to induce expression of both cathepsin-K and MMP-9 in osteoclasts, through its effects on celecoxib-blocked prostaglandin expression (mainly PGE2) by osteoclasts (Zhang, Tanaka et al.

2011). However, although IL-17 is recognised to be involved in the pathogenesis of inflammation-related bone loss in RA through some of these mechanisms, as yet its specific role in bony damage in OA remains to be determined.

The synovial niche

IL-17 is a T-cell-derived cytokine and cells expressing IL-17 have been detected in both RA and OA synovial membranes (Revell, Mayston et al. 1988, Haraoui, Pelletier et al. 1991). IL-17 has also been found in OA synovial fluid and some OA synovial membranes, lending further credence to the hypothesis that it is involved in OA pathogenesis (Chabaud, Durand et al. 1999, Kotake, Udagawa et al. 1999). Increased levels of IL-17 are found in serum and synovial fluid of OA patients, compared to that from controls (Chen B 2014). Both serum and synovial fluids levels of IL-17 have been shown to have a positive correlation with radiographic severity of knee OA (Chen B 2014). However, other groups have demonstrated that levels of IL-17 in synovial fluid are positively correlated with WOMAC pain scores in OA patients but have not found a correlation with KL grade of OA (Liu, Peng et al. 2015). IL-17 has also been shown to stimulate secretion of a number of pro-angiogenic factors by OA synovial fibroblasts, either alone or acting synergistically with TNFα, thereby contributing to the progression of synovial inflammation (Honorati, Neri et al. 2006). IL-17 stimulates the release of IL-6 and CCL7 from synoviocytes via NFκB and PI3 kinase/Akt-dependent pathways (Hurang et al. 2004). In patents having either total hip (THR) or knee (TKR) surgery for OA, 9% were found to have significant levels of IL-17 in their synovial fluid (Snelling SJ 2017). This subset of patients had increased levels of IL-6, leptin, resistin, CCL7 and Nerve Growth Factor (NGF) in their synovial fluid and serum, with corresponding reductions in osteophytes, sclerosis and joint space width (Snelling SJ 2017). The presence of detectable levels of IL-17 in synovial fluid therefore identified a subset of OA patients who were more likely to be younger, female, obese and have rapidly progressive OA, characterised by reduced bony involvement but more synovitis and termed ‘inflammatory OA’ (Garnero, Charni et al. 2006, Conrozier, Ferrand et al. 2007). In this subgroup, IL-17 promotes the production of MMPs, increases osteoclastic and decreases osteoblastic activity (Kotake, Udagawa et al. 1999, Koshy, Henderson et al. 2002, Kim, Park et al. 2014). Studies of inflamed compared to non-inflamed areas of synovium obtained from patients having surgery for knee OA provide further support for the existence of this inflammatory OA phenotype (Deligne C 2015). This group demonstrated that inflamed OA synovium expressed higher levels of IL-17, IL-22, IL-6, IL-23 and TGFβ. However, significant synovitis was found in another subset of these patients who lacked any detectable IL-17 in synovial

fluid, suggesting that another IL-17 independent pathway is involved in the development of synovitis in this subgroup of patients (Deligne C 2015).

DIDNA (dideoxynucleotide) aptamers targeting IL-17RA have been generated and shown, in a murine model of OA, to ameliorate IL-17/IL-17A-mediated IL-6 expression, thereby reducing synovial inflammation (Chen, Li et al. 2011). This raises the possibility that such aptamers may, in the future, represent an additional therapeutic option for the treatment of early OA. Together these findings indicate that IL-17 has an important role in the development of OA, but as yet inhibitors of this cytokine have not been tested as potential future treatments for OA.

IL-18

The chondrogenic niche

IL-18 has emerged as a potentially important cytokine, both in RA and OA, based on several *in vitro* studies and data from animal models (Kapoor, Martel-Pelletier et al. 2011). Firstly, OA chondrocytes spontaneously produce IL-18 in culture and this appears to act synergistically to increase cartilage matrix degradation in response to TNFα (Matsui, Tsutsui et al. 2003). This is supported by data from monolayer cultures of normal, OA and RA chondrocytes where application of IL-18 to the cultures resulted in upregulation of IL-17 receptor α (IL-17Rα) on the surface of the chondrocytes, producing slight increases in expression of MMP-1, 3 and 13, aggrecanase-2 and tissue inhibitor of metalloproteinases-1 (TIMP-1) in a small number of the OA or RA samples (Dai, Shan et al. 2005). This upregulation of catabolic factors results in the chondrocytes undergoing differentiation into an apoptotic chondrocyte phenotype typically seen in the later stages of OA. IL-18 also upregulates the synthesis of COX-2 and TNFα mRNA in chondrocytes, which leads to increased secretion of PGE2 and TNFα respectively (Fu, Liu et al. 2012). Furthermore, studies in IL-18 transgenic mice suggest that high serum levels of IL-18 promote high levels of IL-18 in articular chondrocytes, which results in loss of aggrecan synthesis and cartilage damage (Inoue, Hiraoka et al. 2008). Further *in vitro* and *in vivo* studies reveal that when IL-1 deficient mice are treated with IL-18 by intra-articular injection of mIL-18 adenovirus, no cartilage damage occurs but joint inflammation is still seen (Joosten, Smeets et al. 2004). In contrast, when TNF deficient mice are treated with the same mIL-18 adenovirus, joint swelling and inflammation are reduced but cartilage damage still occurs (Joosten, Smeets et al. 2004). This confirms that joint inflammation and cartilage damage are mediated via two separate pathways: although IL-18 can produce joint inflammation in the absence of IL-1, it

is the subsequent release of IL-1β that is crucial in causing cartilage damage either *in vitro* or *in vivo* (Joosten, Smeets et al. 2004).

The osteogenic niche

To date there is little evidence available regarding the precise effects of IL-18 on the osteogenic components of the OA niche, nor putative mechanisms of action.

The synovial niche

Increased levels of IL-18 are present in both the plasma and synovial fluid of OA patients compared to controls (Wang, Xu et al. 2014). IL-18 upregulates the expression of both COX-2 and TNFα in primary synoviocytes (Fu, Liu et al. 2012). IL-18 levels also correlate with PGE2 production both in OA synovial fluid (Futani, Okayama et al. 2002, Li, Jiang et al. 2009) and in synoviocyte culture supernatants (Weng, Wang et al. 2010), suggesting that this may be one mechanism through which IL-18 exerts its effects on the development of OA. IL-18 levels in synovium, synovial fluid, plasma and cartilage also correlate with disease activity in OA patients (Matsui, Tsutsui et al. 2003). In keeping with this, administration of recombinant rat IL-18 resulted in increased incidence of collagen-induced arthritis in BB rats (Ye, Tang et al. 2004). Conversely, deficiency of IL-18 prevents induction of collagen-induced arthritis in mice (Wei, Leung et al. 2001). A possible role for IL-18 in the pathogenesis of both RA and OA is further supported by the clinical observation that patients receiving anti-TNFα treatment in a clinical trial showed correspondingly reduced levels of circulating IL-18 (Matsui, Tsutsui et al. 2003). Further research is still required to elucidate the exact roles of IL-18 in inflammatory arthritis.

1.9 Strategies for managing inflammation in regenerative medicine

1.9.1 Targeting individual cytokines

There are several potential strategies for targeting the inflammatory component of OA. Firstly, given IL-1 is a key cytokine in OA, reducing levels of IL-1 using specific antagonists (IL-1 receptor antagonist proteins (IRAPs)) would be expected to slow disease progression. In animal models of cartilage damage, intra-articular injection of the IL-1 receptor antagonist (IL-1RA) slowed both the development of joint damage and symptoms (Fernandes, Tardif et al. 1999, Frisbie, Ghivizzani et al. 2002). Disappointingly, intra-articular administration of IL-1RA (anakinra) failed to improve symptoms of knee OA in humans in a randomised, placebo-controlled trial (Chevalier, Goupille et al. 2009). Similarly, a placebo-controlled trial

of intra-articular administration of the autologous IL-1Ra, Orthokin failed to meet its primary endpoint, although patients receiving Orthokin did have a significantly greater improvement in their Knee injury and Osteoarthritis Outcome Score (KOOS) (Auw Yang KG 2008). Therefore, Orthokin is not currently recommended for the treatment of knee OA. Most recently, a small case series of patients with erosive hand OA were treated with a single subcutaneous injection of 160mg of the human monoclonal antibody to IL-1β, canakinumab (Carroll 2015). After 12 weeks of follow up, patients receiving canakinumab had no discernible improvement in either clinical or functional outcomes (Carroll 2015). Several hypotheses have been put forward to explain these results. Firstly, there are lower levels of IL-1α/IL-1β but an elevated IRAP/IL-1β ratio in synovial fluid from OA compared to RA patients (Richette, Francois et al. 2008). The authors of this study postulate that this already elevated level of IRAP in OA synovial fluid accounts for the limited efficacy of intra-articular IRAP treatment. Interestingly, in a study of serum biomarkers in patients with symptomatic knee OA, elevated plasma IL-1Ra levels were predictive of joint space narrowing at 24 months, suggesting that plasma IL-1Ra levels are modestly associated with the severity and progression of symptomatic knee OA (Attur, Statnikov et al. 2015). The other hypothesis is that the half-life of the IRAP within the joint itself is too short (Goupille, Mulleman et al. 2007). This has led to interest in modifying the structure of current commercially available IRAPs to see if prolonging their half-life will improve their clinical efficacy.

One method of achieving prolonged intra-synovial levels of IL-1Ra protein in early phase development involves using a Self-Complementary Adeno-Associated Virus (scAAV) as a vector. Preliminary studies in an equine model of OA confirmed that the scAAVIL-1RA therapeutic vector was able to generate adequate intra-articular levels of IL-1Ra protein long-term (Goodrich, Phillips et al. 2013). A dosing study was successfully carried out in 6 horses and intra-articular levels of IL-1Ra were maintained for up to 8 months with a dose of $5x10^{12}$ vg (Goodrich, Grieger et al. 2015). Further studies to assess the clinical efficacy of this approach are now planned.

More recently, a Phase 1 study of a subcutaneously injected anti-IL-1α/β dual variable domain immunoglobulin (ABT-981) has been carried out in patients with knee OA (Wang, Abramson et al. 2017). This was a dose-ranging and safety and tolerability study and confirmed that the treatment was generally well-tolerated by patients, reached the appropriate target and demonstrated an anti-inflammatory effect with significant reductions in serum IL-1α, IL-1β, MMP-1 induced type I collagen and hs-CRP (Wang, Abramson et al. 2017). Further studies are now planned.

A further option is to combine IL-1 inhibition with growth factor over-expression, a combination termed ‘dual axis therapy’. This improves cartilage healing in animals and could well prove successful in human OA in the future (Haupt, Frisbie et al. 2005, Nixon, Haupt et al. 2005). Both single and double gene transfection of IL-1Ra and TGFβ1 have been tested in a rabbit model of OA (Zhang, Zhong et al. 2015). Injection of IL-1Ra and TGFβ1 expressing cartilage cells into joints was shown to produce a significant reduction in cartilage matrix degradation (Zhang, Zhong et al. 2015). Expression of IL-1Ra and TGFβ in articular tissues was associated with the reversal of OA in the experimental group, validating the use of this combination approach to OA treatment. An alternative strategy is IL-1 knockdown therapy, as this has been shown in animal models to stabilise the course of secondary OA (Nixon, Goodrich et al. 2007).

Given that epigenetic modifications of genes are known to occur in OA cartilage in response to pro-inflammatory cytokines, as discussed earlier, and are potentially reversible, these offer additional possible targets for modifying cytokine effects. So far, HDAC inhibitors have been effective in small animal models, although more selective inhibitors that target single HDACs have yet to be developed (Chen, Bao et al. 2010). Our understanding of the role of miRNAs in the pathogenesis of OA is rapidly advancing and several targets that modulate the effects of IL-1 and TNFα have already been discovered (see previous IL-1 and TNF section and Tables 1.2 and 1.3). Specific inhibitors of individual miRNAs have been developed but have yet to be tested in models of OA. All of the aforementioned strategies can similarly be applied to TNFα and IL-6 inhibition to produce both symptomatic and radiographic benefit, but as yet these have not been the focus of dedicated research within the OA field.

1.9.2 Targeting multiple cytokines

Targeting multiple cytokines represents another potential strategy for treating OA that could be incorporated into tissue engineering constructs. Given the extensive cross-talk that occurs between cytokines, targeting more than one cytokine should produce additive effects, not least as it will overcome the issue of redundancy that can occur when single cytokines are targeted alone. This principle has already been used in the development of a new class of treatment for RA, the janus kinase (JAK) inhibitors. These are a class of oral small molecules that target intracellular signalling pathways downstream to individual cytokines, producing additive effects on cytokine suppression that have already yielded two highly

successful treatments for RA, tofacitinib and baracitinib (Schwartz, Kanno et al. 2017). Additional studies in ankylosing spondylitis are underway.

As IL-1 and TNFα are both integral to the pathophysiology of OA, inhibiting both these factors would seem to offer a pragmatic treatment option. Double gene transfer of IL-1RA and the TNFα blocking gene (TNFR-1) has been effective in reducing cartilage degradation and synovitis in a rabbit model of OA (Wang, Yu et al. 2006). Gene silencing using small interfering RNAs (siRNAs) represents another potential method of inhibiting both of these cytokines. So far, these strategies have not been extended to inhibition of IL-6 but, given the interdependence of this cytokine with both IL-1 and TNFα in OA, combination treatments including IL-6 inhibition could be an effective future therapy. Given that combinations of cytokine inhibitors such as anti-TNF and IL-1Ra have demonstrated increased efficacy for treating RA in animal models, it was hoped these would be similarly effective in humans. However, a clinical trial of combination treatment with anti-TNF (etanercept) and IL-1Ra (anakinra) for RA patients failed to demonstrate any additional treatment over anti-TNF treatment alone and was associated with an increased risk of serious infections and neutropaenia (Genovese, Cohen et al. 2004). Similar concerns were raised following the occurrence of increased serious adverse events including serious infections, without any increase in efficacy, amongst patients receiving either etanercept, adalimumab, infliximab or anakinra together with abatacept (Weinblatt, Combe et al. 2006, Weinblatt, Schiff et al. 2007). For this reason, combinations of biologic agents are not currently recommended for the treatment of RA. This similarly represents a major caveat to using these agents to treat OA, where the risk-benefit ratio would be further negatively skewed. A possible alternative of combining inhibition of pro-inflammatory cytokines with increased levels of anti–inflammatory cytokines (interleukin-4 (IL-4), interleukin-10 (IL-10)) may well be effective in slowing OA progression. Pre-clinical work in a rabbit model of OA where human IL-1RA cDNA and human IL-10 cDNA were transfected using a retroviral vector provides support for this hypothesis (Zhang, Mao et al. 2004). Lapine synoviocytes were transfected and cultured *ex vivo*, before transplantation by intra-articular injection into the knee joints of OA rabbits. IL-1RA alone significantly reduced cartilage breakdown. IL-10 alone was less effective but both genes together produced an additive effect on slowing cartilage breakdown. This approach is currently being tested in the form of Orthokin, an autologous conditioned serum rich in IL-1RA, IL-4, IL-10 and IL-13. Initial results in humans are encouraging but as yet it has only been used in small numbers of patients (Fox and Stephens 2010).

Regarding pre-clinical studies targeting multiple cytokines, one strategy showing considerable promise is intra-articular injection of human articular chondrocytes transfected with cytokine inhibitors and/or growth factors. In a rabbit model of OA, intra-articular administration of human articular chondrocytes transfected with basic fibroblast-like growth factor (bFGF), produced superior chondrocyte proliferation, increased glucosaminoglycan (GAG) and type II collagen content, compared with OA controls (Chen, Qin et al. 2010). Combining transfection with bFGF with one or both of IL-1Ra and IGF-1 transfection produced additive effects on GAG and type II collagen content and tissue inhibitor of TIMP-1 levels, as well as reducing levels of MMP3,-13 and ADAMTS-5.

1.9.3 Upregulating anabolic factors

Instead of targeting the source of inflammation to treat joint damage in OA, an alternative option is to promote healing by adjusting tissue engineering strategies to increase delivery of anabolic factors. Anabolic factors that represent potential targets when devising future treatments for OA can be subdivided into chondrogenic and osteogenic groups. Those relating to chondrocytes include osteogenic protein-1 (OP-1), IGF-1 and SOX-9. The former should restore synthesis of ECM whilst the latter stimulate chondrocyte differentiation. Addition of a growth factor such as transforming growth factor β3 (TGFβ3) could improve cartilage repair even further. This can be achieved using viral vectors to promote alterations in gene expression (Li, Tew et al. 2004, Tew, Li et al. 2005, Cucchiarini, Thurn et al. 2007). Coupled gene transfer of FGF-2 and Sox-9 using an adeno-associated virus vector produces an additive effect, both improving survival and proliferation of normal and OA chondrocytes and increasing matrix production (Cucchiarini, Schetting et al. 2009). Potential osteogenic pathway targets include the osteoprotegerin/receptor activator of nuclear factor-κB/RANK ligand (OPG/RANK/RANKL) system and ephrin B2 (EphB2). Several issues may complicate targeting OPG itself though, as OPG is both a survival factor for tumour cells (Fisher, Thomas-Mudge et al. 2006) and potentially inhibits synovial apoptosis, causing synovial hypertrophy (Kwan Tat, Amiable et al. 2009). Therefore, other levels in this pathway may need to be targeted instead to try and minimise these unwanted effects.

Up-regulation of TGFβ3 as a means of repairing cartilage remains complicated as when TGFβ was overexpressed in murine knee joints by means of an adenoviral vector, synovial hyperplasia and osteophyte formation characteristic of OA were seen, as with local injection (Bakker, van de Loo et al. 2001). Removing the synovial lining cells reduced osteophyte formation, as well as increasing ECM production (Bakker, van de Loo et al. 2001). These

findings were confirmed in a collagenase-induced murine model of OA, in which removal of synovial lining macrophages reduced TGFβ, BMP-2 and -4 production, osteophyte formation and cartilage fibrosis (Blom, van Lent et al. 2004). Intra-articular injection of allogeneic chondrocytes transfected with a retrovirus carrying TGFβ1 cDNA have already been licensed in Korea for the treatment of OA and Phase III trials are about to start in the USA (Evans, Ghivizzani et al. 2018).

Although incorporating an inhibitor to down-regulate TGFβ into tissue engineering strategies would therefore appear to be a viable means of preventing typical osteophyte formation, this has other adverse consequences for the joint. This is based on data from a murine model of OA in which a TGFβ inhibitor (recombinant soluble TGFβ receptor II) was added and osteophyte formation was reduced as expected, but there was also increased loss of proteoglycan from the cartilage and a reduction in cartilage thickness (Scharstuhl, Glansbeek et al. 2002). To incorporate TGFβ inhibition successfully into a bioengineering strategy for OA, it would be necessary to devise a means of targeting TGFβ inhibition purely to the synovial cells and osteoblasts to avoid detrimental effects on the cartilage. One suggested means of achieving this is via local gene therapy with the inhibitory Smads 6 and 7 (Blom, van der Kraan et al. 2007). Another potential strategy would be to combine both IGF-1 and TGFβ, as this has been shown to produce additive anabolic effects on human articular chondrocytes *in vitro* (Seifarth, Csaki et al. 2009).

Another possible target for gene therapy strategies is platelet-derived growth factor (PDGF). PDGF acts as a mitogenic and chemotactic factor for all mesenchymal cells and stimulates meniscal cell proliferation and cartilage synthesis by chondrocytes. The glycosyltransferases are further potential anabolic factors, which catalyse the formation of polysaccharide chains needed for the synthesis of glycosaminoglycan (GAG), one of the major constituents of cartilage matrix (Magdalou, Netter et al. 2008).

Strontium is recognised to have some anabolic effect on bone along with its anti-resorptive effect. The results of the SEKOIA study, a prospective, multicentre, international phase III randomised, double-blind, placebo-controlled trial of the anti-osteoporotic drug strontium ranelate in patients with knee OA (Cooper, Reginster et al. 2012), have shown that strontium reduces joint space narrowing, as well as lessening pain and improving patients' mobility (IOF-ECCEO Conference, Bordeaux, 2012). Strontium reduced cartilage degradation by about a third over the 3 year treatment period. This builds on the data derived from post-hoc analyses of the Spinal Osteoporosis Therapeutic Initiative (SOTI) and the TReatment Of Peripheral OSteoporosis (TROPOS) studies in 2008, suggesting that in

postmenopausal women with osteoporosis and radiographic evidence of spinal OA, taking strontium reduced the radiographic progression of spinal OA and the occurrence of back pain (Bruyere, Delferriere et al. 2008). The precise mechanism of action of strontium in OA is as yet unclear but is likely to involve effects on stem cell differentiation and therefore bone and cartilage repair. This raises the possibility that such agents could in the future, be incorporated into scaffolds to enhance the regenerative capacity of implanted SSCs or chondrocytes.

1.9.4 Downregulating catabolic factors

Dickkopf-1

Dickkopf-1 (Dkk-1) is a direct inhibitor of Wnt/β catenin signalling. It occupies a crucial role in joint remodelling, affecting both bone and cartilage turnover. Comparison of Dkk-1 levels in plasma and synovial fluid from normal and OA patients have shown that Dkk-1 levels are inversely related to OA severity (Honsawek, Tanavalee et al. 2010). Therefore, Dkk-1 may represent a useful future biomarker of OA severity. A recent study in an *in vivo* rat ACLT model of OA showed increased Dkk-1 mRNA and protein expression in OA knees compared with controls (Weng, Wang et al. 2010). Inhibition of Dkk-1 (using a DKK-1 antisense oligonucleotide (DKK-1-AS)) attenuated OA-induced cartilage degradation and subchondral bone loss in knee joints in this experimental model of OA, suggesting that inhibition of Dkk-1 may represent a future therapeutic target for treating OA, although the precise mechanisms of action of Dkk-1 still require further study.

Matrix metalloproteinases

A number of MMP inhibitors have been investigated for use in treating OA. One of these, thymoquinone, has been shown to downregulate MMP-1,-3 and -13 and upregulate TIMP-1, as well as reducing levels of NFκB both in rabbit chondrocytes and in a rabbit ACLT model of OA (Chen, Tang et al. 2010). Trichostatin A (TSA) has also been shown to be effective in reducing the severity of OA in this same ACLT model (Chen, Bao et al. 2010). Once again, its effects appear to be mediated by downregulation of MMP-1,-3 and -13, in this case due to histone deacetylation. Combining this inhibition of histone deacetylation with FGF-2, thus downregulating both anabolic and catabolic genes, has been suggested as a potential means of slowing cartilage turnover (Wang, Song et al. 2009). Although these data from animal models have been encouraging, to date, clinical trials of MMP inhibitors have been disappointing due to lack of efficacy and safety concerns. These centre around the occurrence of significant musculoskeletal side effects with non-selective inhibitors. Focus

has now therefore shifted towards developing selective MMP inhibitors. Several selective MMP-13 inhibitors have now been developed, which reduce cartilage damage in a variety of rat models of cartilage damage, so far without evidence of the musculoskeletal toxicity seen with the non-selective inhibitors (Johnson, Pavlovsky et al. 2007, Gooljarsingh, Lakdawala et al. 2008, Baragi, Becher et al. 2009, Monovich, Tommasi et al. 2009). Another method of inhibiting MMPs is via upregulation of TIMPs, the endogenous regulators of MMP activity. Studies to date suggest that tissue inhibitor of metalloproteinases-3 (TIMP-3) may well be a suitable candidate for treating OA (Kashiwagi, Tortorella et al. 2001, Zhao, Bernardo et al. 2004, Sahebjam, Khokha et al. 2007).

A new selective cFos/activator protein-1 (AP-1) inhibitor T-5224 has been shown to inhibit the expression of MMP-1, MMP-3 and MMP-13, as well as IL-1β, IL-6 and TNFα in IL-1β stimulated human articular chondrocytes (Motomura, Seki et al. 2018). T-5226 also prevented both cartilage destruction and osteophyte formation in the destabilisation of the medial meniscus (DMM) murine model of OA (Motomura, Seki et al. 2018). A herbal compound, sauchinone, has also been shown to inhibit IL-1β-induced MMP-3 and MMP-13 release in human OA chondrocytes, probably mediated via activation of the NFκB pathway (Gao Y 2018).

A new phosphocitrate analog, Carolinas Molecule-01 (CM-01), has recently been suggested as an orally available DMOAD (Sun Y 2018). CM-01 is associated with reduced expression of MMP-1, IL-1β, reduced cell-mediated calcification and increased proteoglycan production in chondrocyte cell cultures (Sun Y 2018). The effects of CM-01 were further examined in the Hartley guinea pig model of post-traumatic OA (Sun Y 2018). CM-01 resulted in a reduction in damage to the articular cartilage, as well as reducing resorption of calcified zone cartilage. These changes were associated with reduced expression of MMP-13, ADAMTS-5, CXCL-5 and COX-2, suggesting that the beneficial effects of CM-01 in OA are associated with downregulation of catabolic pathways in cartilage. These preliminary data therefore support the hypothesis that CM-01 could be a suitable oral treatment for human OA in the future (Sun Y 2018).

Aggrecanases

Loss of aggrecan is one of the first signs of matrix degradation in early OA. The aggrecanases 1 and 2, also known as ADAMTS-4 and -5, are the key enzymes involved in matrix degradation and represent possible targets for OA treatment. Selective inhibitors of ADAMTS-4 or ADAMTS-5 or both are currently in development (Bursavich, Gilbert et al. 2007, Gilbert, Bursavich et al. 2007, Wittwer, Hills et al. 2007, Gilbert, Bursavich et al. 2008).

TIMP-3, mentioned above, has also been shown to act as a potent inhibitor of both ADAMTS-4 and ADAMTS-5.

NFκB inhibitors

Pro-opiomelanocortin (POMC) is a neuropeptide precursor, which can give rise to a variety of anti-inflammatory and immunosuppressive compounds. POMC gene transfer into HTB chondrosarcoma cells and Raw 264.7 macrophages reduces NFκB and IL-1β levels, whilst intra-articular injections of POMC using an adenoviral vector produced significant retardation of OA progression and reduced its severity in the ACLT rat model of OA (Shen, Shiau et al. 2011). This suggests that POMC could represent another novel treatment for OA in the future.

Several other agents that could potentially be used to downregulate the NFκB pathway have also been identified. Overexpression of the high mobility group box 1 inhibitors (HMGB1) box A in IL-1β stimulated human OA chondrocytes has been shown to result in downregulation of IL-1β-mediated activation of the Toll-like Receptor 4 (TLR-4)/NFκB pathway (Fu, Lei et al. 2016). IL-1β-induced production of MMP-1, MMP-3 and MMP-9 was reduced, as well as levels of iNOS and COX-2 (Fu, Lei et al. 2016). The naturally occurring anti-inflammatory and anti-oxidant curcumin also results in downregulation of MMP-13 and upregulation of collagen II in rat articular chondrocytes by inhibiting IL-1β mediated activation of NFκB (Wang, Ma et al. 2017).

Another strategy for inhibiting the NFκB pathway is gene silencing using an adenoviral vector-mediated NFκBp65-specific siRNA (Chen, Lin et al. 2008). This results in inhibition of NFκB activation and reduction of NFκB p65 in knee cartilage and synovium, in the ACLT rat model of OA. It also reduced levels of IL-1 β and TNFα in synovial fluid and correspondingly ameliorated synovial inflammation and cartilage damage in early stage OA This is consistent with previous *in vitro* work demonstrating that NFκBp65-specific siRNA significantly reduced IL-1β and TNFα-induced expression of COX-2, NOS-2 and MMP-9 in cultured rat chondrocytes (Lianxu, Hongti et al. 2006). However, in terms of clinical translation, the feasibility of blocking key physiological pathways such as NFκB without significant side-effects remains to be determined.

Given the multitude of bioactive molecules, the other key step will be determination as to at what stage these molecules should be incorporated into the OA regenerative process. It is not clear if pre-treatment of cells or local delivery with the cells or incorporation onto the scaffold will prove most effective. If these factors are to be combined with a scaffold, careful

consideration must be given to how this can best be achieved, for example by the addition of a reservoir. Further research is still required to clarify these issues.

1.10 Conclusions

OA is the most common form of arthritis worldwide, becoming ever more prevalent with the ageing population. New treatment modalities are therefore much in demand and over recent years the role of tissue engineering in OA has received considerable interest. One of the limiting factors in using tissue engineering strategies to repair arthritic joints is the presence of co-existent inflammation as it is now apparent that the same inflammatory cytokines that are key to the development and progression of RA, namely IL-1, TNFα and IL-6, occupy pivotal roles in the pathogenesis of OA. Therefore, in devising future treatments for OA, it is important to consider how this inflammation can best be inhibited and the implications for not only cartilage but also bone cell function and activity.

For tissue engineering strategies, the key factors to promote bone and cartilage repair are the imbalance between anabolic and catabolic processes and joint inflammation. This can be achieved either by pre-treating the cells with growth factors such as BMP-2, or with inhibitors of catabolic factors such as MMP-13, Dkk-1 or the inflammatory cytokines, prior to seeding them onto the scaffold and implanting them. Alternatively, the scaffold itself can be combined (encapsulation) with growth factors or inhibitors of inflammatory cytokines then seeded with cells ready for implantation. Finally, engineering a scaffold from a material that is pro or anti-inflammatory itself, depending on the patient and the nature of the defect, offers exciting new opportunities, allowing the beneficial effects of inflammation to be harnessed whilst minimising the unwanted effects. To date, the most effective of these strategies appears to be targeting IL-1 together with TNFα. Newer strategies under development include targeting BMP-2, IGF-1, TGFβ, OPG or EphB2.

1.11 Summary

Tissue repair using regenerative medicine strategies represents an exciting new adjunctive treatment to existing medical and surgical treatments for OA. As yet, translation of regenerative medicine techniques into clinical practice remains a distant and desired objective and these techniques have only been used on isolated osteochondral defects in otherwise normal joints. The challenge now is to build upon our rapidly expanding knowledge of the OA niche and use this understanding to modify these techniques with the

aim of intervening earlier on in the disease process, delaying or indeed eventually perhaps even avoiding the need for joint replacement surgery in such patients.

1.12 Current work

This project focuses on characterisation of the inflammatory component of the OA niche and how improved understanding of this, through *in vitro* and *in vivo* studies, will impact on the use of tissue engineering strategies for the future treatment of OA. This series of experiments was designed to build on work being done in the Bone and Joint Group at Southampton University to devise effective tissue engineering strategies for repairing bone defects and determine how these might be applied not just to patients with fracture non-union but also to patients with osteochondral defects due to OA. More specifically, this work will investigate how inflammation present within an osteoarthritic joint modulates these reparative strategies and therefore how they might need adjusting in order to be effective for patients with OA. This will be achieved, firstly by *in vitro* work assessing how the inflammatory cytokines IL-1β, IL-6 and TNFα identified in the preceding literature review as being the main cytokines involved in the pathogenesis of OA, affect the growth and osteogenic differentiation of SSCs. SSCs are one of the key cell types currently being used in tissue engineering strategies. Osteogenic differentiation was chosen as the main outcome measure for this study given the local expertise available in using tissue engineering strategies to produce healing of bone defects.

Once the baseline effects of individual cytokines on the osteogenic differentiation of SSCs have been established, the next step will be to assess the effects of the combination of cytokines present within OA synovial supernatants. This will mimic more closely the inflammatory conditions present within an OA joint. From there, the next objective will be to examine how these individual cytokines, and the combinations present in synovial supernatants, influence the growth and osteogenic differentiation of some of the newer cell types being used in bone tissue engineering, namely embryonic stem cells and induced pluripotent stem cells. Once the effects in *in vitro* cultures have been established, an *ex vivo* model, the organotypic culture system of embryonic chick femora, will be used to assess the effects of individual cytokines and of the synovial supernatants, in a three-dimensional system. Finally, the results from both the *in vitro* and *ex vivo* work will be used to set up an *in vivo* experiment, using the murine subcutaneous implant model of bone development. The latter will come closest to assessing how the individual cytokines and synovial supernatants

might modulate osteogenic differentiation in an OA patient. The overall results will be used to provide information on how the individual cytokines or synovial supernatants affect osteogenic differentiation of SSCs under a variety of culture conditions, with the aim of using this information to advise how future tissue engineering strategies for repairing bone defects, including those seen in OA, should be adapted to overcome these effects of inflammation.

1.13 Overall aim

- To investigate whether the inflammatory cytokines present in OA produce a hostile environment that limits the use of bioengineering strategies to repair damaged joints.

1.14 Objectives

- To determine the effects of inflammatory cytokines present in patients with OA on the normal growth of precursor cells into bone and cartilage and the effect on these processes when such cytokines are inhibited.
- To investigate the effects of inflammatory cytokines from samples of synovial tissue from patients with OA on bioengineering strategies currently under development for the replacement of arthritic joints.
- To examine potential ways of overcoming the perceived detrimental effects of these cytokines on the integrity of such cartilage and bone constructs.

Chapter 2 Materials and Methods

All of the paperwork for the initial design and set up of the study protocol was drafted by me, with oversight from my supervisors. I submitted the original Ethics Application, attended the Local Ethics Committee meeting and answered their queries, submitting revised drafts of the documents where requested, in order to obtain final approval for the study (Appendices A1, A3, A4). I also submitted all the paperwork required for approval by the local Research and Development team and obtained sponsorship from the University of Southampton (Appendix A2). I drafted and defended several Fellowship applications for funding to carry out this research and ultimately was successful in obtaining funding for my doctoral studies in the form of a Cawley Fellowship from the Southampton Rheumatology Trust. Each of the patients recruited to the synovial supernatant part of the study were approached and assessed personally by me and I liaised with the surgical teams to collect their synovial samples on the day of surgery.

Each of the techniques described in this section were taught to me by the technicians and postdocs from within the Bone and Joint Group at the University of Southampton, with the exception of the generation of synovial supernatants, which was taught to me by technicians at the Kennedy Institute. All were selected to demonstrate the chosen outcome of osteogenic differentiation of MSCs in both *in vitro, ex vivo* and *in vivo* experiments and have been previously validated by the group for this purpose. Each of these techniques were practised by me during my pilot work prior to starting my doctoral studies and were subsequently performed by me, with occasional help from the technicians when the volume of overlapping experiments required it. The only exceptions to this were the generation of embryonic stem cells and induced pluripotent stem cells for use in the experiments described in Chapter 5, where I required the specific expertise of my colleagues, as attributed in these sections. Finally, the implantation of the diffusion chambers into the mice for the *in vivo* experiments described in Chapter 6 required an animal license and was therefore carried out by the lab manager Dr Kanczler.

2.1 Tissue culture

2.1.1 Human bone marrow preparation and stromal cell culture

(Tare, Mitchell et al. 2012)

Human bone marrow samples were obtained intra-operatively from patients undergoing primary total hip arthroplasty, with appropriate ethical approval from the Southampton and South West Hampshire Local Research Ethics Committee (LREC-194-99). The samples were taken during reaming of the femur prior to insertion of the femoral component of the prosthesis and placed in a sterile universal. The samples were labelled with patient gender and age and then collected from the operating theatre for processing in the laboratory. Under sterile conditions within a sterile extraction hood (Nuare biological safety cabinet), the bone marrow sample was transferred from the universal tube to a 50ml Falcon tube (Greiner Bio-One, UK). The bone marrow sample was then suspended in 10mLs of alpha modified Eagle's medium (α-MEM – Gibco, UK) and 1% penicillin/streptomycin (P/S – diluted from 100x concentrate, PAA, UK) and shaken vigorously. The bone marrow suspension was then pipetted into a new Falcon tube. The remaining bone marrow sample was then re-suspended in a further 10mls α-MEM+P/S and transferred to the new Falcon tube a further three times. The cells were then pelleted by centrifugation at 1100rpm (250g) for 4 minutes at room temperature. The supernatant was carefully removed and the cell pellet re-suspended in 10mls α-MEM+P/S, using the mechanical drawing action of the pipette to break up the cell pellet. The cell suspension was then pipetted through a cell strainer (0.70µm pore size, Millipore, Ireland) into another Falcon tube. 100µl of the cell suspension was then pipetted into a universal tube and 100µl of 0.1% acetic acid (Sigma-Aldrich, UK) added to disrupt the red blood cells. 30µl of this acetic acid/cell suspension mix was then pipetted into the chamber of a haemocytometer (FastRead 102 Disposable Counting Slide, Immune Systems Ltd, UK) and a cell count performed. The cell count involved placing the haemocytometer under the high power objective of a light microscope and counting the number of cells seen over sixteen small squares, in 3 separate areas chosen at random. A mean cell count was then obtained, multiplied by 10^4 to give the number of cells per ml of cell suspension, then multiplied by ten to give the total number of cells in the 10mls cell suspension. The required cell number was then transferred to cell culture flasks adding additional media as required (5mls in total for a T25 flask (IWAKI SciTech, Japan), 10mls for a T80 flask and 25mls for a T150 flask (SPL LifeSciences, UK). The cells were then transferred to an incubator at 37°C, supplemented with 5%CO_2 for 6 days. On Day 6, the

waste media was washed over the bottom of the flask to mobilise any non-adherent cells or particles, then removed with a pipette. An identical volume of filter-sterilised phosphate buffered saline without calcium or magnesium (PBS) (PAA, UK) was then pipetted into the flask, washed over the adherent cells using a gentle rocking motion and then pipetted out. New media was then added to the culture flasks at the volumes stipulated above and the cells returned to the incubator at 37°C, supplemented with 5%CO_2.

2.1.2 Culture media

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

One unit of α-MEM powder was reconstituted with 1litre of distilled water, together with 2.2g of sodium bicarbonate (Fisher Scientific, UK) and placed on an automatic stirrer for 1 hour to ensure the powder was fully dissolved. The solution was then filter sterilised into an autoclaved glass bottle using a Nalgene filter lid (Fisher Scientific, UK). Standard or basal culture media was α-MEM supplemented with 10% Fetal Calf Serum (Gibco, UK) and 1% penicillin/streptomycin. To produce osteogenic media, 1µM dexamethasone (Sigma, UK) and 100µM of L-ascorbic acid-2-phosphate (Sigma-Aldrich, UK) was added to this basal media. The osteogenic media was made up fresh prior to each use and wrapped in foil to reduce degradation by direct exposure to light.

2.1.3 Media changes

(Tare, Mitchell et al. 2012)

During cell culture experiments, media changes were performed every 2-3 days to replenish the necessary nutrients required for optimal cell growth and differentiation. For cell culture flasks, this involved gently washing the waste media over the bottom of the flask to mobilise any non-adherent cells or particles, then pipetting off the waste media. Filter-sterilised PBS was then pipetted into the flask (5mls for a T25 flask, 10mls for a T80 flask and 25mls for a T150 flask), washed over the adherent cells by a gentle rocking motion, then pipetted out. An identical volume of new culture medium was then added to the flask and it was then returned to the incubator at 37°C, supplemented with 5%CO_2.

For well plates, the lid and one end of the well plate were lifted so that the lid was horizontal but the plate was diagonal. The pipette tip was placed in the bottom corner of the well plate and the media removed, taking care not to disturb the cell monolayer. The plate was then returned to the horizontal and new media pipetted into the well (2mls per well of a

6 well plate; 1ml per well of a 24 well plate). The plates were returned to the incubator at 37°C, supplemented with 5%CO_2.

2.1.4 Cell passaging

(Tare, Mitchell et al. 2012)

To split or 'passage' cells either for re-seeding to increase cell numbers or to set up an experiment with a specified cell density, firstly the cells were removed from the surface of the tissue culture flasks or plates. This was achieved by washing the cells twice with filter-sterilised PBS to remove the previous culture medium. If cells were very confluent or a large amount of extracellular matrix was present, cells were treated first with collagenase (collagenase type IV, Sigma, UK). This involved diluting 100µl of collagenase in 10mls of plain α-MEM, then adding this to the cells according to the size of the flasks (T75 2mls, T150 5mls) and placing them in an incubator at 37°C, supplemented with 5%CO_2. After 20 minutes the cells were rinsed with α-MEM and the excess placed in a Falcon tube. Then 10x trypsin/EDTA (Lonza, UK) was then added to the culture flask (1.5ml per T80 flask, 0.5ml for T25 flask), swirled gently across the bottom of the flask before placing it back in the incubator for 5 minutes. The flask was then removed from the incubator, tapped gently to loosen the cells from the bottom of the flask, checking under the microscope that loosening had occurred. 5-20mls of medium (depending on the flask size) containing FCS to neutralise the enzymes was then pipetted down the side of the flask nearest the cells, swished around and pipetted into another labelled universal, before centrifuging the cells at 1100rpm for 4 minutes. The supernatant was removed and the cell pellet re-suspended in 5-20mls of media (depending on the flask size), then transferred to the new flask(s) or plate(s).

2.1.5 Preparation of synovial cell supernatants

(Protocol courtesy of Prof Brennan, Kennedy Institute of Rheumatology, University of Oxford published as (Brennan, Chantry et al. 1989)

100mg of collagenase A (5mg/ml)(Roche, UK) and 3mg of DNase B (0.15mg/ml)(Sigma-Aldrich, UK) were added to 20mls of prepared media (RPMI 1640 +L-glutamine+HEPES, PAA, UK, with the addition of 1% penicillin/streptomycin) in 2 separate sterile 50ml Falcon tubes. The tubes were inverted several times until no crystals remained then vortexed. The enzyme preparation was then sterile filtered using a 10ml syringe and 0.45µm syringe filter (Millipore, Ireland) into a new 50ml Falcon tube and set aside. A small amount of prepared media was then poured into the base of a medium-sized Petri dish (Sterilin Ltd, UK). The synovium was added to the Petri dish from the sterile universal in which it had been

collected from theatre and, using sterile scissors and forceps, the tissue was cut into small pieces, dissecting off any unwanted tissue (subcutaneous fat/capsule etc). The dissected synovium was then added to the tube containing the enzyme suspension and mixed thoroughly by vortexing. The tissue was then incubated for 1-1.5hours in a water bath, pre-heated to 37°C, until all the clumps of tissue had dispersed. The Falcon tube containing the cell/enzyme suspension was then filled with ice-cold media to stop the digestion reaction and sieved through a sterilised beaker previously covered with 170µm material and autoclaved, allowing the digested synovium to fall through. The cell suspension was then transferred from the beaker into a 50ml Falcon tube and centrifuged at 1000rpm for 10 minutes. The supernatant was discarded and the cells re-suspended in 50ml prepared media before centrifuging again for 10minutes at 1000rpm. This wash step was repeated a further 3 times, with a cell count performed after wash 1 and 3. The synovial cells were then re-suspended in sufficient complete media (RPMI 1640+L-glutamine+HEPES with the addition of 1% penicillin/streptomycin and 10% FCS) to yield a concentration of $1x10^6$ cells/ml. Cells were cultured at $1x10^6$ cells/ml with 1ml of cell suspension per well of a 24 well plate, in an incubator at 37°C supplemented with 5%CO_2. After 48 hours, the supernatants were obtained by covering the plate with Parafilm and centrifuging at 1500rpm for 5 minutes. The supernatant from each well was pipetted off, aliquoted into individual sterile 1.5ml eppendorfs and stored at -80°C.

2.1.6 Setting up organotypic cultures of embryonic chick femurs

(Protocol devised by Southampton group, published as (Smith, Kanczler et al. 2012)

Day 1 fertilised chick eggs were obtained from P.D. Hook Hatcheries (Oxford, UK) and incubate at 38°C in an automatic Hatchmaster A incubator for 11 (E11) days. After this incubation time, embryos were removed from within the eggs and sacrificed by decapitation. Femurs were dissected out of each embryo, soft tissue and muscle removed, and washed in sterile PBS. Control non-cultured femurs were placed onto Millicell inserts (0.4µm pore size, 30mm diameter, Millipore, UK) (two femurs paired from the same chick per insert) and placed into individual wells of 6 well tissue culture plates containing 1ml of media per well, at the liquid/gas interface. Culture media was basal (α-MEM containing 100 units penicillin, 100µg/ml streptomycin and 100µM L-ascorbic acid 2-phosphate), alone or supplemented with IL-1 (10ng/ml), IL-6 (100ng/ml) or TNFα(10ng/ml) (all from Sigma-Aldrich, UK) or aliquots of human synovial supernatants (prepared as described in Section 2.1.5 above). Twelve femurs were cultured per condition at 37°C, supplemented with 5%CO_2 in air, for 10

days, with media changed every 24 hours. After 10 day culture, selected non-cultured and cultured femurs (n=4 for each condition) were used to assess glucosaminoglycan (GAG) content. The remainder were fixed in 4% w/v paraformaldehyde in PBS (PFA, Sigma-Aldrich, UK) for at least 24 hours then imaged radiographically using a Faxitron® Specimen Radiography System (MX-20) (Qados Ltd, Sandhurst, UK) and their lengths measured. Quantitative 3D analysis was then performed using a X-tek BenchTop 160Xi CT scanning system for micro-computed tomography (X-TEK Systems Ltd, Tring, Hertfordshire, UK) (n=6 for each condition). Following Faxitron and microCT analysis, the femurs were then dehydrated through a series of ethanols (50%, 90%, 100%-1, 100%-2), cleared in chloroform and embedded in paraffin wax using an automated Shandon Citadel 2000. 6µm sections were cut from each femur for staining with Alcian blue/Sirius Red or von Kossa to assess proteoglycan and collagen production or mineralisation respectively. The remaining femurs were immunohistochemically stained for type 1 and type 2 collagen to assess for bone and cartilage formation respectively, STRO-1as a skeletal stem cell marker and finally for the proliferation marker proliferating cell nuclear antigen (PCNA).

2.1.7 The subcutaneous implant *in vivo* mouse model of bone formation

(Bolland, Kanczler et al. 2008, Black C.R.; Goriainov V 2015)

Calcium phosphate scaffolds with and without selected MSC populations were generated and implanted sub-cutaneously in severely compromised immunodeficient (SCID) mice for 4 and 12 weeks (Project licence to Prof Oreffo). Male MF-1 *nu/nu* immunodeficient mice (Harlan, Loughborough, UK) were acclimatised for at least a week prior to the start of the experiment. All animals had *ad libitum* access to standard mouse chow at water at all times. All procedures carried out were done with prior ethical approval and in accordance with the regulations laid down in the Animals (Scientific Procedures) Act 1986, UK. Mice were anaesthetised via the intraperitoneal route using fentanyl-fluanisone (Hypnorm) (Jansson-Cilag Ltd) and midazolam (Hypnovel) (Roche) in sterile water in a ratio of 1:1 and a dose of 10ml/kg. Perforated capsules containing either scaffold alone (n=3), scaffold plus HBMC (n=3) or scaffold+HBMC+chosen cytokine (IL-1β, IL-6 or TNFα) or cytokine inhibitor (n=3 for each condition). Vybrant® (Invitrogen, UK) - labelled cells were used to ensure that the implanted cells could be detected following *in vivo* study and to prove that new tissue formation was a result of implanted cells rather than host recruited cells. At the end of the 4 or 12 week period, mice were euthanased and the subcutaneous implants removed. Thereafter, skeletal tissue formation was assessed using a combination of histological

staining (A/S, von Kossa), immunofluorescence for type 1 and type 2 collagen and microCT, as previously published (Bolland, Kanczler et al. 2008).

2.2 Cell proliferation assay- WST-1

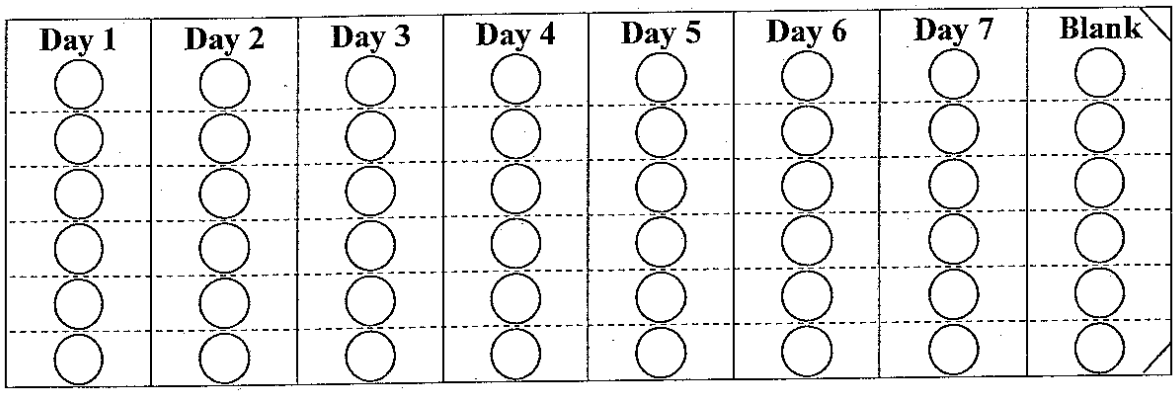
2.2.1 Rationale for using the assay

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

WST-1 (4-{3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate) (Roche, UK) was used to quantify cell proliferation and viability as an outcome measure in some of the experiments outlined above. This was a colorimetric assay based on the cleavage of a tetrazolium salt (WST-1) by mitochondrial dehydrogenases in metabolically active cells, to yield formazan, a dark red dye. The presence of this dye was then detected using an ELISA plate reader.

2.2.2 WST-1 assay

Passage 0 (P0) or passage 1 (P1) cells were obtained from adult human bone marrow samples as described earlier and plated out across a 48 well plate at a seeding density of $1x10^3$ cells per well and labelled as shown below;



The cells were then placed in an incubator at 37°C, supplemented with CO_2. After 24 hours, a 1:10 dilution of WST-1 in basal culture medium was prepared (1 0.5ml aliquot in 4.5ml of media). All the media was then removed from the Day 1 and Blank labelled wells and 400µl of WST-1 solution was pipetted into each well. The plate was then returned to the incubator for 4 hours. At the end of the incubation period, the plate was removed from the incubator, wrapped in blue roll and transferred to the reader tray of an ELX-800 Universal Microplate Reader (spectrophotometer) (Bio-Tek Instruments Inc.). The plate was then read at

λ=450nm, using KC4 3.4 Rev 21 software. Data was saved for future analysis and the well plate returned to tissue culture, the WST-1 solution in the Day 1 and Blank wells replaced with basal media and the plate returned to the incubator. The same process was repeated every 24 hours for a total of 7 days.

2.3 Histological analysis

2.3.1 Cell viability staining

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

Cell viability was assessed using live/dead staining with cell tracker green (CTG (CMFDA (5-chloromethylfluorescein diacetate) (Invitrogen, UK) and ethidium homodimer (EH-1) (Invitrogen, UK). Using this method, viable cells stained green when viewed under a fluorescence microscope, whilst non-viable cells stained orange.

Preparation of reagents

1 vial of CTG (50µg per aliquot, final working concentration 10µg/ml; Ex_{λ} 492nm, Em_{λ} 517nm) and 1 aliquot of EH-1 (1mg/ml stock solution, diluted to final working concentration 5µg/ml and stored in 25µl aliquots; Ex_{λ} 568nm, Em_{λ} 580-620nm) were removed from the -20°C freezer and allowed to thaw. 10µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) was added to the vial of CTG to dissolve it, then the CTG solution and contents of the EH-1 vial were added to 5mlsof tissue culture medium and mixed gently. To label the cells, 200µl of the CTG/EH-1/culture medium was added to each well of a 24 well plate, before placing the plate in an incubator at 37°C, supplemented with 5%CO_2, for 1-1.5 hours. At the end of this period the plate was removed from the incubator, the labelling solution pipetted off and replaced with fresh tissue culture media. The plate was the placed back in the incubator for a further 45 minutes. The media was then pipetted off and the cells washed with 1xPBS, then fixed in ice cold 95% ethanol for 5 minutes, before washing again with 1xPBS. The plate was covered with foil and stored at 4°C until images could be taken using the fluorescence microscope.

2.3.2 Alkaline phosphatase staining

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

Ethanol fixing of cells for histology

Cells were rinsed twice in cold PBS using a volume appropriate to the size of the culture well (6 well plate, 2mls per well; 24 well plate 1ml per well). A sufficient volume of cold 95%

ethanol (Diluted analytical grade reagent absolute ethanol, Fisher Scientific, UK) was then added to cover the base of each well completely and left in contact with the cells for 10 minutes, resulting in fixation of the cells. The cells were rinsed a further two times with cold PBS and left to air dry.

Preparation of reagents

ALP stain was prepared by adding 400µl of Napthol AS-MX Phosphate Alkaline Solution (Sigma-Aldrich,UK) to 9.6mls of distilled water. 2.4mg of Fast Violet B salts (Sigma-Aldrich, UK) were then added to the Napthol solution just prior to use.

ALP staining

Staining solution was added to the cells (600µl per well for a 6 well plate; 300µl per well for a 24 well plate) after fixation in ethanol as described above. The plate was wrapped immediately in blue towel roll and placed in an incubator at 37°C, supplemented with 5%CO_2, until a prominent purple stain was seen within the wells (usually 30-60 minutes). The reaction was stopped by rinsing the wells with distilled water. The plate was allowed to air dry and stored in the fridge until the images could be photographed under the light microscope.

2.3.3 Alcian Blue/Sirius red staining

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

Slides were warmed at room temperature for 30 minutes. The slides were de-waxed and dehydrated by passing them through two sets of Histoclear (Fisher Scientific, UK) for 7 minutes each, then through descending concentrations of methanol (100%-1, 100%-2, 90%, 50%) for 2 minutes each. Haematoxylin (Weigert's Haematoxylin A and B solution) was then applied to the slides using a pastette and left on for 10 minutes. The slides were rinsed in a water bath for 10 minutes then dipped 3 times in acid/alcohol (20ml hydrochloric acid + 2 l 50% methanol) before rinsing again for 5 minutes. 0.5% Alcian blue 8GX (to stain cartilage matrix and proteoglycans) was then applied to the slides and left on for 10 minutes. The slides were rinsed in a water bath for 1 minute, stained with molybdophosphoric acid for 10 minutes and then rinsed in a water bath for 1 minute and drained. 1% Sirius red F3B (to stain the collagen in the matrix) was then added to the slides for 1 hour then the sections were rinsed in a water bath for 1 minute and drained. The sections were dehydrated by passing them through increasing concentrations of methanol (50%, 90%, 100%-1, 100%-2) for 30 seconds each, followed by two sets of Histoclear for 30 seconds each. The slides

were mounted in DPX mountant (Bios Europe, UK) in the fume cupboard and left to dry overnight before imaging.

2.3.4 Von Kossa staining

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

Slides were de-waxed and dehydrated by passing them through two sets of Histoclear for 7 minutes each, then through descending concentrations of methanol (100%-1, 100%-2, 90%, 50%) for 2 minutes each. 1ml of silver nitrate was added to each slide using a pastette and the slides were placed under UV light for 20 minutes. The slides were washed thoroughly in running water for 5 minutes and arranged on rods over a water bath. Sodium thiosulphate was then added to the slides and left on for 8 minutes before rinsing the slides in running water for 5 minutes. The slides were counterstained with Alcian Blue for 1 minute, rinsed with water and then counterstained with van Gieson stain. After 5 minutes the sections were then blotted on paper towels to remove excess stain and dehydrated by passing them through increasing concentrations of methanol (50%, 90%, 100%-1, 100%-2) for 30 seconds each, cleared in two sets of Histoclear for 30 seconds each. The slides were then mounted in DPX in the fume cupboard and left to dry overnight before imaging.

2.3.5 Immunostaining for collagen I and II

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

Cells were fixed with ethanol as described above then rinsed with fresh PBS at room temperature. The samples were then blocked with 1% bovine serum albumin (BSA Fraction V, pH 7) (PAA, UK) in PBS for 45 minutes at room temperature (600µl per well for a 6 well plate; 300µl per well for a 24 well plate). The blocking solution was removed and the wells rinsed with 1% BSA in PBS. Wells were labelled for primary antibody, isotype control and negative control. The primary antibody was diluted in 1%BSA in PBS and placed on the samples to be stained at the volumes shown above. For type 1 collagen staining the primary antibody was polyclonal rabbit type 1 collagen (gift from Larry Fisher, NIH, Maryland, USA), prepared at a 1:300 dilution. For type 2 collagen staining, the primary antibody was polyclonal rabbit type 2 collagen (Merck Millipore, UK), prepared at a 1:500 dilution. The samples were incubated at room temperature for 2 hours, or alternatively overnight in the cold room at 4°C on a rocking bed mixer. The samples were rinsed in distilled water then washed twice in PBS, for 5 minutes each time. The PBS was removed and the fluorescent secondary antibody (anti-rabbit IgG Alexa Fluor 594)(Invitrogen, UK), diluted 1:100 in

1%BSA in PBS at an identical volume to that used for the primary antibody, added in a darkened area. The plate was wrapped in foil and incubated at room temperature for 1 hour. The wells were rinsed with PBS, then washed with PBS for 5 minutes. The PBS was then removed and DAPI solution added, diluted 1:100 in PBS, at the same volumes as for the antibodies above, for 5 minutes. The DAPI solution was then removed and the cells rinsed 3 times with PBS. Finally, the cells were covered with PBS, wrapped in Parafilm (Bemis, USA) and visualised under the fluorescence microscope using a TRITC filter. Images were taken from this microscope with an AxioCam HR using AxioVision Version 4.1 software.

2.4 Biochemical analysis

2.4.1 Alkaline phosphatase assay

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

Sample preparation for biochemistry assays

Samples were prepared for biochemistry assays by rinsing them twice in cold, non-sterile PBS (6 well plate- 2mls per well, T25- 5mls per well, T80- 10mls per flask, T150-25mls per flask), followed by fixing them in cold 95% ethanol for 10 minutes. The cells were then rinsed twice more with PBS before being left to air dry. 0.05% Triton X (diluted from 100x concentrate) (Sigma-Aldrich) was added to the cells (600µl per well for a 6 well plate, 250µl per well for a 24 well plate), using a cell scraper (Greiner Bio-One GmbH, Germany) to remove cells from the surface of the plate. The plate was then sealed and placed in a -20°C freezer for 30 minutes or overnight. The plate was thawed in the incubator at 37°C, supplemented with 5%CO_2, for 30 minutes. This freeze/thaw process was repeated 3 times in total and the samples were ready for use.

Preparation of reagents

Assay buffer was made using 10mls 2-AMP alkaline buffer solution (1.5M) (Sigma-Aldrich, UK), 20mls distilled water and 60µl Igepal CA-630 (Sigma-Aldrich, UK). ALP substrate was prepared with 60mg p-nitrophenol phosphate (Sigma-Aldrich, UK), 15ml 2-AMP alkaline buffer solution and 30mls distilled water. These reagents were used to produce a set of ALP standards to use in the assays as shown overleaf. Before starting a set of experiments fresh ALP standards were made to minimise variability. These were then stored at 4°C.

ALP standard (p-nitrophenol) (µl)	Assay buffer (mls)	nmols/ml
5	4.995	10
25	4.975	50
50	4.950	100
100	4.900	200

Table 2.1 Composition of ALP standards for biochemistry assay

ALP assay

10µl samples were placed in individual wells of a clear, flat-bottomed 96 well plate (Corning Inc, USA). Each sample was performed in triplicate. 100µl of each ALP standard was placed in individual wells and these were done in duplicates. For the control wells, 10µl of 0.05% Triton X was used. 90µl of ALP substrate was added to all samples and the blank well and the plate was placed in the incubator at 37°C, supplemented with 5%CO_2 until the samples developed a yellow colouration. The time required for this colour change to occur was recorded for use in later calculations. 100µl of 1M sodium hydroxide (VWR International Ltd, UK) was then added to all wells, including the standards, to stop the reaction. The colorimetric change was measured using an ELX 800 Universal microplate reader using KC4 3.4 Rev 21 software (λ=405nm) (Bio-Tek Instruments Inc.).

2.4.2 DNA assay

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

Samples were prepared for DNA biochemistry assays using the protocol described above.

Preparation of reagents

1xTris-EDTA (TE) was prepared by diluting 100xTE (Sigma-Aldrich, UK) with distilled water at a 1:100 ratio.

PicoGreen working solution was obtained by thawing PicoGreen dsDNA reagent (Invitrogen, UK) at room temperature and then diluting it in 1xTE at a 1:200 dilution. The tube containing the working solution had to be wrapped in foil to protect it from the light as it

was a fluorescent probe. The fluorimeter sensitivity setting of each PicoGreen batch was calibrated before use to reduce variation. DNA stock solution was prepared by diluting herring sperm DNA (BDH Lab Supplies, UK) in distilled water to obtain a concentration of 10mg/ml. DNA standards were then made using these reagents as shown below:

DNA stock (2µg/ml)	**1XTE**	**DNA concentration (ng/ml)**	**Final DNA concentration with PicoGreen (ng/ml)**
0µl	100µl	0	0
1	98	40	20
10	90	200	100
50	50	1000	500
75	25	1500	750
100	0	2000	1000

Table 2.2 Composition of DNA standards for biochemistry assay

DNA standards were made in sufficient quantities for each batch of experiments to reduce variability.

DNA assay

Samples previously prepared in 0.05% Triton X as described above, were loaded into individual wells of a black plastic, flat-bottomed 96 well plate (Corning Inc, USA). 10µl of each sample was placed in a well and each sample was performed in triplicate. 90µl of 1xTE was then loaded into each sample well. DNA standards at the concentrations shown above were also loaded into individual wells. These were loaded in duplicate. 100µl of PicoGreen working solution was added to all wells, including the standards. The assay plate was then transferred to a FLX Fluorimeter (Bio-Tek Instruments, Inc.), read at λ=485/430nm and the results recorded for later analysis.

2.5 Molecular analysis

2.5.1 Trizol method

(Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012)

2.5.1.1 RNA extraction

The flasks of cells were placed onto a tray of ice. All the culture media was tipped out of the flasks into Virkon and the cells were washed with PBS. All the PBS was removed from the flasks, first tipping then drying the flasks on tissue to remove any drips. 1ml of Trizol (Invitrogen, UK) was added to each flask and the flask was tipped from side to side to ensure that the whole surface covered with cells was coated sufficiently. A cell scraper was used to remove the cells from the surface of the flask and a 10ml pipette was used to wash cells to the bottom of the flask. The cells from each flask were added to individually labelled 1.5ml diethyl pyrocarbonate (DEPC) - treated eppendorfs (Greiner Bio-One, UK) and placed immediately in a -80°C freezer.

2.5.1.2 RNA isolation

RNA samples were allowed to thaw and incubated for 5 minutes at room temperature. 200µl of chloroform (VWR International, France) was added to each sample. The samples were vortexed to mix the reagents thoroughly, then incubated for a further 2-3minutes at room temperature and centrifuged at 12500rpm for 15 minutes at 4°C. At the end of centrifugation the samples had separated into 3 layers; the top aqueous layer containing the RNA was then pipetted off into a new 1.5ml DEPC treated eppendorf. 500µl of isopropanol (Sigma-Aldrich, UK) was added to each sample and the tubes were vortexed, incubated at room temperature for 10 minutes, then centrifuged at 12500rpm for 10 minutes at 4°C. The RNA was now visible as pellet on the hinge side of each tube. The supernatant was pipetted off and the RNA pellets washed with 1ml of 75% ethanol. The tubes were vortexed briefly to lift the pellet off the side of the tube, then centrifuged at 7500rpm for 5 minutes at 4°C. All the ethanol was removed using a fine 10µl pipette tip and each individual pellet dissolved in 30µl of warmed DPEC treated water. The concentration and purity of the RNA in each sample was determined by analysis of 1µl of each sample in a Nanodrop 1000 (ThermoScientific, UK), using Nanodrop 1000 Version 3 6.0 software. Samples were stored at -80°C.

2.5.1.3 RNA clean up

Using the Nanodrop readings obtained above, the volume of RNA sample required to yield 5µg of RNA was calculated. Samples were prepared as shown below:

.....µl	Volume of RNA sample containing 5µg of RNA
5µl	10xDNase 1 buffer
2.5µl	RNAase-free DNase (1U/µl) –used straight from freezer and added to the side of the tube
.....µl	Ultra-purified water to make up volume to 50µl
50µl	Final reaction volume

Table 2.3 Example of the composition of the reaction mixture for RNA clean up

The reagents were mixed well and incubated in a water bath pre-heated to 37°C for 20 minutes. 100µl of binding buffer was added to each sample, followed by 150µl of 95% ethanol and mixed well. This mixture was transferred to individual Zymo-Spin columns for each sample, placed within collection tubes and centrifuged at 12000rpm for 1 minute at 4°C. The flow through was discarded from each collection tube. 400µl of RNA Prep Buffer was then added to each column and centrifuged at 13000rpm for 1 minute at 4°C. The flow through was discarded. 800µl of RNA Wash Buffer was added to each column and centrifuged at 13000rpm for 30 seconds. The flow through was discarded. This wash step was repeated with a further 400µl of RNA Wash Buffer and the flow through was discarded. The spin columns were then centrifuged in the emptied collection tubes at 13000rpm for 2 minutes, removed from their collection tubes and transferred to RNase-free tubes. 10µl of ultra-purified water, pre-heated in a water bath at 65°C, was added directly onto each column, allowed to soak for 5 minutes and then centrifuged at 13000rpm for 45 seconds. The flow through (eluted RNA) was kept for each sample and a further 6µl of pre-heated ultra-purified water was added to the columns, left to soak for 5 minutes and then centrifuged at 13000rpm for 1 minute. The concentration and quantity of eluted RNA from each column was then measured using the Nanodrop as described above. The RNA was either used straight away for cDNA synthesis or stored at -80°C.

2.5.1.4 cDNA synthesis

The volume of clean RNA sample required to provide 2µg of clean RNA was calculated from the Nanodrop values obtained at the end of the RNA clean up as described above. RNA/primer mixtures were prepared for each RNA sample in 0.5ml DEPC treated eppendorfs according to the formula shown below. Each component was added in the order shown and the final mixture was then kept on ice.

..µl	Volume of DNA-free RNA sample required to yield 2µg clean RNA
1µl	10mM dNTP
1µl	Oligo(dT) (500µg/ml)
..µl	Volume of ultra-purified water required to give total volume per sample of 10µl
10µl	Total reaction volume per tube

Table 2.4 Example of RNA/primer mixture for cDNA synthesis

The samples were then incubated in a water bath at 65°C for 5 minutes and moved back onto ice for 1 minute. Another reaction mixture was prepared as shown below.

2µl	10x RT Buffer
4µl	25mM $MgCl_2$
2µl	0.1M DTT
1µl	RNase OUT Recombinant RNase Inhibitor
1µl	Reverse Transcriptase (Superscript 2)
10µl	Total reaction volume per tube

Table 2.5 Example of the reaction mixture for cDNA synthesis

10µl of the above reaction mixture was added to each RNA/primer mixture and pipetted up and down to mix the reagents. The samples were incubated in a water bath, pre-heated to 42°C, for 50 minutes. The reactions were then terminated by placing the tubes in a water bath pre-heated to 70°C, for 15 minutes. The samples were chilled on ice and stored at -20°C.

2.5.1.5 Real-time PCR

The desired primers for a PCR run were selected and placed on ice to thaw at least an hour in advance. All primers were used at a 5µM working concentration, obtained by diluting 10µl of 100µM primer stock with 190µl of ultra-purified water. MasterMix was prepared for each gene to be tested, according to the formulae shown below, where *n* was the number of samples to be tested:

$2_{\text{(running samples in duplicate)}} \times (n+1_{\text{(for negative control)}}) + 2_{\text{(for loss)}}$ = total number of reactions required

For each reaction:

25µl	2x Sybr green Master Mix
5µl	Forward primer 5µM
5µl	Reverse primer 5 µM
13µl	Ultra-purified water
48µl	Total reaction volume

Table 2.6 Example of the MasterMix components for rt-PCR

Samples and MasterMix were kept on ice throughout the preparation stage. For each gene of interest, 48µl of Master Mix was pipetted into individual wells of a 96 well PCR plate (Star Lab, UK), followed by 2µl of the relevant cDNA sample. Once the plate was completed it was sealed with DNA/RNA/RNase-free optical adhesive film (Applied Biosystems, UK) and placed in a rt-PCR analyser (7500 Real Time PCR System, Applied Biosystems, UK). The plate layout was entered using 7500 software version 2.0.5. A 2 hour PCR run was performed and standard curves recorded. Analysis was performed using the above software, giving CT values for each gene. Delta CT (dCT) values were then calculated by subtracting the CT for the housekeeping gene (β actin unless stated otherwise) from that of the gene of interest. ddCT values were then calculated by subtracting the lowest dCT value of the samples from all samples run in that same plate. Relative expression of a given gene was calculated using the following formula:

Relative expression = 2^(-ddCT)

Average and standard deviations were then calculated across all groups.

2.5.2 VILO method

(Cheung, Sposito et al. 2014)

2.5.2.1 RNA purification

350µl of RLT buffer per well of a 6 well plate, or 600µl per flask, was added, pipetted up and down and transferred to an RNase/DNAse free tube using a filter tip. The RNA was either stored at -80°C or processed immediately.

For RNA isolation, an equal volume of 70% ethanol to that of RLT buffer used was added to the homogenised lysate and mixed well by pipetting. Up to 700µl of sample was then added to the spin column, placed within a collection tube. The tubes were centrifuged at 10000rpm for 15 seconds. The flow through from the columns was then discarded. 700µl of Buffer RW1 was added to each spin column before centrifuging at 10000rpm for 15 seconds. The flow through from the columns was then discarded. 500µl of Buffer RPE was added to each of the spin columns and the tubes were centrifuged at 10000rpm for 2 minutes. Each spin column was placed in a new 2ml collection tube and the old collection tubes containing the flow through were discarded. The new tubes were centrifuged at 13000rpm for 1 minute. Each spin column was placed in a new 1.5ml collection tube and 30µl of RNase-free water added directly to the spin column membrane. The tubes were centrifuged at 10000rpm for 1 minute. The eluate from each column was added directly to the spin column membrane and the tubes centrifuged at 10000rpm for a further 1 minute to increase the concentration of RNA obtained. The concentrations of RNA in each sample were calculated using the Nanodrop as described above.

2.5.2.2 cDNA synthesis

The volume of RNA sample needed to obtain 500ng of RNA was calculated using the spreadsheet shown overleaf.

RNA Quantification	**M72 P1 + cytokines**
RNA Amount Required (µg)	0.5

Sample (10 µl)	**260/280**	**260/230**	**ng/µl**	**µg/µl**	**vol for 0.5 µg RNA**	**1:5 dilution**	**vol H_2O**
M72 P1 Basal	2.02	1.67	353.36	0.3534	1.4		5.6
M72 P1 BIL-1	2.02	1.56	446.19	0.4462	1.1		5.9
M72 P1 BIL-6	2.04	1.37	279.5	0.2795	1.8		5.2
M72 P1 BTNF	2.08	1.32	755.52	0.7555	0.7	3.5	3.5
M72 P1 Osteo	2.03	1.67	395.59	0.3956	1.3		5.7
M72 P1 OIL-1	2.06	2.00	524.12	0.5241	1.0		6.0
M72 P1 OIL-6	2.04	1.33	336.49	0.3365	1.5		5.5
M72 P1 OTNF	2.07	2.14	1039.6	1.0396	0.5	2.5	4.5

This volume was placed in an RNA/DNase-free tube and ultra-purified water added to give a total volume of 7µl in each tube. A master mix of reagents was made as shown for n+2 reactions, where *n* was the number of samples to be tested:

VILO Reaction Mix	2µl per sample
VILO 10xSuperscript	1µl per sample
Aliquot	3µl into each tube containing 7µl of diluted RNA
Total volume	10µl per tube

Table 2.7 Composition of the MasterMix used for cDNA synthesis

The master mix was pipetted up and down to mix the reagents and sample and briefly spun down using the Minispin (Technico Mini Microcentrifuge, FisherScientific, UK). The samples were placed in a thermocycler (Veriti 96 well Thermal Cycler; Applied Biosystems, UK) and run through using the VILO RT software program using a 10µl reaction volume. This program heated the samples to 25°C for 10 minutes, 42°C for 120 minutes, 85°C for 5 minutes, holding them at 4°C. At the end of the cycle, samples were diluted 1:5 with ultra-purified water (40µl of ultra-purified water added to each 10µl cDNA sample) to prepare the cDNA samples for use in a real-time polymerase chain reaction (rt-PCR).

2.5.2.3 Real-time PCR

This was performed according to the same protocol described above for the Trizol method.

2.6 ELISAs

Cytokine concentrations of each synovial supernatant obtained were obtained by ELISA using separate Quantikine® ELISA kits (R&D Systems, UK) for each cytokine as detailed below.

2.6.1 IL-1β ELISA

All standards and reagents were prepared as directed by the manufacturers. 200µl of standard, control or sample as indicated (in duplicate) was added to each well, covered with an adhesive strip and incubated at room temperature for 2 hours. Each well was aspirated and washed 3 times, 200µl of Conjugate added to each well, covered with a new adhesive strip and incubated for a further hour at room temperature. Each well was aspirated and washed 3 times and 200µl of Substrate Solution added to each well and incubated for 20 minutes at room temperature, taking care to protect the samples from the light. 50µl of Stop Solution was added to each well and the microplate was read at 450nm (with the wavelength correction set at 540 or 570nm), within 30 minutes.

2.6.2 IL-6 ELISA

All standards and reagents were prepared as directed by the manufacturers. 100µl of Assay Diluent RD1W was added to the required number of wells of the microplate provided.

100µl of standard, control or sample as indicated (in duplicate) was added to each well, covered with an adhesive strip and incubated at room temperature for 2 hours. Each well was aspirated and washed 4 times, 200µl of Conjugate added to each well, covered with a new adhesive strip and incubated for a further 2 hours at room temperature. Each well was aspirated and washed 4 times and 200µl of Substrate Solution added to each well and incubated for 20 minutes at room temperature, taking care to protect the samples from the light. 50µl of Stop Solution was added to each well and the microplate read at 450nm (with the wavelength correction set at 540 or 570nm), within 30 minutes.

2.6.3 TNFα ELISA

All standards and reagents were prepared as directed by the manufacturers. 50µl of Assay Diluent RD1F was added to the required number of wells of the microplate provided. 200µl of standard, control or sample as indicated (in duplicate) was added to each well, covered with an adhesive strip and incubated at room temperature for 2 hours. Each well was aspirated and washed 4 times, 200µl of Conjugate added to each well, covered with a new adhesive strip and incubated for a further hour at room temperature. Each well was aspirated and washed 4 times and 200µl of Substrate Solution added to each well and incubated for 20 minutes at room temperature, taking care to protect the samples from the light. 50µl of Stop Solution was added to each well and the microplate read at 450nm (with the wavelength correction set at 540 or 570nm), within 30 minutes.

2.7 Image capture and analysis

Images of samples in culture flasks or wells were captured using a Zeiss Axiovert 200 inverted microscope using Zeiss AxioVision software version 4.1. Light microscopy images were taken using an Axiocam HR camera and fluorescence images were captured using Axiocam MR. Images of slides were examined and captured using an Olympus BX-51/22 dotSlide digital virtual microscope and images created using OlyVIA 2.1 software (Olympus Soft Imaging Solutions, GmBH).

2.8 Data analysis plan

The study protocol was designed so that individual experiments were performed in triplicate, in keeping with accepted practice for cell culture experiments. Having reviewed the

published literature from both our group and others performing cell culture experiments with SSCs, it was decided to present the data from cell culture work using both digital camera and microscopy images to allow macro and microscopic views of cell cultures. Biochemical results were to be presented both visually, using cell culture and microscopy images, as well as graphically. For the graphical representation, it was decided to present both individual experimental results and summative (n=3) results, analysed as per the statistical methods section below. Similarly, for the PCR results, it was decided to present both individual experimental results and summative (n=3) results, analysed as per the statistical methods section below. ELISA results were likely to be fewer in number so it was decided to summarise them on individual graphs for each cytokine, with each individual supernatant represented by its own data point. Linear regression was to be used to assess for any association between cytokine concentration in the supernatant and ALP specific activity as a surrogate measure for osteogenic differentiation.

Demographic data was to be collected on all patients providing synovial supernatant samples for analysis, including age, sex, medication history, onset and duration of OA, ESR and CRP values, severity of OA (using Kellgren-Lawrence grading of radiographs). These variables were to be inputted into Stata to obtain summary statistics and perform a multivariate analysis, accepting that this may be limited by small patient numbers and that the findings would only be generalisable to the selected cohort of patients with OA severe enough to require joint replacement surgery.

2.9 Statistics

Statistical calculations within this thesis were performed using GraphPad Prism software version 7.06. Data were expressed as mean +/- standard deviation, followed by the number of experiments performed, usually n=3 unless specified otherwise. Comparisons for biochemistry and PCR data were carried out using one-way analysis of variance (ANOVA). If the ANOVA was significant, the Tukey-Kramer multiple comparison test was used as a post hoc test. For all analyses, a *p* value less than 0.05 was considered significant.

Chapter 3 The effects of cytokines on HBMSC cultures and immunoselected SSC cultures

3.1 Establishing baseline state

3.1.1 Introduction

One of the defining features of multipotent stem cells, as discussed earlier, is their ability to differentiate into multiple lineages. For skeletal stem cells (SSCs) this includes bone, cartilage, smooth muscle, fat, connective tissue and, more debatably, neural tissue, skeletal muscle and beta pancreatic cells (Figure 3.1).

The main focus of this work is on the effects of inflammatory cytokines on the osteogenic differentiation of human bone marrow stromal cells (HBMSCs) and immunoselected SSCs (Stro-1+ve cells). Osteogenic differentiation was selected as the outcome measure for the study as there was considerable local expertise in evaluating the osteogenic differentiation of both HBMSCs and SSCs within a variety of tissue engineering constructs being developed for the repair of bone defects. A number of different protocols have been developed to facilitate osteogenic differentiation of these cells. All of these include dexamethasone and ascorbic acid, but various other factors may also be included. A standardised protocol for osteogenic media has been developed locally, which includes the addition of both dexamethasone and ascorbic acid (Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012). Various other factors including BMP-2 and TGFβ have also been shown to potentiate osteogenic differentiation but were not routinely added to cultures in these experiments. Osteogenic differentiation has been confirmed with this protocol via demonstration of alkaline phosphatase (ALP) staining of the cultured cells (an early marker of osteogenic differentiation); Alizarin O and Sirius red (A/S) staining for mineralised osteoid (a marker of late stage osteogenic differentiation); ALP production on biochemical assays and up-regulation of ALP, collagen 1, osteocalcin and Runx-2 messenger RNA (mRNA) (osteogenic gene expression), in previously published studies (Tare, Kanczler et al. 2010, Tare, Mitchell et al. 2012). Fetal femur cell cultures were also included to assess whether the findings from HBMSCs and immunoselected SSCs in culture would be generalisable to other cell populations used locally for the repair of bone and cartilage defects. With this baseline data in place, these outcomes could then be used to evaluate the effects of the inflammatory cytokines IL-1β, IL-6 and TNFα, on the osteogenic differentiation of SSCs *in vitro.*

Strontium has been shown to have beneficial effects in slowing the progression of OA, both in terms of reducing pain, improving patient's mobility and reducing joint space narrowing (Cooper, Reginster et al. 2012). However, the mechanism of these effects is unclear. Tissue engineering strategies have been developed locally to augment impaction bone grafting in revision hip surgery and for the repair of bone defects (Bolland, Kanczler et al. 2008, Tare, Kanczler et al. 2010, Dawson JI 2014, Black C.R.; Goriainov V 2015). If these are able to be expanded to the repair of osteochondral defects such as those seen in OA, strontium could be a potentially useful bioactive factor to incorporate into such scaffolds. Within this work, a sub-study was therefore devised, aimed at establishing whether strontium had an effect on the osteogenic differentiation of cytokine modulated SSCs and thereby a potential role as a bioactive molecule for incorporation into future tissue engineering strategies for OA.

On completion of the experimental work in this chapter, a standardised experimental protocol for assessment of the effects of the inflammatory cytokines IL-1β, IL-6 and TNFα on basal and osteogenic differentiation of HBMSCs and immunoselected SSCs, including validated outcome measures was established. This was then used to inform the design of the synovial supernatant experiments that form the basis of the work described in Chapter 4. The data generated were also used to determine the optimal concentrations of the inflammatory cytokines IL-1β, IL-6 and TNFα for use in later *ex vivo* and *in vivo* studies (Chapters 6). This baseline *in vitro data* forms an important first step towards optimising existing bone tissue engineering strategies in development locally to take account of the effects of inflammation on SSC growth and differentiation, so that they can be used successfully in patients with OA in the future.

3.1.1.1 Overall aim

- To investigate whether the inflammatory cytokines present in OA produce a hostile environment that limits the use of bioengineering strategies to repair damaged joints.

3.1.1.2 Objectives

1. To demonstrate the differentiation of HBMSCs and immunoselected SSCs extracted from human bone marrow samples under basal and osteogenic conditions and define appropriate outcome measures.

2. To assess the effects of inflammatory cytokines known to be involved in osteoarthritis on the differentiation of HBMSCs and immunoselected SSCs under basal and osteogenic conditions using the same outcome measures.

3. To assess the effects of the addition of strontium to HBMSCs and immunoselected SSCs under basal and osteogenic conditions, with or without the addition of inflammatory cytokines, using the same outcome measures.

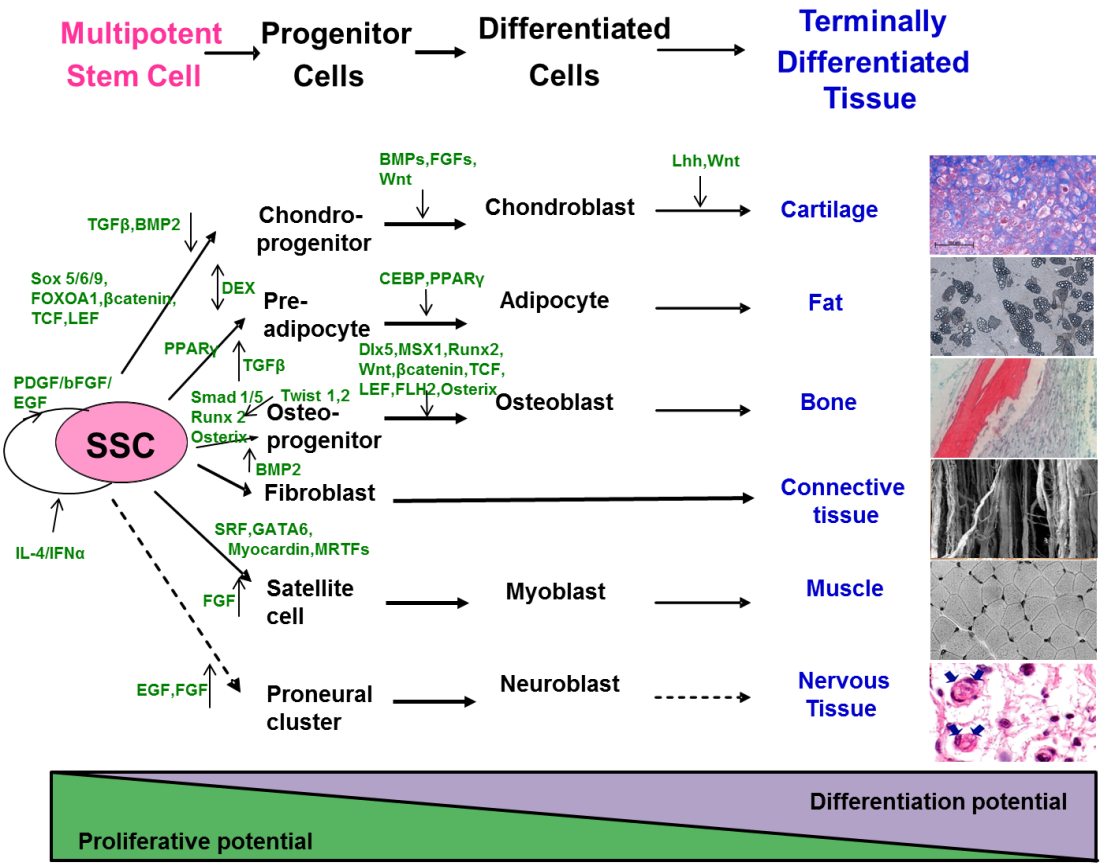


Figure 3.1 Stromal lineage differentiation from skeletal stem cells (SSCs)
- adapted from (Tare, Babister et al. 2008, Charbord, Livne et al. 2011)

3.1.2 Experimental set up

3.1.2.1 HBMSC cultures in basal and osteogenic media over 7 days

Cultures of human bone marrow stromal cells (HBMSCs) were set up following preparation of bone marrow samples according to the protocol described in Section 2.1.1. The cells were cultured initially in basal media for 6 days, washed with PBS and fresh media applied. Further media changes were carried out every 2-3 days (as detailed in Section 2.1.3) until the cells reached 95% confluence. The cells were then released from the surface of the tissue culture plastic by treatment with collagenase followed by trypsin (detailed in Section 2.1.4). The cell suspension obtained was centrifuged (1100rpm for 4 minutes), the cell pellet re-suspended in 10mls of basal media and a cell count performed using a haemocytometer (Section 2.1.1). Cells were seeded at $1x10^5$ cells per well of a 6 well plate in either basal or osteogenic media. Single wells were labelled for used in histological analysis (ALP, type I and type II collagen) and ALP and DNA biochemistry assays, whilst 3 wells in each media were reserved for PCR analysis due to the larger cell numbers required. Cultures were run for a total of 7 days prior to analysis.

3.1.2.2 Stro-1 positive SSC cultures in basal and osteogenic media over 7 days

A similar protocol was used for cultures with Stro-1 positive cells, seeding at $1x10^5$ cells per well of a 6 well plate, with duplicate wells for ALP staining and cell viability (CTG) staining, and triplicate wells for ALP and DNA biochemistry assays, in each of basal and osteogenic media. T75 flasks were seeded at $2.5x10^5$ cells for PCR in each of basal and osteogenic conditions respectively. All cultures were carried out over 7 days with media changes every 2-3 days as previously specified.

3.1.2.3 HBMSC cultures in basal and osteogenic media over 21 days

Temporal studies were undertaken to demonstrate the effects of different culture conditions on HBMSCs in basal or osteogenic media over 21 days. HBMSC were seeded at $1x10^4$ cells per well of a 12 well plate, with wells in triplicate for each of ALP staining, CTG staining, ALP biochemistry and DNA biochemistry, in each of basal and osteogenic media. A T75 flask was seeded at $5x10^5$ cells for each of the 3 different time points, in each of basal and osteogenic media, for use in PCR.

3.1.3 Results

3.1.3.1 Histology

HBMSCs were imaged using both a digital camera (Figure 3.2A) whilst in P0 cultures, in tissue culture flasks and by light microscopy using a phase setting (Figure 3.2B). Cells showed typical fibroblastic cell morphology and growth that was comparable with previous cultures carried out within our laboratory and reported in the literature. Excellent cell viability at the end of 7 and 21 day cultures was confirmed by CTG/EH-1 staining (Figure 3.2C) – viable cells shown in green, non-viable in orange, nuclei stained blue. All cultures in osteogenic media produced increased ALP (pink/purple) staining compared to basal cultures (Figure 3.2D), reflecting osteogenic differentiation.

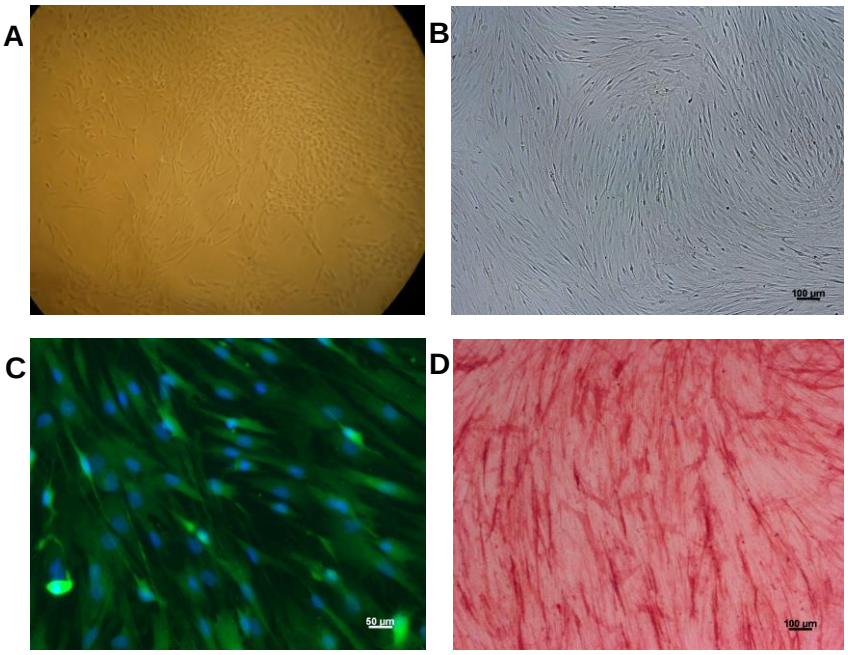


Figure 3.2 HBMSC images

A: HBMSCs in culture; **B:** Phase images of HBMSC at confluence (scale bar represents 100µm); **C:** Viability staining of HBMSC using cell tracker green (CTG) and ethidium homodimer (EH) (scale bar represents 50µm); **D:** Staining of HBMSC for alkaline phosphatase production (ALP) (scale bar re-presents 100µm).

3.1.3.2 Biochemistry

As expected, in both unselected HBMSC and Stro-1 positive cell cultures the use of osteogenic media (supplemented with ascorbate and dexamethasone) resulted in higher levels of ALP production than basal media (Figures 3.3 and 3.4). This confirmed that these media supplements were sufficient to produce osteogenic differentiation.

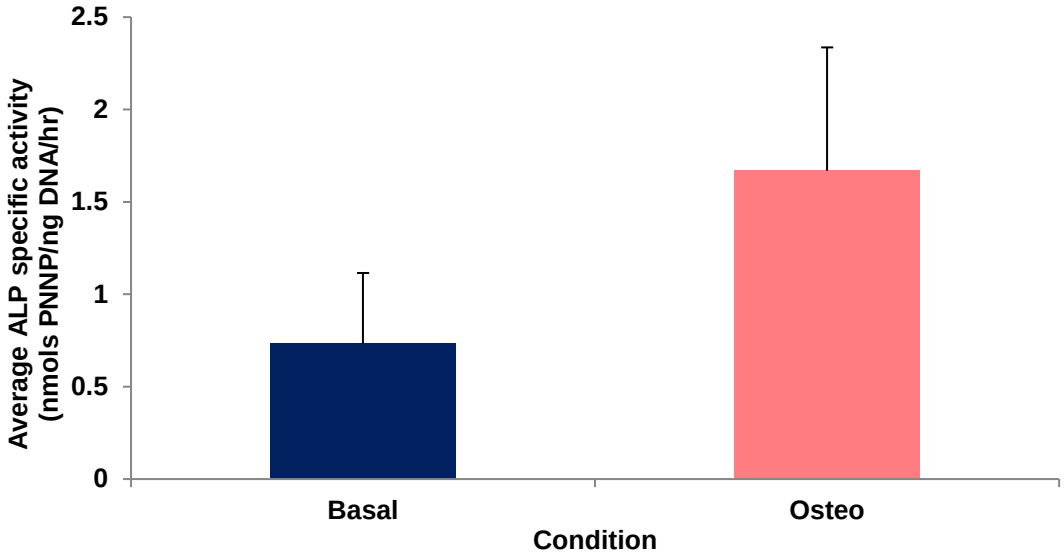


Figure 3.3 ALP specific activity of HBMSC at Day 7 (F47).
Data presented as mean ALP specific activity +/- SD for a single experiment performed in triplicate. Basal=Basal media alone, Osteo=Osteogenic media alone. Differences between the groups did not reach statistical significance.

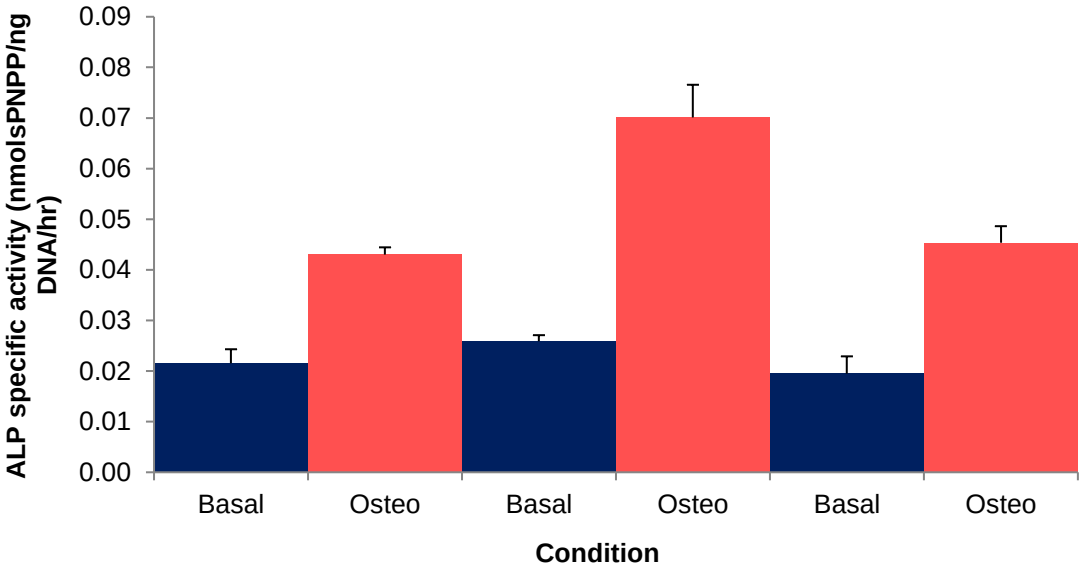


Figure 3.4 ALP specific activity of Stro-1 positive HBMSCs at Day 7.
Data presented as mean +/- SD for a single experiment performed in triplicate. Basal=Basal media alone, Osteo=Osteogenic media alone. Differences between the groups did not reach statistical significance.

Critically, this effect was maintained from early through to late phase differentiation, as shown by increasing levels of ALP production during the multiple time point experiment lasting 21 days described above (Section 3.2.1.3) the results of which are summarised in Figure 3.5.

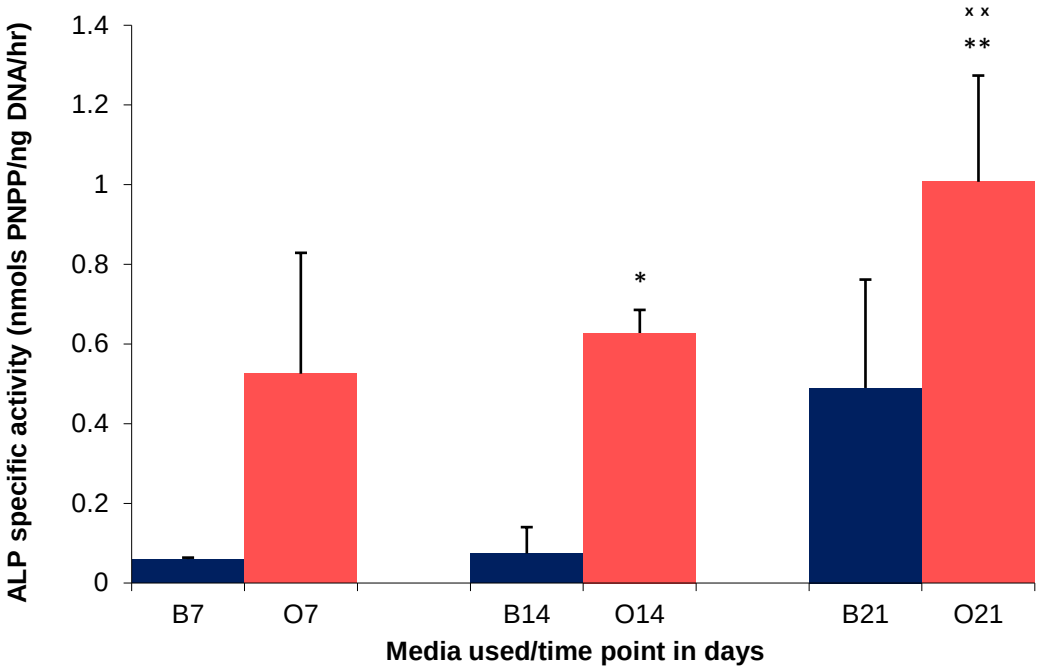


Figure 3.5 ALP specific activity of HBMSC over a 21 day time course.
Data presented as mean +/- SD for a single experiment performed in triplicate. B7=Basal media at Day 7, O7=Osteogenic media at Day 7, B14=Basal media at Day 14, O14=Osteogenic media at Day 14, B21=Basal media at Day 21, O21=Osteogenic media at Day 21. *p<0.05, **p<0.01 vs Basal Day 7 values, ˣˣp<0.01 vs Basal Day 14 values.

3.1.3.3 Molecular biology

Rt-PCR results mirrored the findings of the biochemical assays, confirming increased ALP production under osteogenic conditions compared with basal conditions (Figure 3.6). Collagen 1 expression was lower than anticipated, as was Runx-2, but collagen 2 and Sox-9 expression (chondrogenic markers so served as negative controls) were both negative.

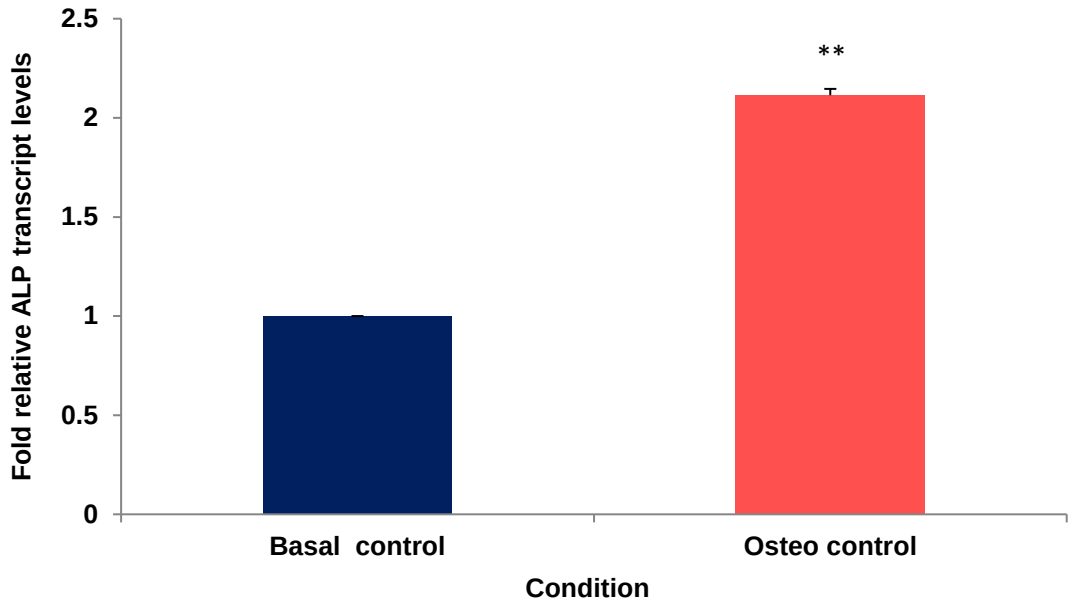


Figure 3.6 Expression of ALP by HBMSC at Day 7 (F61).
Data presented as mean ALP gene expression relative to β actin expression +/- SD for a single experiment performed in duplicate. Basal control=Basal media alone, Osteo control=Osteogenic media alone. **p<0.01 vs basal control.

3.2 Assessing the effects of adding cytokines

3.2.1 Experimental set up

Initial dose-ranging studies for the cytokines IL-1β, IL-6 and TNFα were performed. Human bone marrow cells were extracted and grown to confluence as described above. Cells were then seeded at $5x10^5$ cells per well of a 6 well plate, into 3 separate plates, one per cytokine to be tested. Basal media was added to a total of 2mls per well. Commercially available IL-1β, IL-6 and TNFα (Sigma-Aldrich, UK) were then diluted according to the manufacturer's instructions and added to individual wells at concentrations of 0.01, 0.1, 1, 10 and 100ng/ml for IL-1 and TNFα and 1, 10, 100 and 1000ng/ml for IL-6. A control well containing basal media alone was included on each plate. Media changes were carried out every 2-3 days for a total of 7 days. A WST-1 assay (described in full in Section 2.2.2) was performed every 24 hours to allow measurement of cell proliferation and hence calculation of a dose response curve for each cytokine. This then enabled optimum concentrations to be determined for each cytokine as follows: IL-1β 10ng/ml, IL-6 100ng/ml, TNFα 10ng/ml.

3.2.2 Single passage experiments

Cultures of human bone marrow stromal cells (HBMSCs) were set up following preparation of bone marrow samples according to the protocol described in Section 2.1.1. The cells were cultured initially in basal media for 6 days, washed with PBS and fresh media applied. Further media changes were carried out every 2-3 days (as detailed in Section 2.1.3) until the cells reached 95% confluence. The cells were then released from the surface of the tissue culture plastic by treatment with collagenase followed by trypsin (detailed in Section 2.1.4). The cell suspension obtained was centrifuged (1100rpm for 4 minutes), the cell pellet re-suspended in 10mls of basal media and a cell count performed using a haemocytometer (Section 2.1.1). Cells were then seeded at $1x10^5$ cells per well of a 24 well plate in either basal or osteogenic media. Each of these two groups were then subdivided further into 4 groups: controls (no additional factors); IL-1β (10ng/ml); IL-6 (100ng/ml); TNF (10ng/ml). For each of these 8 conditions, single wells were labelled for use in histological analysis (ALP, type I and type II collagen) and ALP and DNA biochemistry assays. The remaining cells were seeded at $1x10^5$ cells per T25, into 8 separate T25s (one for each of the 8 conditions being used) for use in PCR analysis. Media changes were performed every 2-3 days. Cultures were run for a total of 7 days prior to analysis.

3.2.3 Multiple passage experiments

In order to ascertain whether the effects of cytokines persisted over time, several multiple passage experiments were carried out. The initial set up was identical to that described in Section 3.2.1.1 above, except that the proportion of cells remaining after setting up the plates and flasks were placed in 2x T150s. Following expansion the cells were used for experimentation.

3.2.4 Summary of experiments

HBMSC +cytokines Single passage experiments	HBMSC + cytokines Multiple passage experiments	SSCs +cytokine cultures	Fetal femur +cytokine cultures	HBMSC+ cytokines+ Strontium	SSCs+ cytokines+ Strontium
F61	F74 (P1-P2)	F72	H1123	F72	F84
F47	F76 (P1-P5)	F78	H1326	F78	M78
F78	M72 (P1-P5)	F54	H1134	F54	M82
A3					

Table 3.1 Summary of cytokine experiments performed

3.2.5 Results

3.2.5.1 HBMSC cultures

Histology

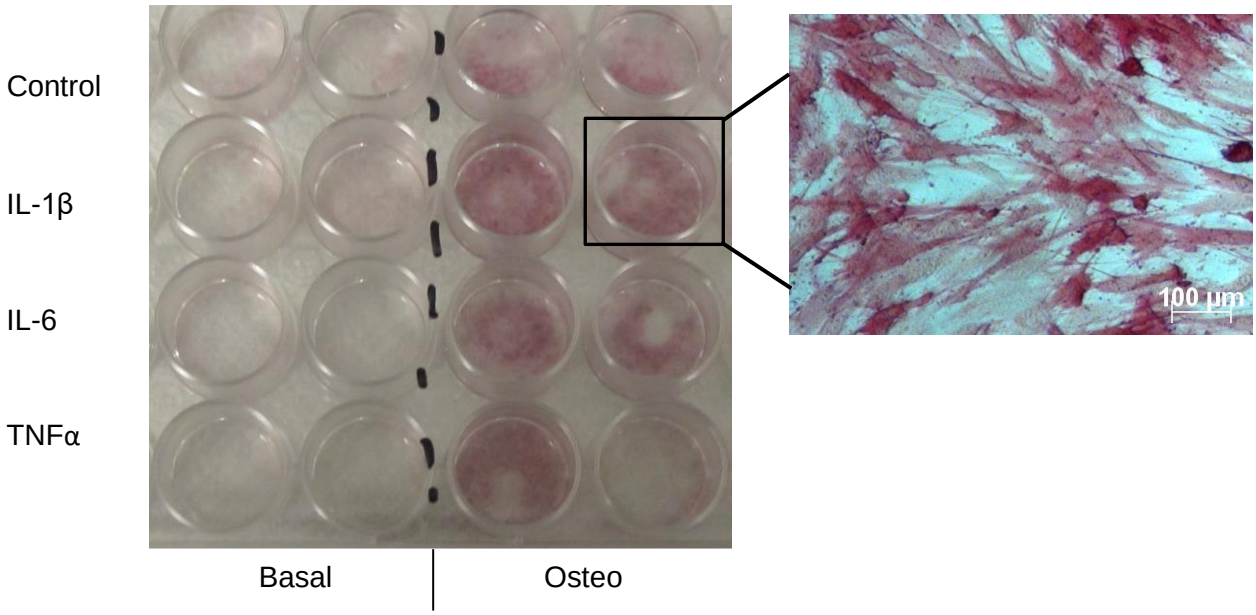


Figure 3.7 Alkaline phosphatase staining of HBMC with or without cytokines
- showing characteristic pink/purple staining under osteogenic conditions, with additive effects of IL-1 and IL-6 and to a lesser extent with TNFα. Main plate overview taken with a digital camera at maximal zoom; insert – high power magnification of selected well under a light microscope.

Biochemistry

In initial proof of concept experiments (n=3), addition of IL-1β to the culture media produced an additive osteogenic effect compared to basal cultures, which was statistically significant ($p<0.05$) (Figures 3.8-3.11). TNF produced a slight additive osteogenic effect, which was less marked than that observed for IL-1β and this was only statistically significant in one of the three experiments (Figure 3.8). IL-6 appeared to have a negligible effect on ALP specific activity. However, there was notable inter-sample variation, accounting for the large standard deviations seen with the summary plots (Figure 3.11). This may well reflect inter-patient variations in cytokine levels and further work is underway to gather data on CRP levels and OA scores for patients to analyse and attempt to account for these differences by quantifying disease activity.

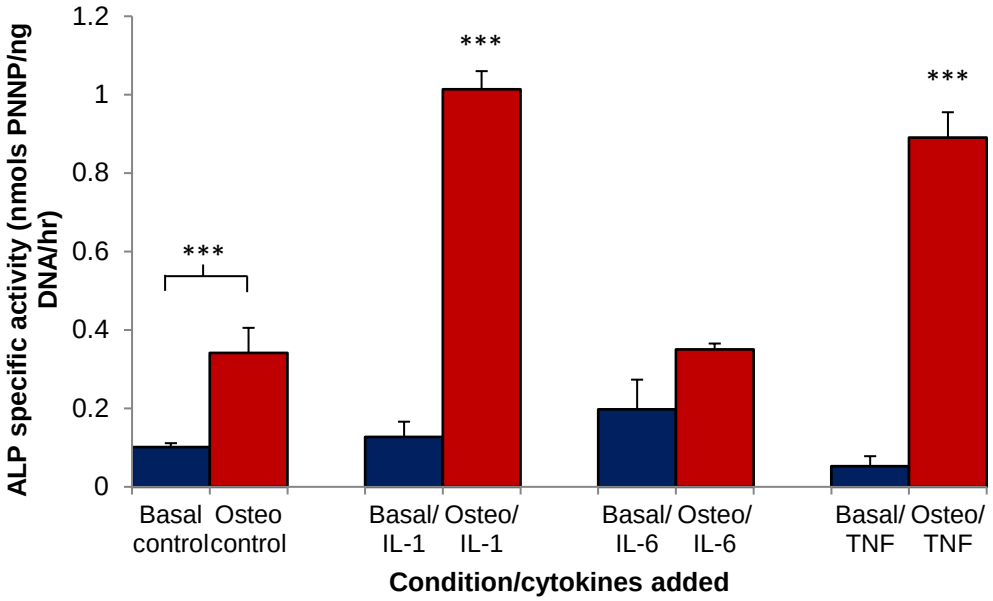


Figure 3.8 ALP specific activity of HBMSC with or without cytokines (F88).

Data presented as mean ALP specific activity +/- SD for a single experiment performed in triplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. ***p<0.001 compared to osteogenic control sample.

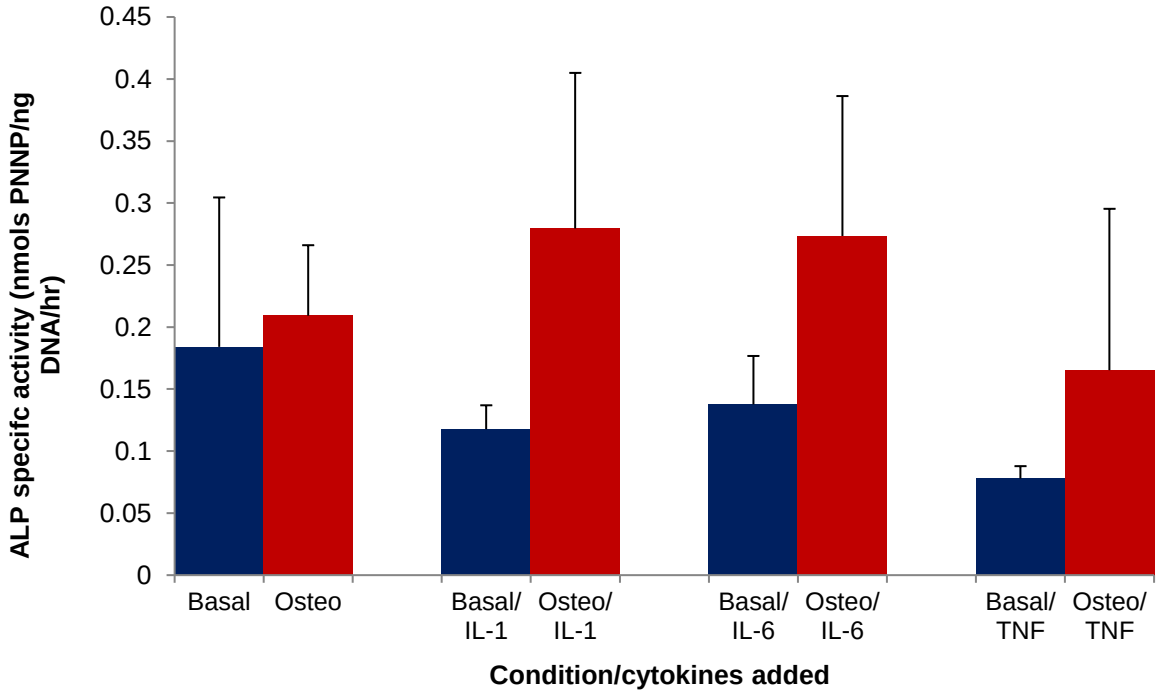


Figure 3.9 ALP specific activity of HBMSC with or without cytokines (F78).

Data presented as mean ALP specific activity +/- SD for a single experiment performed in triplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. Differences between the groups did not reach statistical significance.

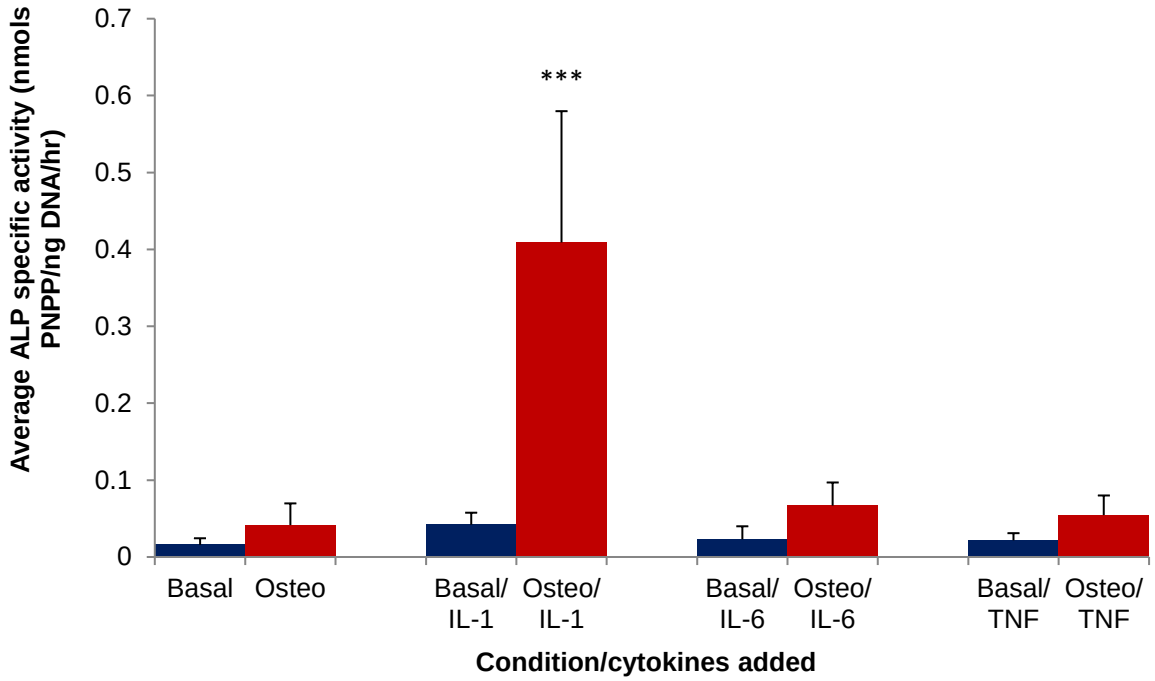


Figure 3.10 ALP specific activity of HBMSC with or without cytokines (A3).
Data presented as mean ALP specific activity +/- SD, for a single experiment performed in triplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. $^{***}p<0.001$ versus osteogenic control sample.

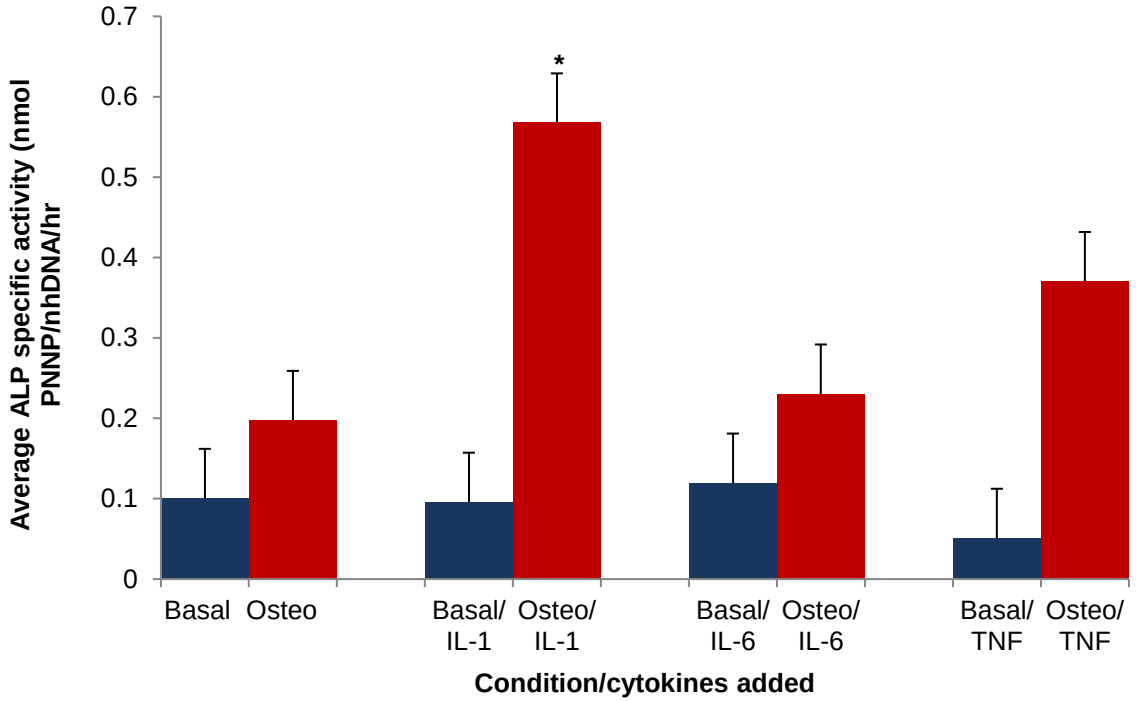


Figure 3.11 ALP specific activity of HBMSC with or without cytokines.
Data presented as mean ALP specific activity +/- SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone. Basal/IL-1 =Basal media+IL-1, Osteo/IL-1=Osteogenic media+IL-1, Basal/IL-6= Basal media+IL-6, Osteo/IL-6= Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. $^{*}p<0.05$ versus osteogenic control sample.

In the second set of experiments (n=4) where cells were seeded at $5x10^4$ cells per well of a 24 well plate for the biochemistry assays, similar patterns were observed: IL-1β had an additive osteogenic effect on HBMSC cultures, although the magnitude of this effect varied between samples (Figure 3.12-3.15). In contrast, TNFα triggered down-regulation of ALP specific activity to a varying extent. Once again, the addition of IL-6 had the least impact on osteogenic differentiation, except in one isolated sample (Figure 3.15). Importantly these patterns were maintained when cells were passaged out to and including passage 5 (Figures 3.13-3.15).

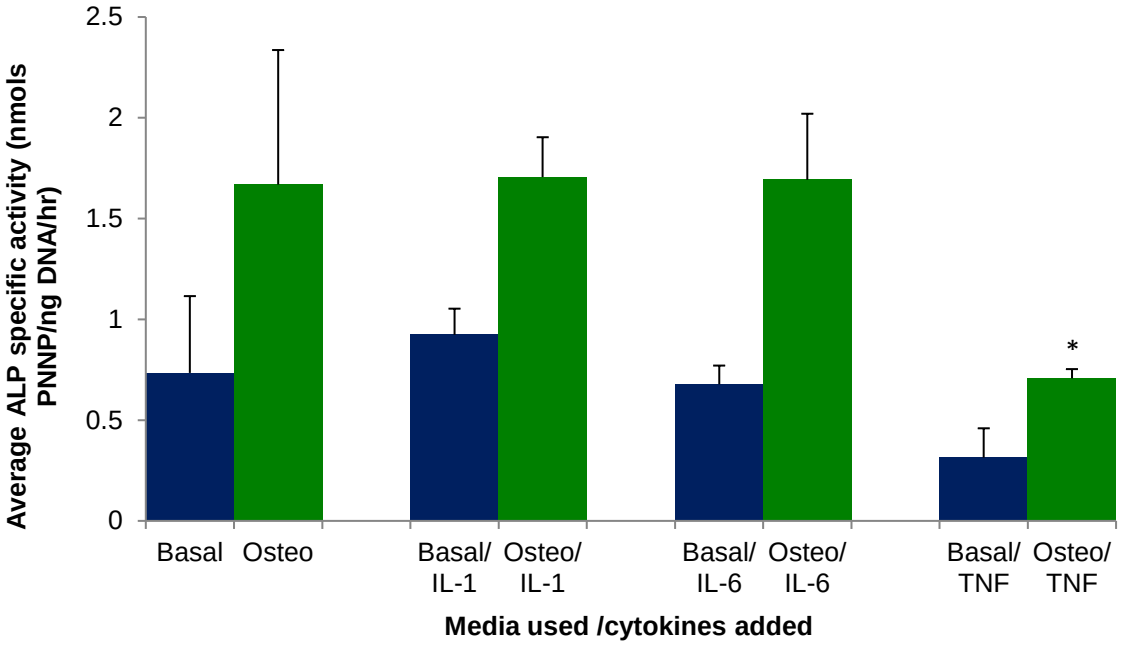


Figure 3.12 ALP specific activity of HBMSC with or without cytokines (F47).
Data presented as mean +/- SD for a single experiment performed in triplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media+IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. $^*p<0.05$ versus osteogenic control sample.

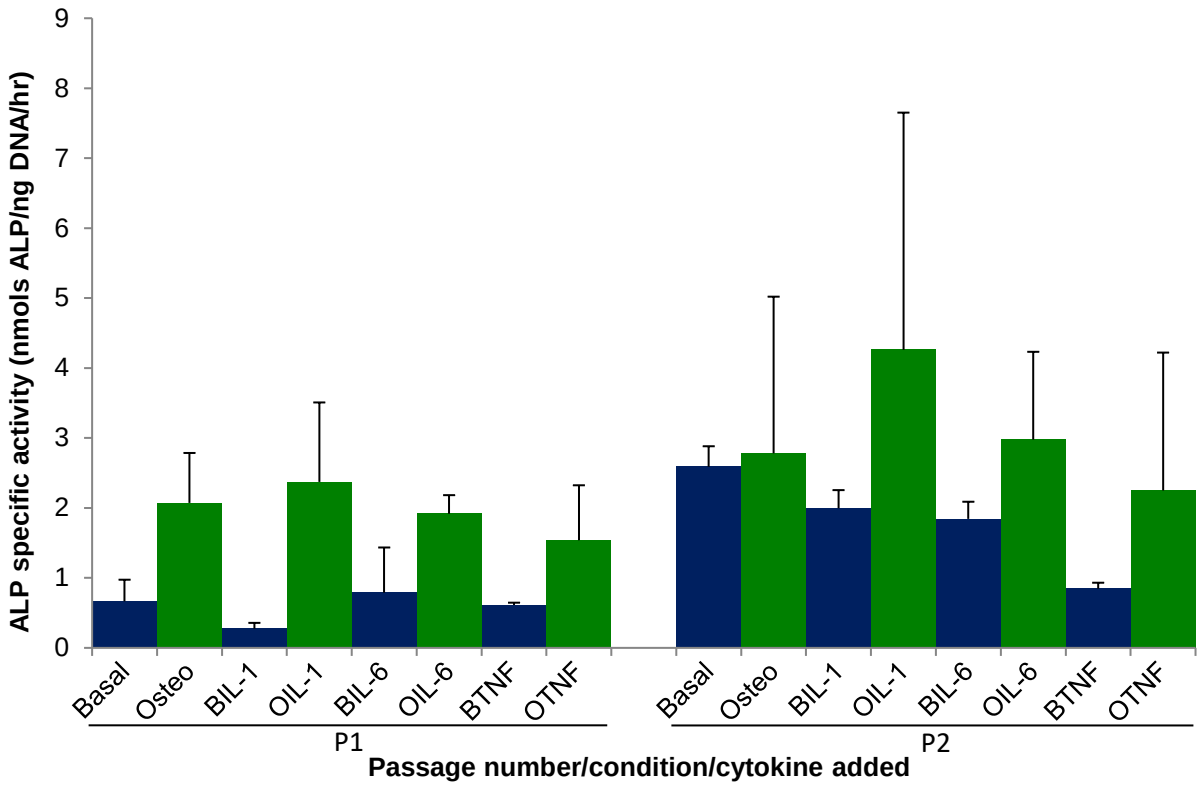


Figure 3.13 The effects of increasing passage number on ALP specific activity of HBMSC with or without cytokines (F74).
Data presented as mean ALP specific activity +/- SD for a single experiment performed in triplicate. P1=passage 1, P2=passage 2, Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. Differences between the groups did not reach statistical significance.

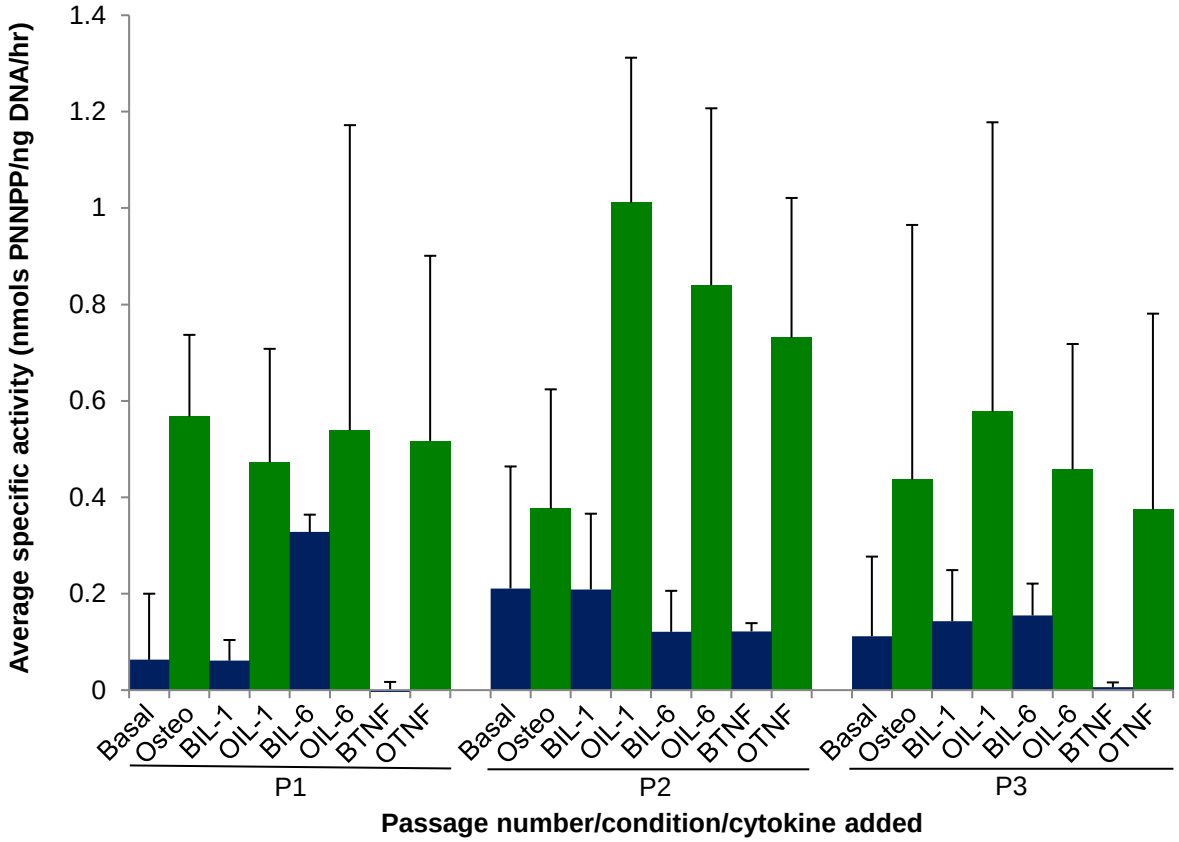


Figure 3.14 The effects of increasing passage number on ALP specific activity of HBMSC with or without cytokines (F76).
Data presented as mean ALP specific activity +/- SD, for a single experiment performed in triplicate. P1=passage 1, P2=passage 2, P3=passage 3, Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. Differences between the groups did not reach statistical significance.

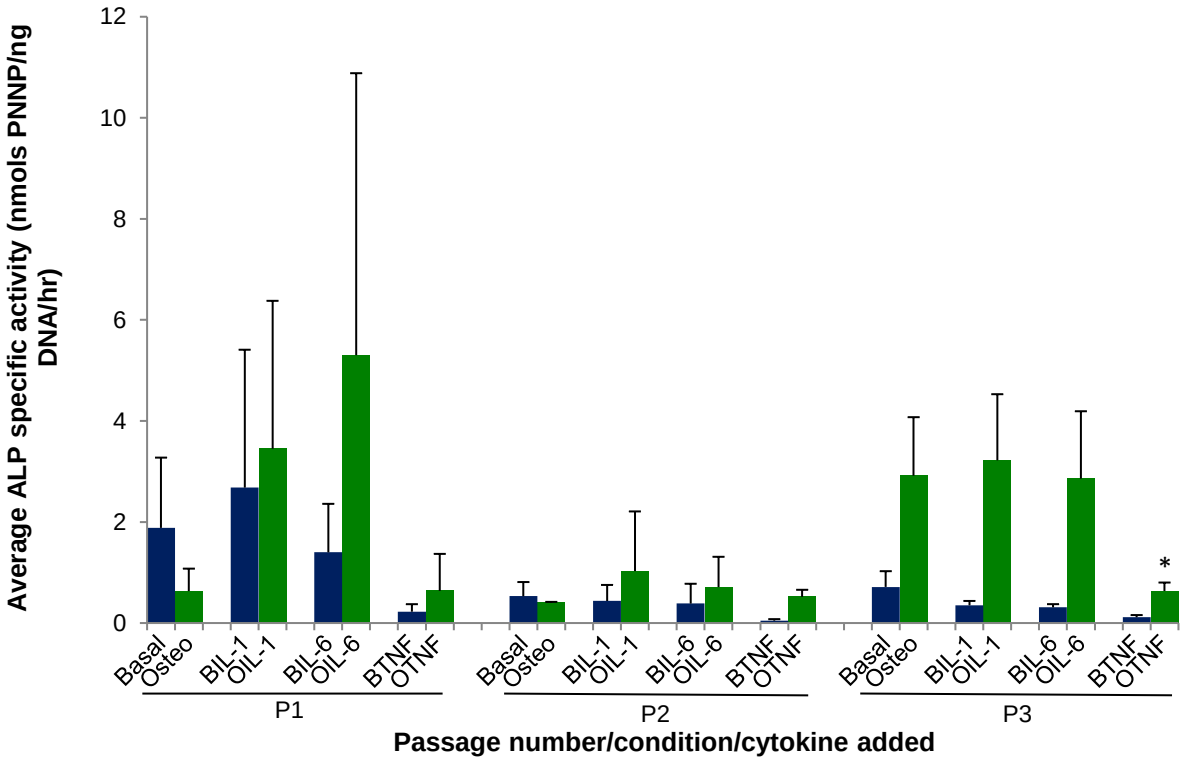


Figure 3.15 – The effects of increasing passage number on ALP specific activity of HBMSC with or without cytokines (M72).
Data presented as mean ALP specific activity +/- SD for a single experiment performed in triplicate. P1=passage 1, P2=passage 2, P3=passage 3, Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. *p<0.05 versus osteogenic control sample.

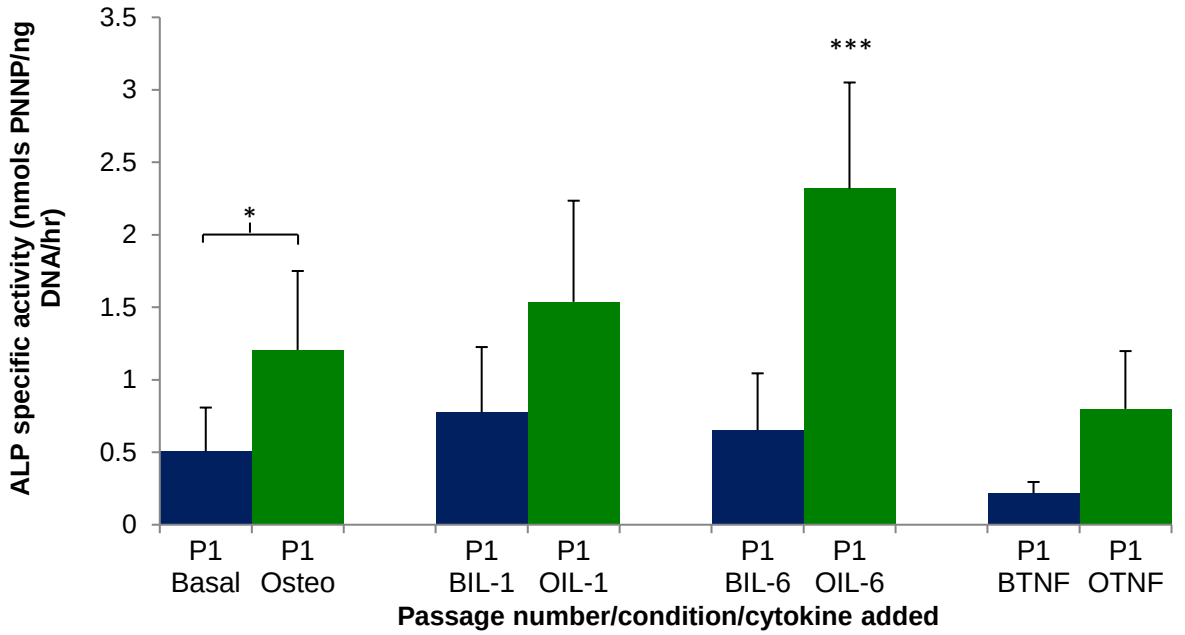


Figure 3.16 ALP specific activity of HBMSC from 4 OA patients with or without cytokines.
Data presented as mean ALP specific activity +/- SD n=4. P1= passage 1, Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. *p<0.05, ***p=<0.001 versus osteogenic control sample.

Molecular biology

The rt-PCR results largely concurred with the biochemistry assay data, with some inter-sample variation (Figures 3.17-3.19). The addition of IL-1β stimulated an increase in osteogenic gene expression (exemplified by an increase in ALP messenger RNA), IL-6 produced little change, while TNFα suppressed osteogenic gene expression. This observation was maintained across multiple passages of the same cells (Figures 3.20 and 3.21).

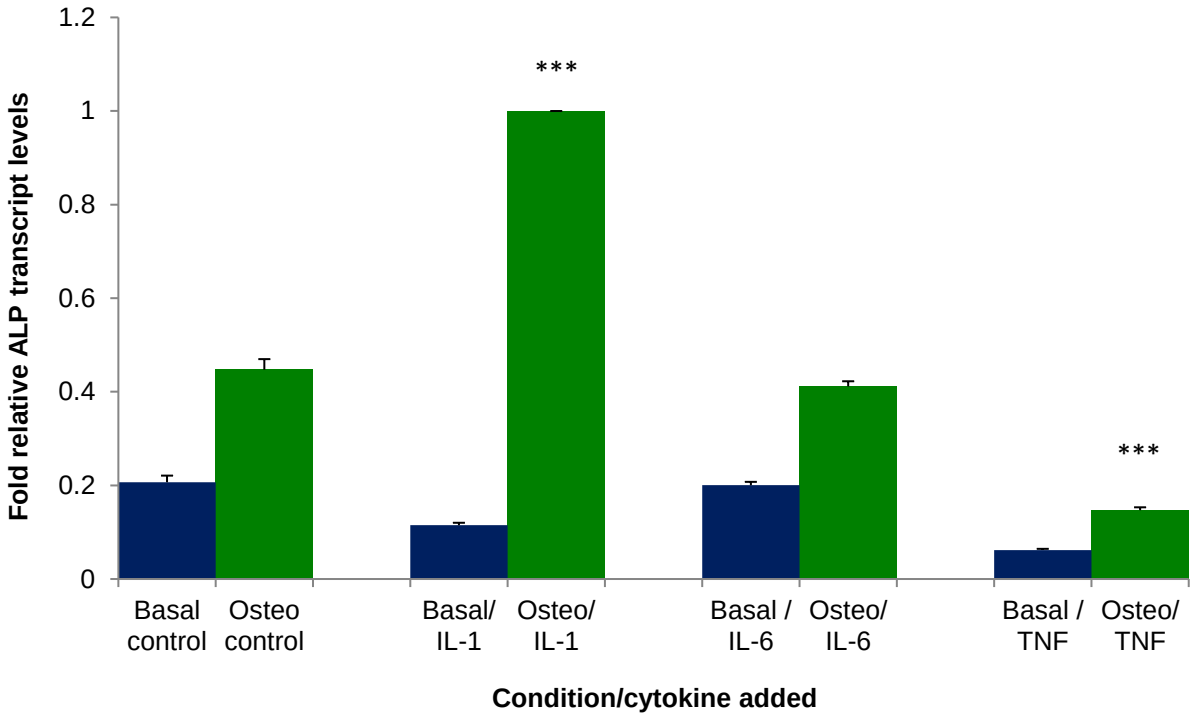


Figure 3.17 ALP expression of HBMSC with or without cytokines at Day 7 (F61)
Data expressed as mean ALP expression relative to β actin +/- SD for a single experiment performed in duplicate, Basal control=Basal media alone, Osteo control=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF.***p<0.001 versus osteogenic control sample.

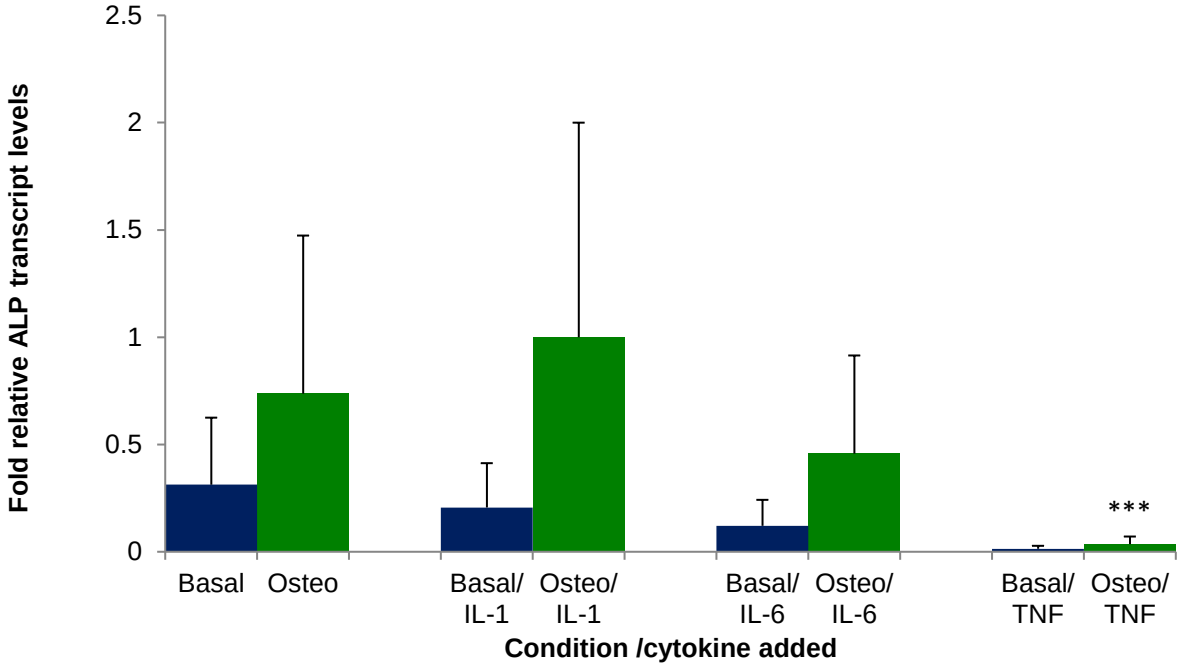


Figure 3.18 ALP expression for HBMSC at Day 7 (F47).

Data presented as ALP expression relative to β actin +/- SD for a single experiment performed in duplicate, Basal=Basal media alone, Osteo=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF $^{***}p<0.001$ relative to osteogenic control.

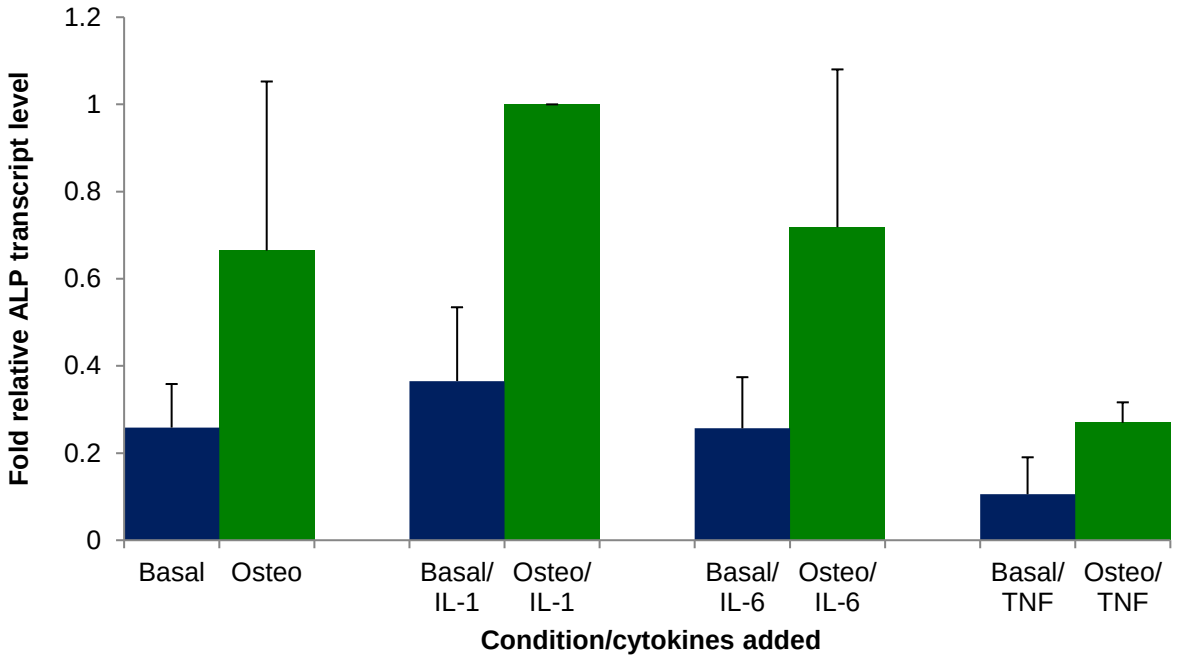


Figure 3.19 ALP expression for HBMSC at Day 7 (M72 P1).

Data presented as mean ALP expression relative to β actin +/- SD for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. Differences between the groups did not reach statistical significance.

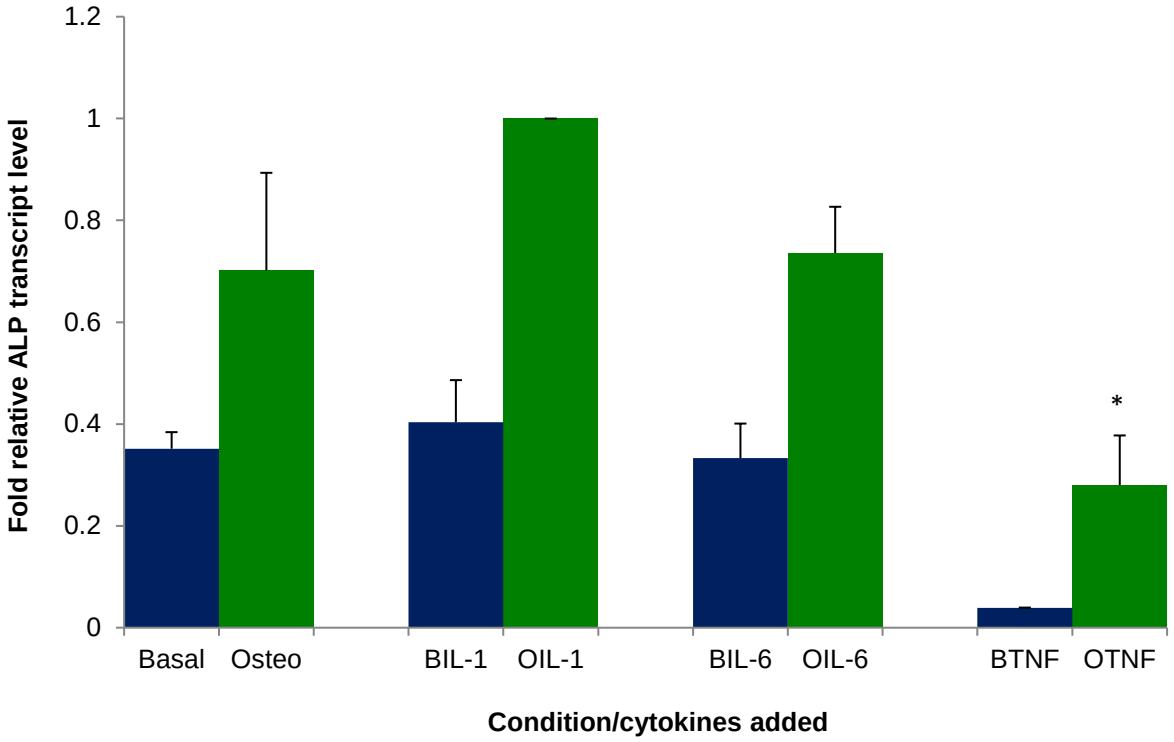


Figure 3.20 ALP expression for HBMSC at Day 7 (M72 P2).
Data presented as mean ALP expression relative to β actin +/- SD for a single experiment performed in duplicate, Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF, *p<0.05 relative to osteogenic control.

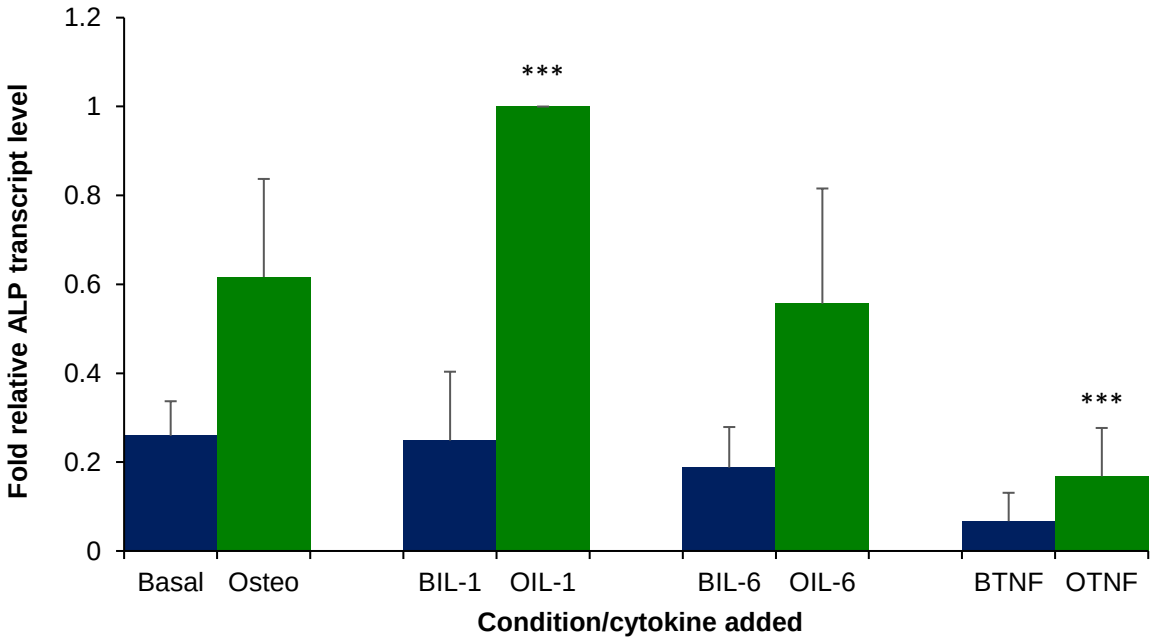


Figure 3.21 ALP expression of HBMSCs at Day 7(F61, F47 and M72).
Data expressed as mean ALP expression relative to β actin expression +/- SD, n=3, Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF
***p<0.001 versus osteogenic control sample.

3.2.5.2 Stro-1 +ve SSCs cultures

Biochemistry

IL-1β had an additive osteogenic effect on SSC cultures (Figures 3.22-3.23). In contrast, TNFα triggered down-regulation of ALP specific activity to a varying extent. The addition of IL-6 had the least impact on osteogenic differentiation. Overall, the same pattern was seen with the Stro-1 +ve sub population of HBMSCs in culture (Figures 3.22-3.23) as with the unselected HBMSC cultures but in this instance did not reach statistical significance.

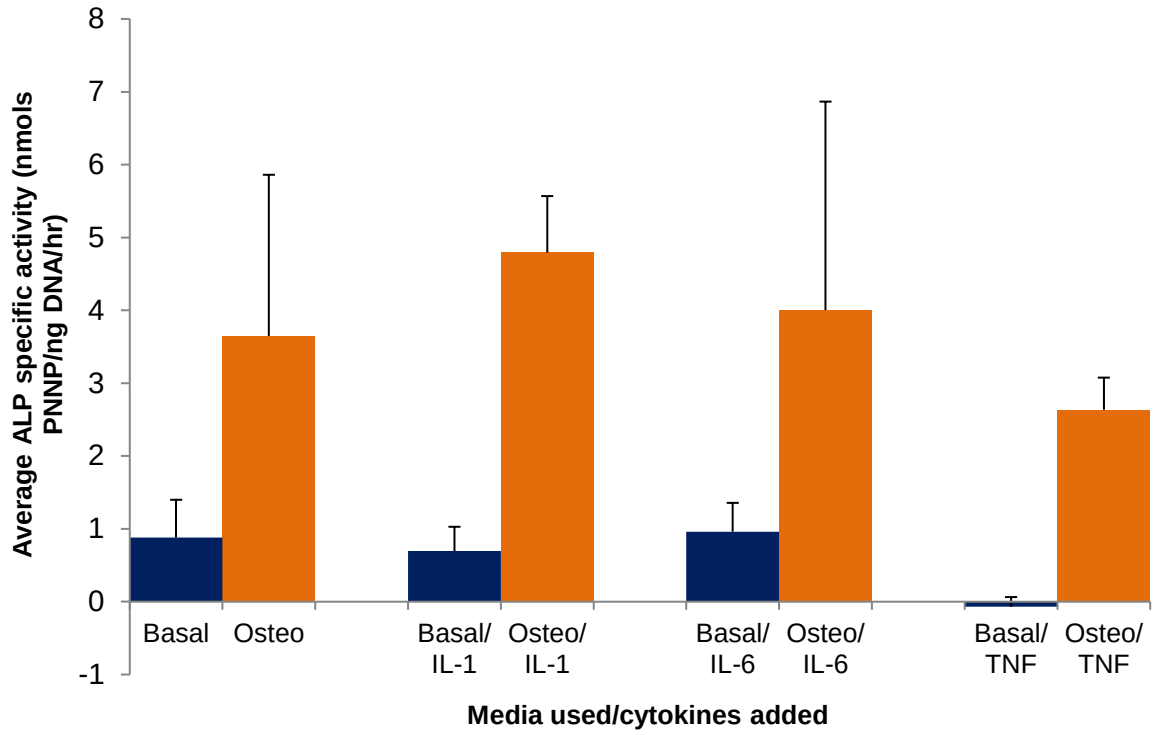


Figure 3.22 ALP specific activity of Stro-1 positive SSCs with or without cytokines (F72).
Data presented as mean ALP specific activity +/- SD, for a single experiment performed in triplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. Differences between the groups did not reach statistical significance.

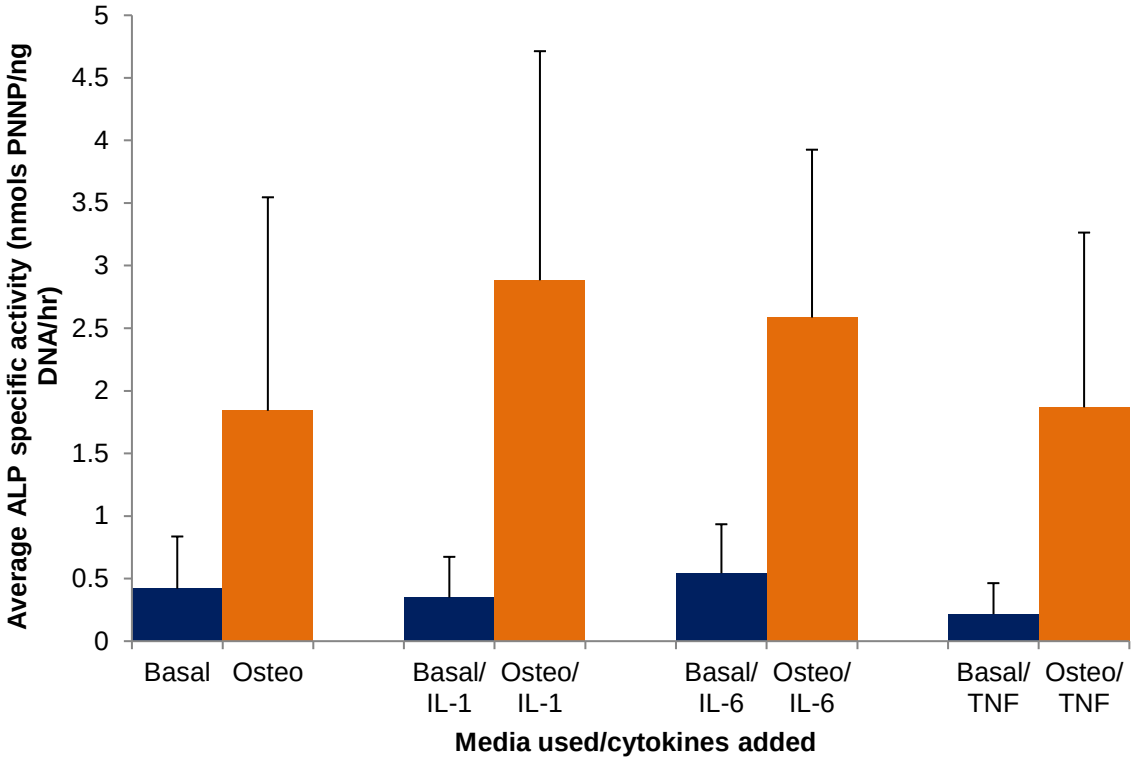


Figure 3.23 ALP specific activity of Stro-1 positive SSCs with or without cytokines.
Data presented as mean ALP specific activity +/- SD, n=3. Basal control=Basal media alone, Osteo control=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. Differences between the groups did not meet statistical significance.

Molecular biology

Similar results were seen with Stro-1 positive cell cultures as with unselected HBMSC with regard to the effects of IL-1β and TNFα (Figures 3.24). IL-1β produced a stimulatory effect on osteogenic gene expression, whilst TNFα had an inhibitory effect. IL-6 exerted an additional osteogenic effect in this set of cultures, although this did not reach statistical significance and was not apparent when data from additional Stro-1 positive cultures was analysed (Figure 3.25). The additive osteogenic effect of IL-1β was still evident and highly statistically significant in these additional Stro-1 positive cell cultures (Figure 3.25).

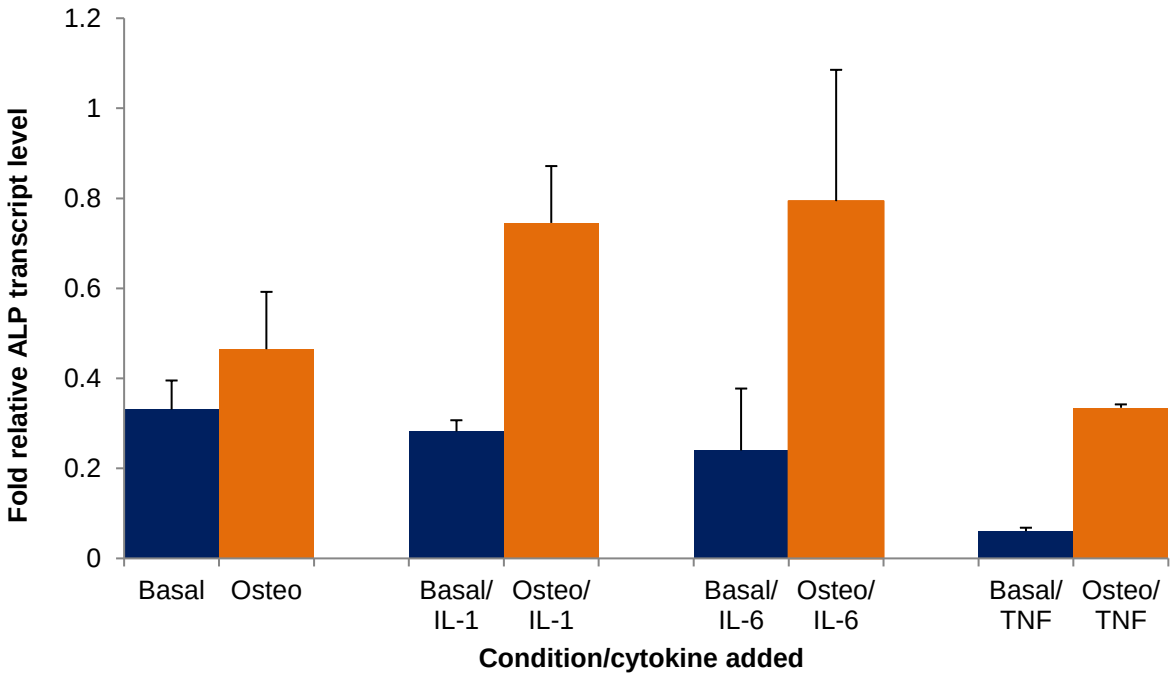


Figure 3.24 ALP expression of Stro-1 positive SSCs at Day 7(F72).
Data expressed as mean ALP expression relative to β actin expression +/- SD, for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. Differences between the groups did not meet statistical significance.

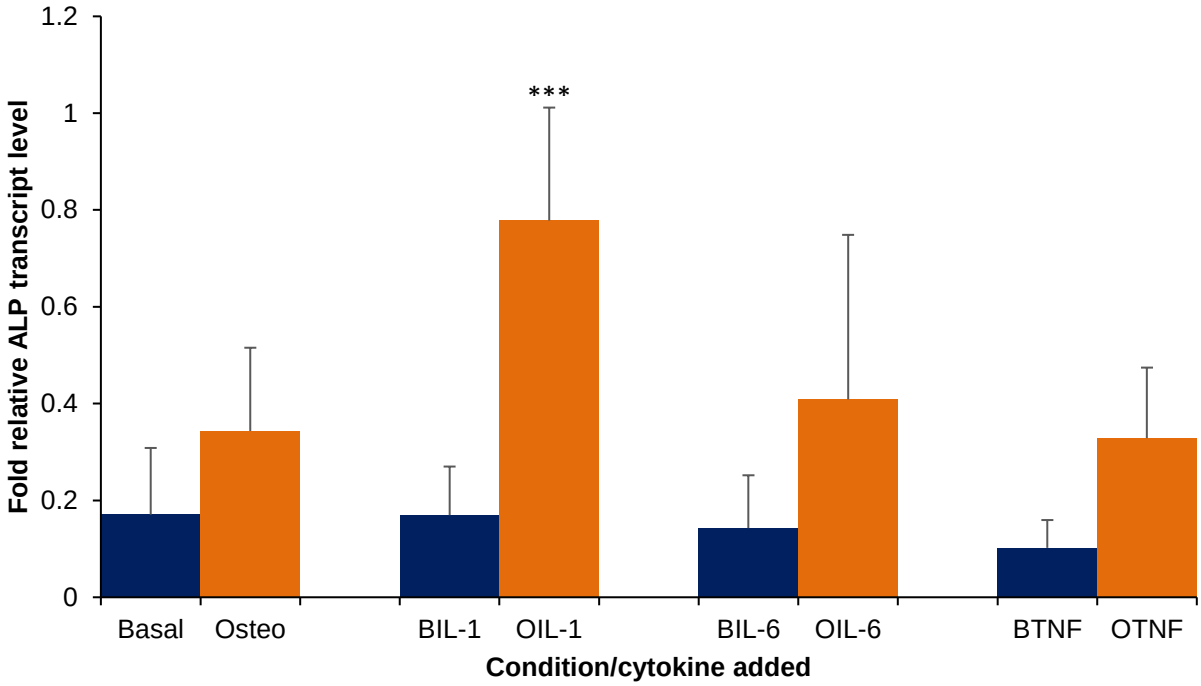


Figure 3.25 ALP expression of Stro-1 positive HBMSCs at Day 7 (F72, F78 and F54).
Data expressed as mean ALP expression relative to β actin expression + SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. ***p<0.001 versus osteogenic control sample.

3.2.5.3 Fetal femur cultures

Biochemistry

With fetal femur cultures, which served as a positive control for these studies, again an additive osteogenic effect was seen with IL-1β but this was more marked for IL-6 (Figure 3.26). The inhibitory effect of adding TNFα was more apparent in fetal femur cultures in comparison to HBMSC cultures.

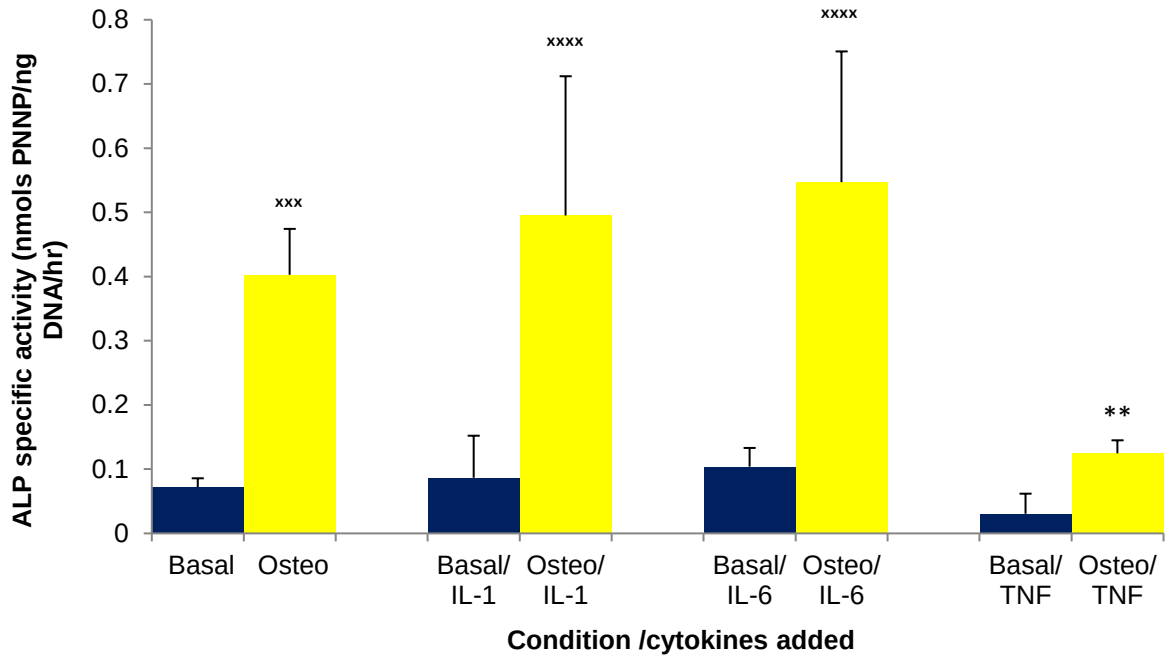


Figure 3.26 ALP specific activity of 3 fetal femur cell samples at Day 7 with or without cytokines. Data presented as mean ALP specific activity + SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. $^{**}p<0.01$ versus osteogenic control sample, $^{xxx}p<0.001$ versus basal control, $^{xxxx}p<0.0001$ versus basal control.

Molecular biology

For the fetal femur cultures (n=3) summarised in Figure 3.27, there was a decrease in ALP gene expression with IL-1β, although this did not reach statistical significance. IL-6 and TNFα produced comparable trends to those observed in standard HBMSC cultures as detailed above.

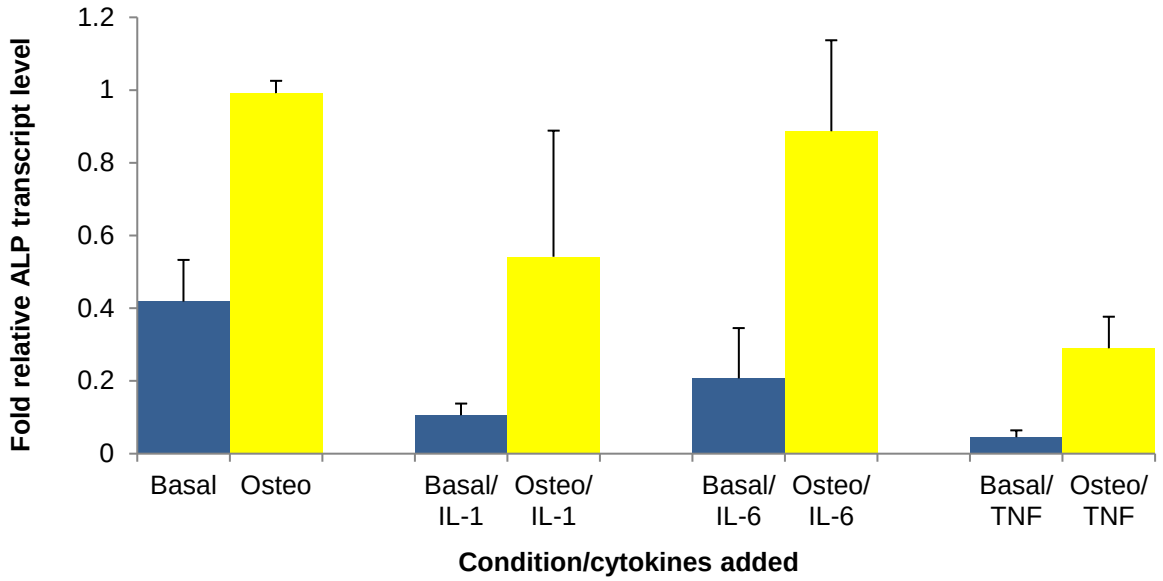


Figure 3.27 ALP expression of fetal femur cells at Day 7.
Data expressed as mean ALP expression relative to β actin expression +/- SD, n=3. Basal control=Basal media alone, Osteo control=Osteogenic media alone, Basal/IL-1=Basal media+IL-1, Osteo/IL-1=Osteogenic media +IL-1, Basal/IL-6=Basal media+IL-6, Osteo/IL-6=Osteogenic media+IL-6, Basal/TNF=Basal media+TNF, Osteo/TNF=Osteogenic media+TNF. Differences between the groups did not meet statistical significance.

3.2.5.4 Summary of results

Cultures	Effects of IL-1β on ALP production	Effects of IL-1β on osteogenic gene expression	Effects of IL-6 on ALP production	Effects of IL-6 on osteogenic gene expression	Effects of TNFα on ALP production	Effects of TNFα on osteogenic gene expression
Unselected HBMSCs	+	+	+/-	+/-	-	-
Stro-1 +ve SSCs	++	+	+	+	-	-
Fetal femur cells	+	-	+	+	--	-

Table 3.2 Summary of the effects of inflammatory cytokines on the different cell cultures under study

'+' = stimulatory effect, '-' = inhibitory effect, '+/-'=negligible effect

3.2.6 Discussion

In the current study, HBMSCs extracted from human bone marrow samples were shown to proliferate and to display the typical fibroblast cell morphology under basal culture conditions. The addition of osteogenic media to the cultures resulted in the HBMSCs undergoing osteogenic differentiation, confirmed by positive staining for ALP and upregulation of early markers of osteogenic differentiation on qRt-PCR. Addition of the inflammatory cytokines IL-1β, IL-6 and TNFα modulated HBMSC function, with IL-1β exerting an additive osteogenic effect on osteogenic differentiation. This effect was seen not only in unsorted HBMSC cultures, but also in the Stro-1 positive cultures. TNFα had a similar additive effect on osteogenic differentiation in some cultures, but the effect was less potent than for IL-1β. IL-6 overall had negligible effect on osteogenic differentiation in HBMSC, generally producing levels of ALP production or gene expression comparable with the effects of osteogenic media alone. However, in the Stro-1 positive cell cultures (Figure 3.25) and in the fetal femur cultures (Figure 3.19) it appeared to have some additive osteogenic effect. This finding was replicated with increased *n* numbers in terms of ALP production (Figure 3.18), suggesting that this is a true variation between culture types. Further work will therefore be needed to elucidate the mechanism through which this effect occurs.

The central role of the pro-inflammatory cytokines IL-1β, IL-6, TNFα and IL-17 in the inflammatory arthritides such as RA is well-recognised and has recently been established for OA. Given this knowledge it might be anticipated that these cytokines would have a detrimental effect on the growth and differentiation of SSCs. Indeed, a study of osteogenic differentiation of murine SSCs demonstrated that both IL-1β and TNFα suppressed the differentiation of SSCs along the osteoblastic lineage (Lacey, Simmons et al. 2009). IL-1β reduced cell proliferation, attenuated ALP production and suppressed the expression of the osteoblastic genes *ALP, alpha 1 procollagen, Runx2* and *Osterix*. TNFα similarly reduced ALP production and the expression of not only *ALP, alpha 1 procollagen, Runx-2* and *Osterix* but also *Osteopontin* and *Osteonectin*. Overall, these findings suggested that IL-1β and TNFα inhibited the development of bone from primary murine SSCs.

In contrast, in human SSCs cultures, addition of IL-1β (0.1-1ng/ml) or TNFα (0.1-10ng/ml) resulted in increased ALP activity and mineralisation (alizarin red staining) compared to controls (Ding, Ghali et al. 2009). The range of IL-1β used was slightly lower (0.1-1ng/ml) than the concentration of IL-1β used in our experiments, whereas the range of TNFα used was directly comparable with our work (Williams et al, Conference abstracts from BSR 2011,

2012). Interestingly, despite these findings, levels of *Runx2* expression and osteocalcin secretion were both reduced in these cultures, which suggested that Runx2 was not involved in mineralisation. Use of osteoblast like cells with a predominant negative Runx-2 expression failed to inhibit mineralisation, supporting the authors' hypothesis that mineralisation was Runx2 independent. Reduced levels of collagen expression were also found in osteoblasts from SSC cultures. These findings provided one plausible explanation for the reduced levels of *Runx2* and *Osteocalcin* gene expression seen in the HBMSC/cytokine cultures described above on molecular analysis, when ALP expression and activity was preserved.

Further evidence of the stimulatory effects of TNFα on osteogenic differentiation of SSCs comes from a study investigating the effects of TNFα on the differentiation of human adipose tissue derived stromal cells (hADSCs) (Cho, Shin et al. 2010). TNFα was found to increase osteogenic differentiation in a dose-dependent manner, over the first 3 days in culture. A combination of strategies including NFκB inhibitors, ERK inhibitors and overexpression of miR-146a were then used to determine the molecular mechanisms responsible for these effects. These data suggested that TNFα increased osteogenic differentiation of hADSCs through activation of NFκB and a resultant increase in the expression of transcriptional coactivator with PDZ-binding motif (TAZ).

The data presented here are in keeping with those from a study examining the effects of various cytokines (IL-1β, IL-6, TNFα and IL-17) on the differentiation of human SSCs into osteoblasts (Sonomoto, Yamaoka et al. 2012). This work also demonstrated that IL-1β was the most potent cytokine for enhancing osteogenic differentiation in cells exposed to osteogenic media. Through measurement of the expression of the Wnt signalling molecules β-catenin, Wnt3a, Wnt 5a, Wnt 7b and Wnt 10b, the investigators were able to determine that IL-1β significantly upregulated expression of *WNT5A* alone. This suggested that the effects of IL-1β on osteogenic differentiation could be mediated through the non-canonical Wnt pathway. This was confirmed through gene knockdown strategies and the use of small interfering RNAs (siRNAs) to *WNT5A* and *ROR2* (a component of the WNT5A cell surface receptor), which confirmed that silencing either one or both of these genes resulted in significant reductions in ALP activity and osteogenic gene expression. Therefore, current evidence suggests that IL-1β stimulates osteogenic differentiation of human SSCs via the mWnt5a-Ror2 pathway. This pathway represents a possible target for future RA therapies as the investigators suggest and, taking into account the significant role IL-1β is now known

to occupy in OA, agents targeting this pathway may also be a therapeutic option for the treatment of OA in the future.

3.3 Assessing the effects of adding strontium to cytokine modulated HBMSCs

3.3.1 Experimental set up

Cultures of HBMSCs were set up following preparation of bone marrow samples according to the protocol described in Section 2.1.1. The cells were cultured initially in basal media for 6 days, washed with PBS and fresh media applied. Further media changes were carried out every 2-3 days (as detailed in Section 2.1.3) until the cells reached 95% confluence. The cells were then released from the surface of the tissue culture plastic by treatment with collagenase followed by trypsin (detailed in Section 2.1.4). The cell suspension obtained was centrifuged (1100rpm for 4 minutes), the cell pellet re-suspended in 10mls of basal media and a cell count performed using a haemocytometer (Section 2.1.1). Cells were then seeded at $1x10^4$ cells per well of a 24 well plate in either basal or osteogenic media. Each of these two groups were then subdivided further into 4 groups: controls (no additional factors); IL-1β (10ng/ml); IL-6 (100ng/ml); TNF (10ng/ml).

Strontium was added to all cultures (except controls) at final concentrations of 0.1mM, 1mM or 10mM. For each of these conditions, single wells were labelled for use in histological analysis (ALP, type I and type II collagen) and ALP and DNA biochemistry assays. The remaining cells were seeded at $1x10^5$ cells per T25, into 8 separate T25s (one for each of the 8 conditions being used) for use in PCR analysis. Media changes were performed every 2-3 days. Cultures were run for a total of 7 days prior to analysis. Cell viability was confirmed using cell tracker green.

Cultures were set up with both unselected HBMSCs for 3 experiments and then with Stro-1 positive cells for a further 3 experiments.

3.3.2 Results

3.3.2.1 Histology

Control					
B	BIL-1	BIL-6	BTNF	O	OIL-1
		0.1mM Sr			
OIL-6	OTNF	B	BIL-1	BIL-6	BTNF
				1mM Sr	
O	OIL-1	OIL-6	OTNF	B	BIL-1
BIL-6	BTNF	O	OIL-1	OIL-6	OTNF

Figure 3.28 ALP staining of HBMSC + cytokines + Sr experiment (F67).
Alkaline phosphatase staining of HBMC with or without cytokines showing characteristic pink/purple staining under osteogenic conditions, with additive effects of IL-1 and IL-6 and increasing concentrations of strontium. Main plate overview taken with a digital camera at maximal zoom.
C=control experiment rows, 0.1mM= 0.1mM Strontium experiment, 1mM=1mM Strontium experiment. B= Basal media alone, BIL-1= Basal media+IL-1, BIL-6= Basal media+IL-6, BTNF= Basal media+TNF, O= Osteogenic media alone, OIL-1 = osteogenic media+IL-1, OIL-6= osteogenic media+IL-6, OTNF= osteogenic media+TNF.

3.3.2.2 Biochemistry

Strontium had no adverse effects on the growth of HBMSCs. Optimal cell growth was observed at 0.1-1mM concentrations of strontium. There was an additive osteogenic effect of strontium seen numerically across the osteogenic control experiments but this was not universal so did not meet statistical significance in the summary analyses. Under control conditions, IL-1β produced an additive osteogenic effect, as did IL-6. This reached statistical significance for IL-1β in the Stro-1 +ve HBMSC experiments but not for IL-6 (Figure 3.29). TNFα exerted a modest inhibitory effect.

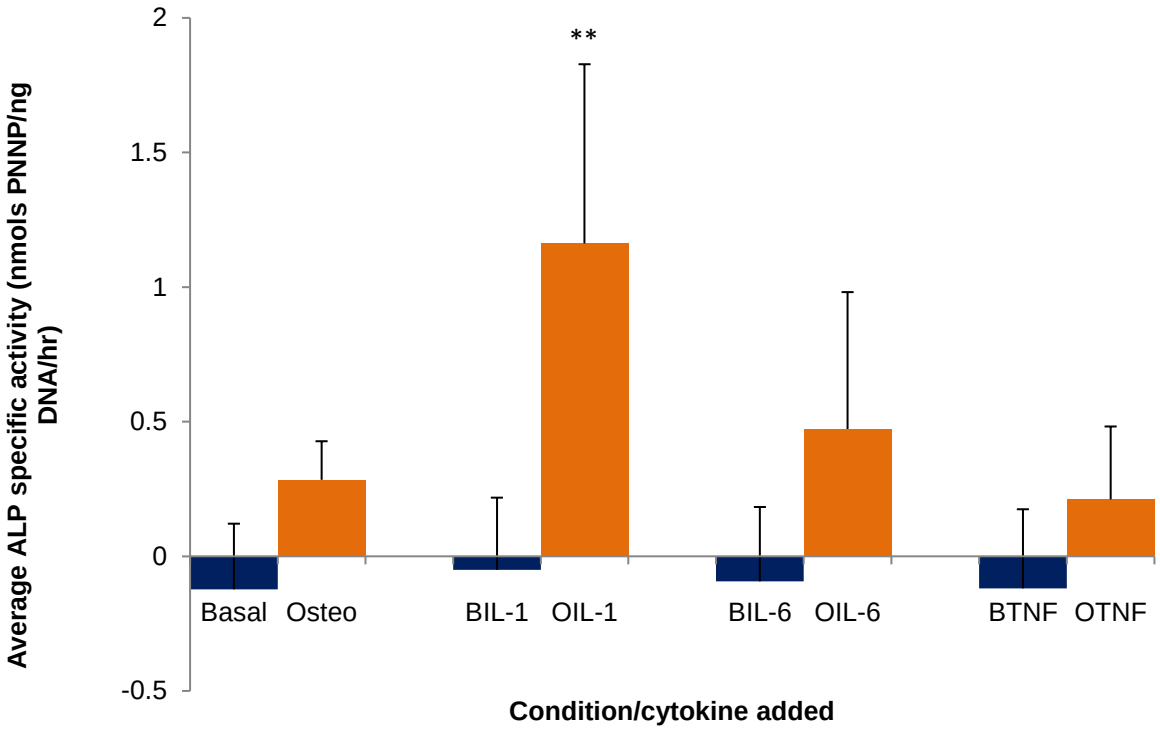


Figure 3.29 ALP specific activity of Stro+ve HBMSC at Day 7, with or without cytokines, control experiments.
Data presented as mean +/- SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. **p<0.01 versus osteogenic control sample.

The additive osteogenic effects of IL-1β and IL-6 persisted when strontium was added to the cultures, as did the inhibitory effect of TNFα (Figures 3.30 and 3.31). Very low values for ALP production were reported in these experiments under basal conditions, which would be expected in comparison to the osteogenic conditions. Once normalised as part of the analysis used to determine values for ALP specific activity, this resulted in negative values in the basal groups.

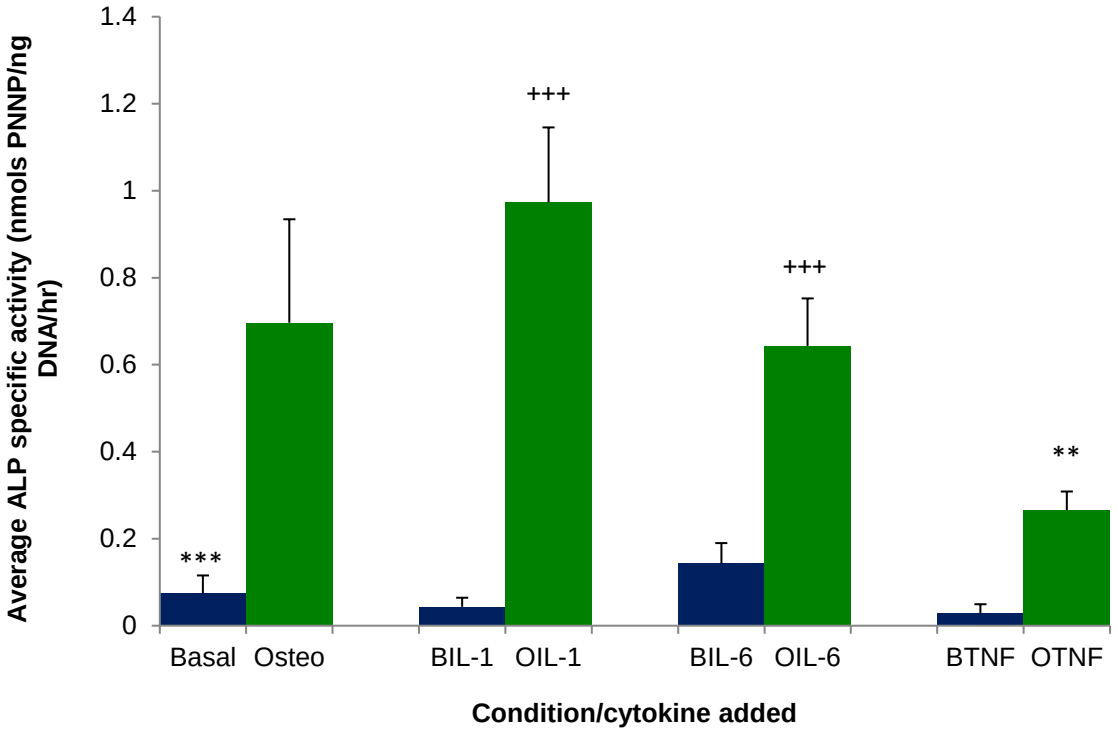


Figure 3.30 ALP specific activity of unselected HBMSC at Day 7, with or without cytokines and 0.1mM strontium (F67 P2).
Data presented as mean +/- SD, for a single experiment performed in triplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF.
**p<0.01, ***p<0.001 versus osteogenic control sample; $^{+++}$p<0.001 versus basal control.

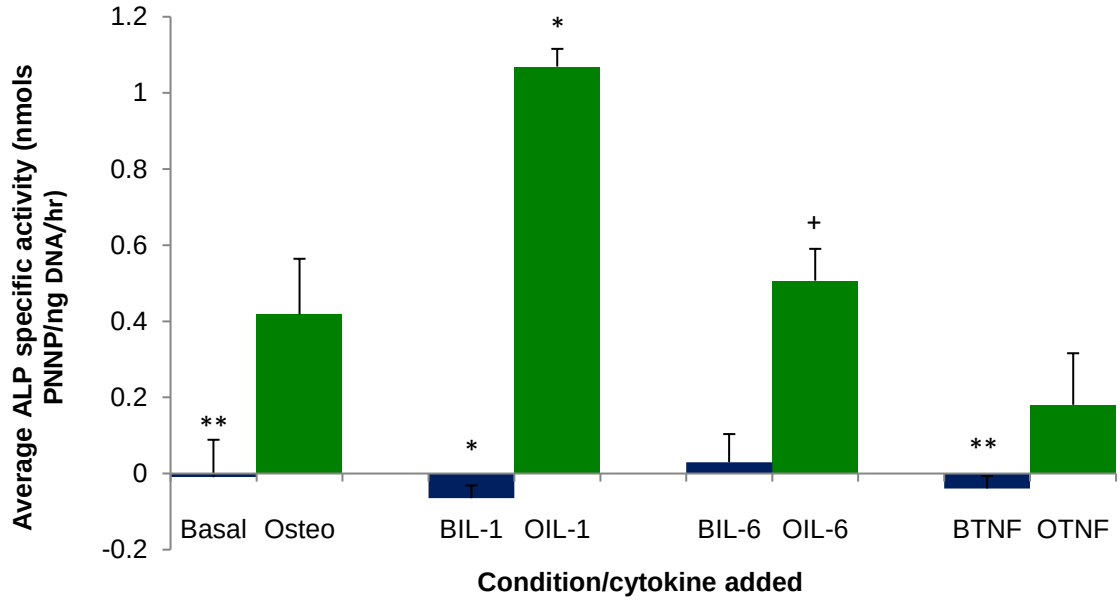


Figure 3.31 ALP specific activity of unselected HBMSC at Day 7, with or without cytokines and 1mM strontium (F67 P2).
Data presented as mean +/- SD, for a single experiment performed in triplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF.
*p<0.05, **p<0.01 versus osteogenic control sample; $^{+}$p<0.05 versus basal control.

This additive osteogenic effect of IL-1β was most marked for the Stro-1+ve cell experiments at the 0.1mM concentration of strontium (Figure 3.32).

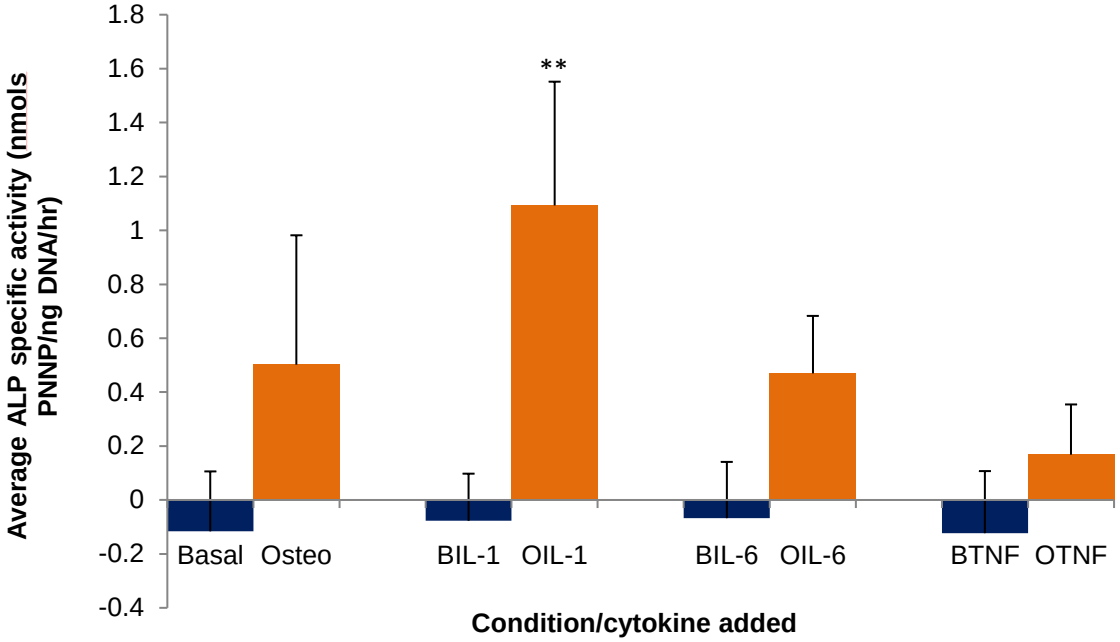


Figure 3.32 ALP specific activity of Stro-1 +ve HBMSC at Day 7, with or without cytokines and 0.1mM strontium.
Data presented as mean +/- SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. **p<0.01 versus osteogenic control sample.

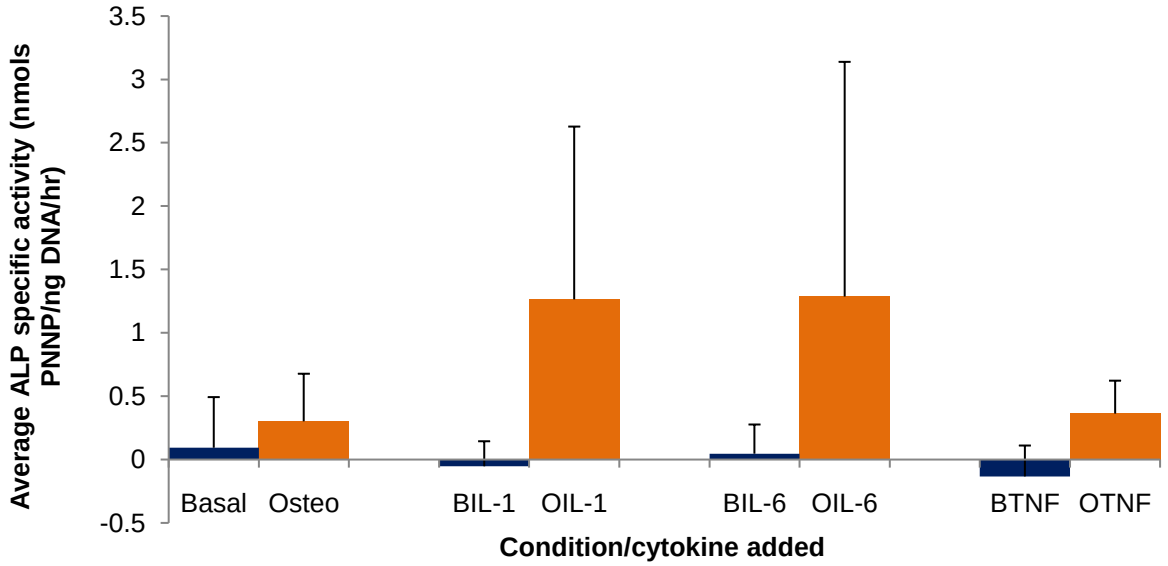


Figure 3.33 ALP specific activity of Stro-1 positive HBMSC at Day 7, with or without cytokines and 1mM strontium.
Data presented as mean +/- SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. Differences between the groups did not meet statistical significance due to high intersample variation.

3.3.2.3 Molecular biology

In contrast to the histology and biochemical data discussed above, molecular analysis revealed a significant inhibitory effect of IL-1β on ALP gene expression in the strontium containing cultures, at both the 0.1mM and 1mM concentrations of strontium (Figures 3.35 and 3.36). Although there was a trend to this in the Stro-1 positive HBMSC control cultures, this did not attain significance, suggesting that it was potentially related to the inclusion of strontium in the cultures (Figure 3.34). TNFα produced an inhibitory effect in all cultures, in keeping with findings on histology and biochemical analysis (Figures 3.34-3.36). This effect attained statistical significance in both of the strontium groups. IL-6 produced a significant increase in ALP gene expression in the 0.1mM strontium cultures, which was then abolished in the 1mM strontium cultures (Figure 3.35 and 3.36).

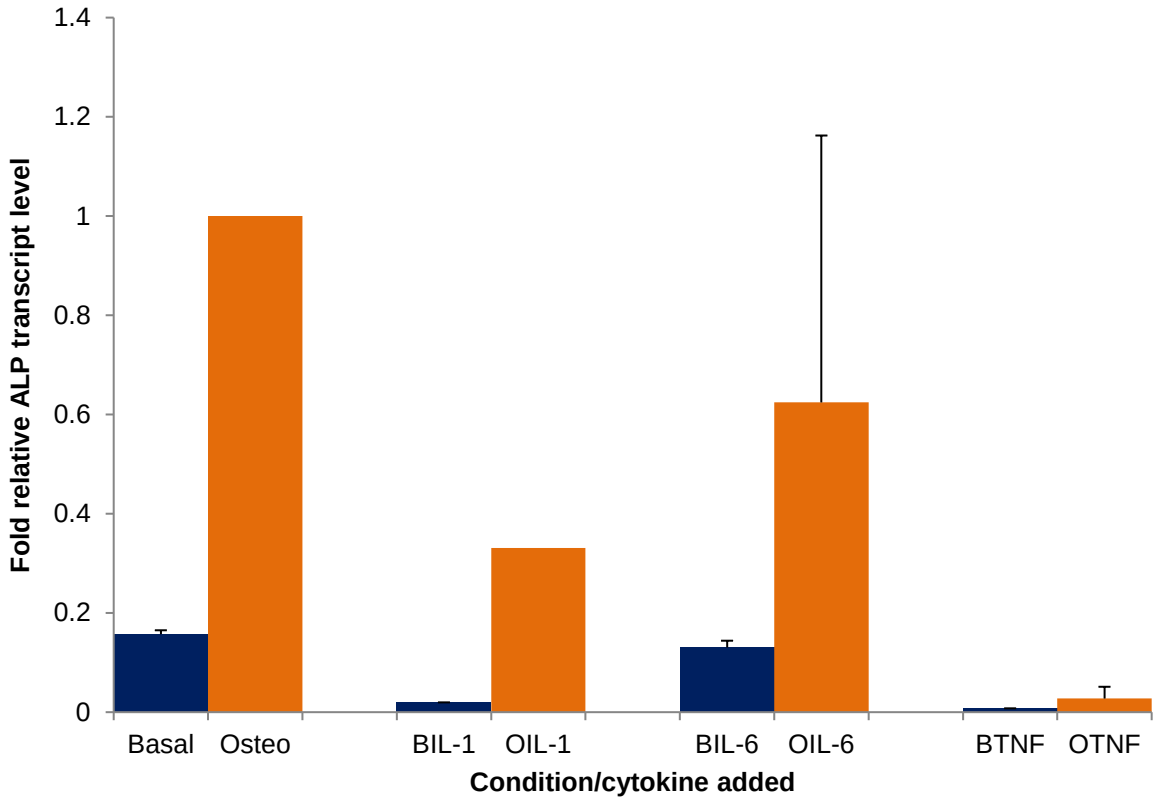


Figure 3.34 ALP expression of Stro-1 positive HBMSC at Day 7, with or without cytokines, control experiment

Data presented as mean ALP expression relative to β actin +/- SD, for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. Differences between the groups did not meet statistical significance.

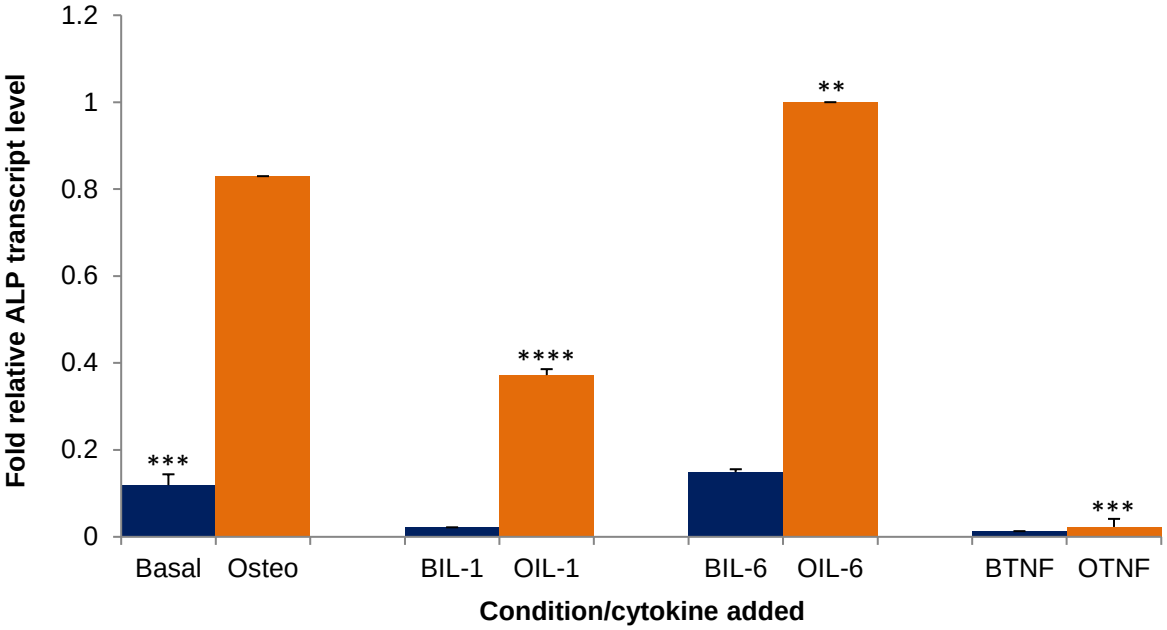


Figure 3.35 ALP expression of Stro-1 positive HBMSC at Day 7, with or without cytokines and 0.1mM strontium

Data presented as mean ALP expression relative to β actin +/- SD, for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. **p<0.01 ***p<0.001 ****p<0.0001 versus osteogenic control sample.

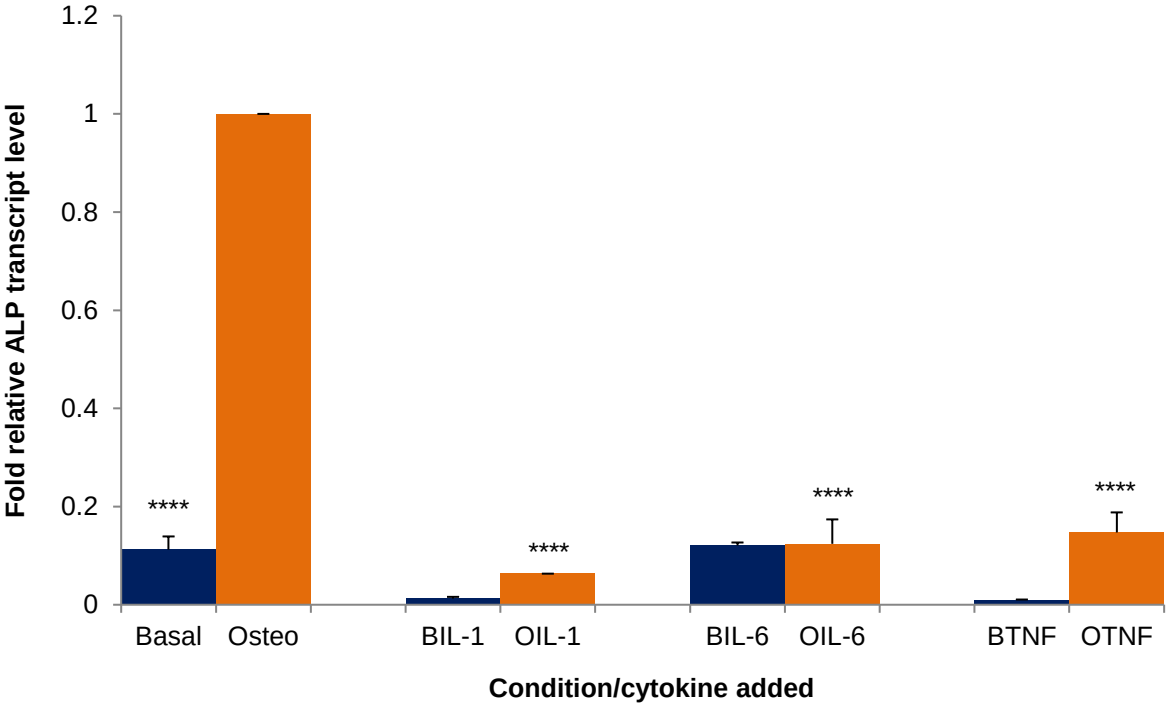


Figure 3.36 ALP expression of Stro-1 positive HBMSC at Day 7, with or without cytokines and 1mM strontium

Data presented as mean ALP expression relative to β actin +/- SD for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. ****p<0.0001 versus osteogenic control sample.

When the expression of other osteogenic genes was examined, including collagen-1 (COL-1), this showed more consistency with the histological and biochemical results described above. Although not achieving statistical significance, addition of IL-1β produced an additive osteogenic effect in terms of increasing collagen-1 gene expression, in both control and strontium experiments (Figures 3.37-3.39). The effect was most marked in the 0.1mM strontium cultures, attenuating slightly in the 1mM cultures. TNFα produced a mild inhibitory effect on collagen-1 gene expression in the strontium cultures, most marked at the 1mM concentration of strontium. IL-6 produced an increase in collagen-1 gene expression in control and 1mM strontium cultures.

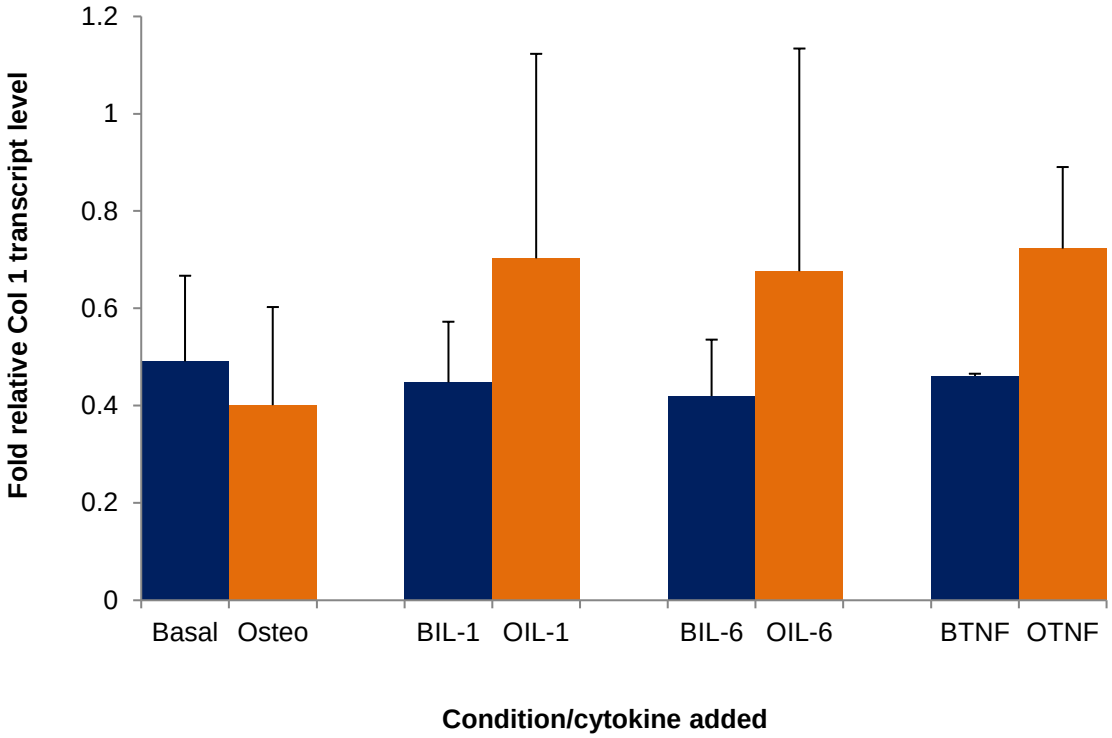


Figure 3.37 Collagen 1 expression of Stro-1 positive HBMSC at Day 7, with or without cytokines, control experiment

Data presented as mean Col-1 expression relative to β actin +/- SD, for a single experiment performed in Duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. Differences between the groups did not meet statistical significance.

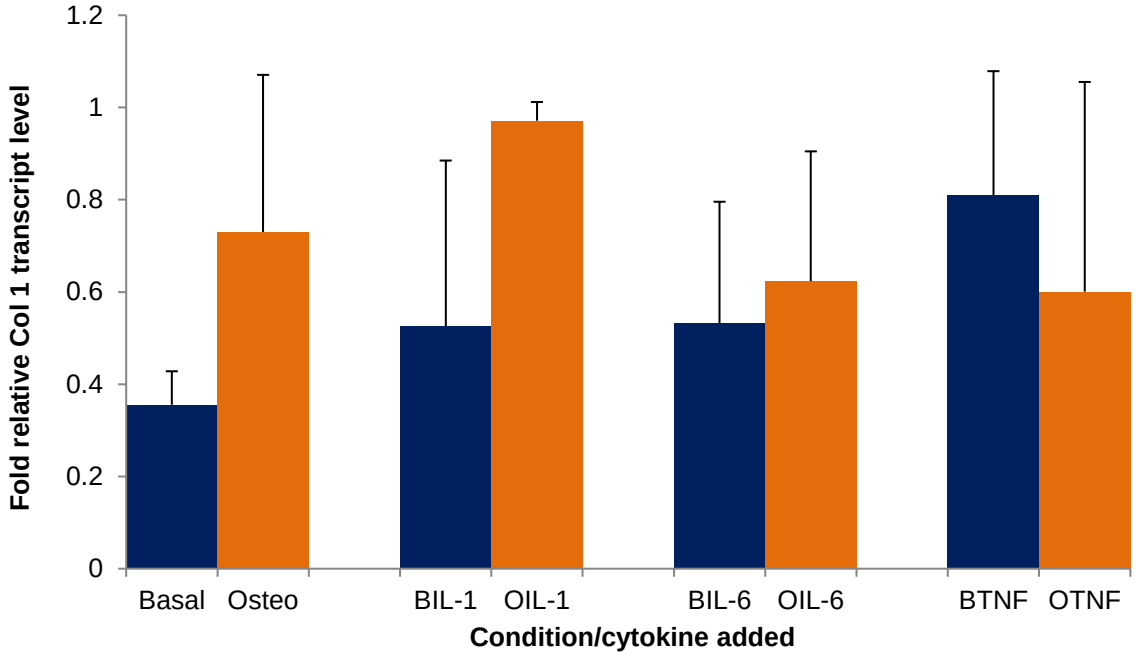


Figure 3.38 Collagen 1 expression of Stro-1 positive HBMSC at Day 7, with or without cytokines and 0.1mM strontium

Data presented as mean Col 1 expression relative to β actin +/- SD, for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. Differences between the groups did not meet statistical significance.

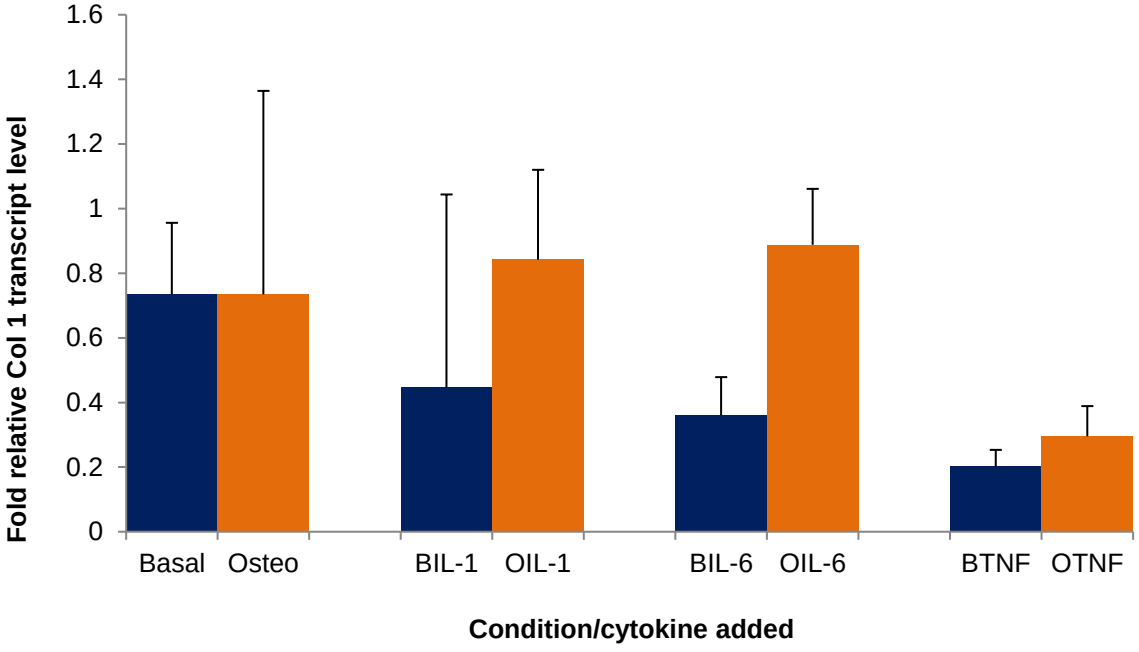


Figure 3.39 Collagen 1 expression of Stro-1 positive HBMSC at Day 7, with or without cytokines and 1mM strontium

Data presented as mean Col-1 expression relative to β actin +/- SD, for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media +IL-1, BIL-6=Basal media+IL-6, OIL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. Differences between the groups did not meet statistical significance.

3.3.2.4 Discussion

The addition of strontium to HBMSC in culture produced an additive osteogenic effect as assessed by ALP staining (Figure 3.28). There was a trend to significance on biochemistry and PCR analyses but this did not attain statistical significance. The additive osteogenic effects of IL-1β, described in the baseline cytokine experiments (Section 3.2), were maintained in the strontium experiments and were most marked on biochemical analysis of the Stro-1 positive HBMSC cultures containing 0.1mM strontium (Figure 3.32). Data from rt-PCR were somewhat conflicting with reduced ALP gene expression in control and strontium cultures (Figures 3.34-3.36) but a trend to an additive osteogenic effect with increased collagen 1 gene expression (Figures 3.37-3.39). IL-6 had some stimulating effect on ALP production in one of the Stro-1 positive HBMSC cultures but this was not significant in the summary data. IL-6 significantly increased ALP gene expression in the 0.1mM strontium HBMSC cultures but not the 1mM strontium cultures, where it had a significant inhibitory effect. It had a small stimulatory effect on collagen 1 gene expression in both control and 1mM strontium cultures, less so in the 0.1mM strontium cultures. TNFα was inhibitory in terms of results from ALP staining, measurement of ALP production and ALP and collagen 1 gene expression, in keeping with data from baseline cytokine experiments (Section 3.2).

There has been increasing interest in using strontium to treat osteoarthritis over recent years, given its known efficacy in osteoporosis and mixed anti-resorptive and anabolic effects on bone. Support for this hypothesis came from some post hoc analyses of the SOTI (Spinal Osteoporosis Therapeutic Intervention) and TROPOS (Treatment of Peripheral Osteoporosis) trials (Bruyere, Delferriere et al. 2008). 22% of these patients had OA symptoms at baseline. Of these patients, 42% of those in the strontium treated group had a lower overall OA score over a 3 year period, with a 33% reduction in disc space narrowing score. 34% of patients had less back pain. This led to the development of a phase III multicentre, randomised double-blind, placebo-controlled trial, SEKOIA (Strontium Ranelate Efficacy in Knee Osteoarthritis Trial). The results of the SEKOIA trial demonstrated that patients with knee OA receiving either 1g or 2g strontium ranelate per day had significantly less progression of their knee OA, as defined by reduced joint space narrowing and improvement of symptoms in the 2g/day group (reduced WOMAC scores including pain and physical functioning domains) (Cooper, Reginster et al. 2012). This suggested that strontium may have a role as a potential disease-modifying OA drug (DMOAD). However, in 2014, the

European Medicines Agency (EMA) Pharmacovigilance Risk Assessment Committee (PRAC) investigated reports of some serious adverse events, including cardiovascular events. As a result, since that time strontium's use has been restricted to those without cardiovascular risk factors and a history of or risk factors for venous thromboembolism. Since then, there has been increased interests in how the beneficial effects of strontium on bone might be incorporated into tissue engineering strategies to deliver local benefit within the affected joint whilst minimising any risks associated with systemic treatment with strontium.

Given these findings regarding the beneficial effects of strontium in both OA and osteoporosis, it was a logical progression to investigate whether strontium could be of benefit in tissue engineering strategies to stimulate osteogenic differentiation. The unselected HBMSC and Stro-1 positive cultures presented here offered an ideal system in which to assess not only the effects of strontium on osteogenic differentiation of SSCs but also how inflammatory cytokines, present in variable concentrations in OA and RA patients, might influence the degree of benefit the patient experienced after treatment. Strontium had no adverse effects on cell proliferation but there was a dose-response relationship between strontium and the degree of stimulation of osteogenic differentiation. The optimal dose was the lower 0.1mM concentration in the majority of the cultures assessed. IL-1β still exerted an additive osteogenic effect when strontium was added the SSC cultures and likewise, TNFα retained its inhibitory effect. Reviewing the literature allowed comparison of our results with work published to date, validating the doses of strontium chosen, as well as providing insights into potential mechanisms of action of both strontium alone and in combination with cytokines (Figures 3.40-3.41).

The precise mechanisms through which strontium exerts a beneficial effect in OA were initially unclear but are now thought to involve the following: inhibition of subchondral bone resorption through effects on osteoprotegerin (OPG) (Narmazi 2012), RANKL and matrix metalloproteinases (MMP-2 and MMP-9) (Tat, Pelletier et al. 2011); direct actions on cartilage; stimulation of proteoglycan synthesis and thereby cartilage matrix formation (Reginster, Beaudart et al. 2013, Riera, Martimbianco et al. 2017) (Figure 3.41). In terms of harnessing these beneficial effects of strontium to create tissue engineering based treatments for OA, multiple strategies have been investigated including coating scaffolds, creating novel alloys, coating bioceramics with it, or incorporating strontium into glass beads, bone cements or hydroxyapatite gels. Analysis of the effects of these novel materials on

animal and human SSC growth and differentiation has given crucial insights into the pathways involved in the modulation of SSCs by strontium.

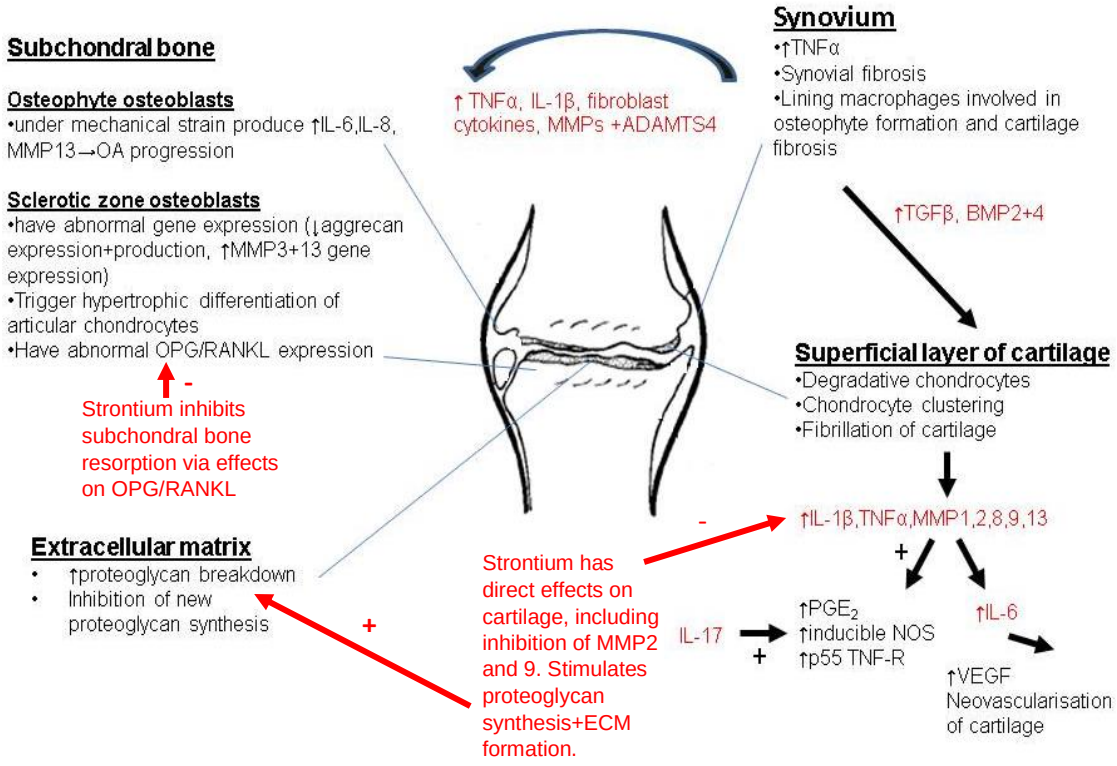


Figure 3.40 Summary of suggested pathways in the pathophysiology of OA that are thought to be influenced by strontium
(adapted from (Williams, Edwards et al. 2012)).
TNFα – tumour necrosis factor alpha; IL – interleukin; MMP – matrix metalloproteinase; ADAMTS-4 – ADAM metallopeptidase with thrombospondin type 1 motif, 4; TGFβ – transforming growth factor beta; BMP – bone morphogenetic protein; PGE2 –prostaglandin E2; iNOS – inducible nitric oxide synthase; p55 TNF-R – p55 tumour necrosis factor receptor; VEGF – vascular endothelial growth factor.

Cultures of rat bone marrow-derived SSCs in osteogenic or adipogenic media for 1-2 weeks, with the addition of 0.1mM or 1mM strontium showed a dose dependent increase in osteoblastic differentiation and a reduction in adipogenic differentiation compared to controls (Li, Li et al. 2012). This was demonstrated through increased expression of ALP, increased levels of mRNA expression of core binding factor α1 (Cbaf1), Runx-2, bone sialoprotein (BSP) and osteocalcin (OCN) in the cultures with osteogenic media and strontium. This increase in ALP production with the addition of strontium is in keeping with the changes in ALP production for the HBMSC and strontium cultures described above (Section 3.3.2.2), using the same concentrations of strontium. Further experiments using rat BMSC cultures incorporating 0.1 - 3mmol/L concentrations of strontium also demonstrated increased ALP

activity and increased formation of calcium nodules (Li, Wang et al. 2011). Addition of strontium to these cultures was also found to be associated with a dose-dependent increase in bone morphogenetic protein expression (BMP-7). When BMP-7 was suppressed using the inhibitor Noggin, this led to a reduction in ALP expression and calcium nodule formation, thus providing confirmation of the importance of BMP-7 in mediating the osteogenic effects of strontium on BMSCs. Another group performed further studies in which rat SSCs were cultured with strontium at concentrations between 0.1 and 10mmol/L (Lv, Huang et al. 2013). The same increase in ALP expression and mineralised nodule formation occurred with the addition of strontium as in the studies mentioned above. A significant increase in Runx-2 expression and phosphorylation of Smad 1,5 and 8 was seen, peaking at 1mmol/L strontium. Addition of Noggin or Smad 1 siRNA to these cultures resulted in abrogation of this increase in ALP expression and mineralised nodule formation, indicating that bone morphogenetic BMP-2/ Smad signalling, as well as BMP-7, are crucial to the strontium-induced stimulation of osteogenic differentiation of rat BMSCs. Other studies in BMSCs from ovariectomised rats, where the addition of strontium was once again associated with increased expression of ALP, COL-1, Runx-2, OCN, BMP-2, BSP and Osteoprotegerin (OPG), together with reduced RANKL, suggested strontium concentrations of 0.25-0.5mM might be optimal for local application via tissue engineering strategies (Guo, Wei et al. 2016).

Stimulatory effects of strontium on osteogenic differentiation have also been reported recently in human adipose-derived SSC cultures (Aimaiti, Maimaitiyiming et al. 2017). Here, the addition of 25-500μM concentrations of strontium was associated with increased ALP expression, calcium deposition and increased expression of Runx-2, ALP, Collagen 1 and Osteocalcin on rt-PCR. Interestingly, in these hADSSC cultures, as opposed to the rat BMSSC cultures described above, concentrations of strontium between 1000 - 3000μM appeared to exert an inhibitory effect on these parameters, mediated through phosphorylation of ERK1/2 signalling (Aimaiti, Maimaitiyiming et al. 2017). This builds on previous work on the effects of strontium on the RAS/MAPK signalling pathway (Peng, Zhou et al. 2009). This demonstrated that strontium significantly increased osteoblast-related gene expression and ALP production in osteogenic-differentiating SSCs. Both transcriptional activity and phosphorylation of Runx-2 significantly increased in strontium treated SSCs (Peng, Zhou et al. 2009). Further functional studies showed that the Rat Sarcoma viral oncogene homolog (RAS), an upstream regulator of ERK 1/2 and p38, was activated by adding strontium to SSCs in culture. Either selective inhibition of ERK 1/2 +p38 or siRNA -mediated knockout of RAS, attenuated this strontium -induced stimulation of

osteoblast-related gene expression via Runx-2. This confirmed that strontium promotes osteogenic differentiation through activation of the RAS/MAPK signalling pathway and the downstream transcription factor Runx-2 (Peng, Zhou et al. 2009). The more recent data demonstrating that there is a dose-dependent effect of strontium on these pathways (Aimaiti, Maimaitiyiming et al. 2017), could explain the attenuation of the increase in ALP production and ALP gene expression seen at the higher 1mM concentration of strontium in some of our HBMSC cultures described above (Sections 3.3.2.2 and 3.3.2.3).

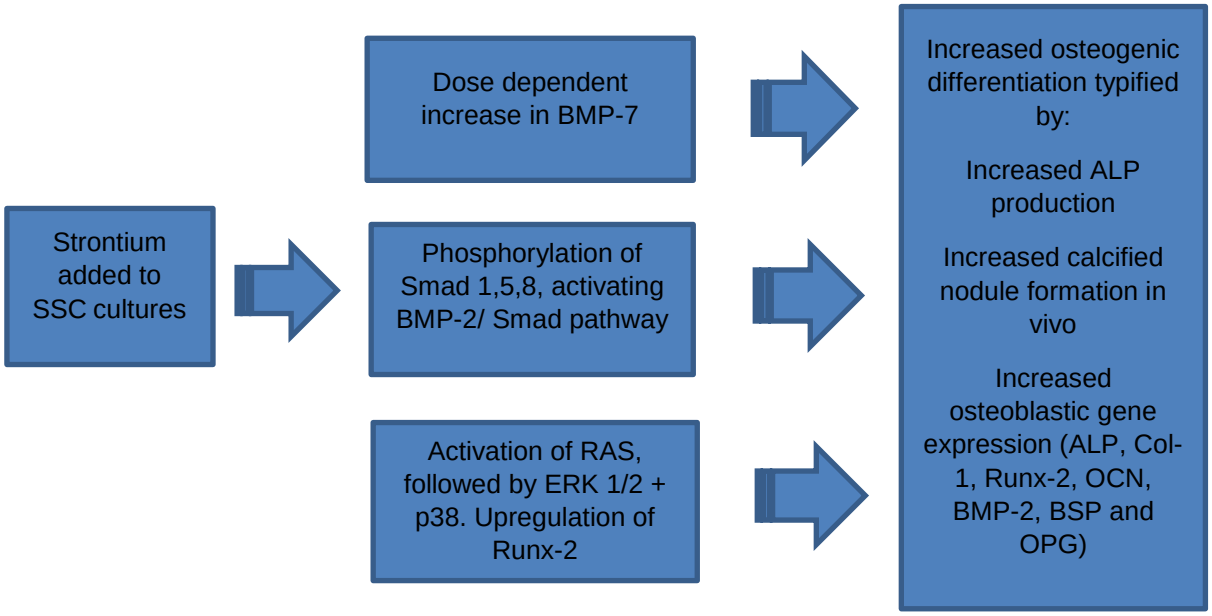


Figure 3.41 Schematic diagram summarising the proposed mechanisms through which strontium exerts its stimulatory effects on osteogenic differentiation of SSCs
BMP-7 - bone morphogenetic protein -7; BSP - bone sialoprotein; ERK 1/2 - extracellular signal-regulated kinase 1/2; OPG - osteoprotegerin; RAS - rat sarcoma viral oncogene homolog; Smad-1 – mothers against decapentaplegic homolog 1.

Another group assessed the effects on HBMSC growth and differentiation of introducing strontium ions either as a dissolved salt (as in our HBMSC experiments) or incorporating them into a bioactive calcium phosphate coating (Birgani, Malhotra et al. 2016). Both forms of strontium increased ALP activity in a dose dependent manner and increased expression of ALP, BSP, BMP-1, OPN and OCN mRNA. This supports the strategy of harnessing the beneficial effects of strontium on osteogenic differentiation by incorporating strontium ions directly into a scaffold for use in tissue engineering strategies. Further studies incorporating strontium into hydroxyapatite monohybrid scaffolds with 0, 1, 5 and 10% strontium concentrations showed excellent adhesion, spreading and proliferation of HBMSCs,

especially at the 5% concentration (Lei, Xu et al. 2017). This was mirrored by increases in ALP production, extracellular matrix mineralisation and expression of ALP and COL-1 on rt-PCR. The precise concentration of strontium for optimal effects on osteogenic differentiation therefore seems to vary dependent on cell type and culture/scaffold properties with a suggested range from 0.025mM – 1mM being favoured at present.

As well as using strontium as a bioactive factor, the properties of the scaffold/implant can be used to promote osteogenic differentiation. A recent example of utilising this approach is the creation of a strontium containing nanolayer on plasma sprayed calcium silicate coating for orthopaedic implants (Hu, Li et al. 2017). The nanotopography itself promoted growth of the HBMSC into a polygonal osteoblastic shape and stimulated collagen expression via activation of integrin β1. The addition of strontium lead to increased ALP production and matrix mineralisation, indicative of increased osteogenic differentiation. Analysis of gene expression revealed that strontium mediated these effects through activation of an extracellular calcium-sensitive receptor (CASR). These nanotopographical cues, together with activation of CASR, then led to increased expression of Runx-2, resulting in increased expression of the other genes involved in osteoblastic differentiation including BMP-2, BSP, OPN and OCN. Simultaneously, IL-6 and the RANKL/RANK pathway was downregulated, inhibiting osteoclastogenesis (Hu, Li et al. 2017).

There is also evidence from both *in vitro* and *in vivo* studies in human SSCs that strontium exerts an influence over the Wnt/β catenin pathway (Yang, Yang et al. 2011). Incorporating strontium into human SSC cultures resulted in increased extracellular matrix gene expression, consistent with osteogenic differentiation and was shown to be due to activation of the Wnt/β catenin pathway. When the SSCs were seeded onto a collagen-strontium substituted hydroxyapatite scaffold and implanted into a rat calvarial defect model, histology and μCT showed improvement in bone quantity in the transplanted area, both in terms of new and remodelled bone, and extracellular matrix deposition. Increased levels of β catenin were found within this newly formed bone (Yang, Yang et al. 2011).

In summary, the baseline cytokine studies described in Section 3.2. demonstrated *that IL-1β produced an additive osteogenic effect on HBMSC in culture. IL-6 had a negligible effect and TNFα produced a milder additive osteogenic effect in some cultures.* Data from the literature supports the role of IL-1β as the most potent cytokine for enhancing osteogenic differentiation in cells exposed to osteogenic media, mediated via the mWnt5a-Ror2 pathway (Sonomoto, Yamaoka et al. 2012). In this substudy, strontium produced a mild stimulatory effect on the osteogenic differentiation of HBMSC. Data from the literature reviewed above

suggests that this stimulation of osteogenic differentiation is mediated through a variety of mechanisms including: BMP-2/Smad signalling; stimulation of BMP-7; RAS/MAPK signalling and the Wnt/β catenin pathway. IL-1β was still able to produce a significant osteogenic effect in terms of ALP staining, ALP production and Col-1 gene expression on rt-PCR, although expression of other osteoblastic genes was less consistent. TNFα produced a significant inhibitory effect on osteogenic differentiation in the strontium cultures, suggesting that this potentially interfered with one of the signalling pathways mentioned above, through which strontium mediates its effects.

Overall, this suggests that *IL-1β and strontium have potential roles as bioactive factors* for incorporating into tissue engineering strategies to promote osteogenic differentiation and provide a targeted treatment for early to moderate OA in the future.

Summary

- As in baseline cytokine experiments, IL-1β produced an additive osteogenic effect on HBMSC in culture.
- IL-6 had a negligible effect, whilst TNFα exerted an inhibitory effect on osteogenic differentiation of HBMSC in culture.
- Strontium itself produced a mild stimulatory effect on osteogenic differentiation of HBMSC.
- IL-1β, both in current work and in the literature, is the key cytokine for enhancing osteogenic differentiation in OA.
- Effects on osteogenic differentiation thought to be mediated through multiple mechanisms including: BMP-2/Smad signalling; stimulation of BMP-7; RAS/MAPK signalling and the Wnt/β catenin pathway.
- Both IL-1β and strontium have a role as potential bioactive factors that could be harnessed to improve the success of future targeted tissue engineering strategies for the treatment of OA.

3.3.2.5 Limitations and future work

Due to a finite supply of bone marrow samples to generate cultures from, the maximal n number for any given experiment was 3, which resulted in high standard deviations if there was considerable inter-sample variability in results. This made some the analyses more heterogeneous and challenging to interpret. Despite good laboratory practice, infections of

incubators and cultures, either one's own or those of colleagues, did on occasion result in loss of a whole experiment, which could not then be repeated.

In order to confirm the stimulatory effect of IL-1β, and the inhibitory effect of TNFα in HBMSC cultures, further studies will be needed to establish the effects of inhibiting these individual inflammatory cytokines on both unselected HBMSC and Stro-1+ve cell differentiation. However, the work summarised herein provides sufficient supporting evidence of the important roles that both IL-1β and strontium have in modulating osteogenic differentiation in OA, to open up an exciting avenue of future research aimed at incorporating this combination of bioactive factors into targeted tissue engineering strategies for the treatment of OA.

Chapter 4 The effects of synovial membrane supernatant on differentiation of HBMSCs

4.1 Introduction

Skeletal stem and progenitor cells (SSCs), often referred to as mesenchymal stem cells (MSCs), from a variety of tissue sources have been increasingly investigated as a future treatment strategy for OA (Sakaguchi, Sekiya et al. 2005, Alegre-Aguaron, Desportes et al. 2012, Lee, Hah et al. 2015, Garcia, Wright et al. 2016, Lee, Park et al. 2016, Pagani, Borsari et al. 2017, Feng, Luo et al. 2018, Jia, Liang et al. 2018). However, the inflammatory milieu now recognised to exist within OA joints represents a potential caveat to the use of such tissue engineering strategies in patients with arthritis. Despite this, as yet, little work has been done to elucidate the effects of pro-inflammatory cytokines on tissue engineering strategies. In Chapter 3, the effects of the individual pro-inflammatory cytokines IL-1β, IL-6 and TNFα on the growth and differentiation of both HBMSC and SSCs were established. However, in any given arthritic joint it is the combinations of cytokines and their interactions that bring about joint damage and synovial supernatants provide a unique means of accessing this cocktail of cytokines. The current experiments were therefore designed to assess the effects of synovial supernatants from patients with OA on HBMSCs in culture, working on the basis that this would be a more accurate *in vitro* equivalent of the inflammatory milieu within an OA joint. The data generated will then be used to move on to assessing the effects of synovial supernatants on other cell types currently being used for bone and cartilage tissue engineering (Chapter 5) and finally in *ex vivo* and *in vivo* models of bone formation (Chapter 6).

4.2 Overall aim

- To investigate whether the inflammatory cytokines present in OA produce a hostile environment that limits the use of bioengineering strategies to repair damaged joints.

4.3 Objectives

1. To determine the cytokine profiles of synovial supernatants from a cohort of OA patients undergoing joint replacement surgery.

2. To assess the effects of synovial supernatants from these OA patients on the growth and differentiation of HBMSCs, immunoselected SSCs and fetal femur cells in basal and osteogenic conditions.

Methods

4.4 Preparation of synovial supernatants from OA patients

4.4.1 Patient recruitment

Individuals were selected from Orthopaedic lists of patients due to undergo elective primary total hip or knee replacement surgery or synovectomy at Southampton General Hospital. Full ethical approval for the study was obtained from the Isle of Wight, Portsmouth and South East Hampshire Local Research Ethics Committee (LREC reference number: 08/H0501/58) (Appendix A1). Individuals were approached to participate in the study by Dr Williams in the Orthopaedic Pre-assessment clinics at Southampton General Hospital. Patients were given a copy of the Participant Information Sheet (Appendix A2) about the study and asked to confirm that they wished to take part in the study either by telephone or by returning a reply slip. Those who agreed to participate were contacted by telephone by Dr Williams and an appointment arranged in the Wellcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital. Informed consent was obtained from each patient prior to undertaking any part of the study protocol (Version 1, 01.04.2008, Appendix A3). The diagnosis of OA was determined by history and clinical examination by Dr Williams to ensure patients meet the ACR criteria. Demographic data and records of current drug therapies were recorded. The only samples used were surplus synovial tissue that would be removed routinely as part of hip or knee replacement surgery and discarded.

4.4.2 Sample collection

Synovial samples were collected at the time of surgery in a sterile universal. The sample was then transferred immediately to the laboratory and stored in RPMI 1640+L-glutamine+HEPES media (PAA, UK) at 2-8°C prior to processing. Samples were always processed within 24 hours to avoid degradation.

4.4.3 Preparation of synovial supernatants

(Protocol courtesy of Prof Brennan, Kennedy Institute of Rheumatology, Oxford)

Synovial supernatants were prepared according to the Kennedy Institute protocol described in Section 2.1.5 and summarised diagrammatically in Figure 4.1:

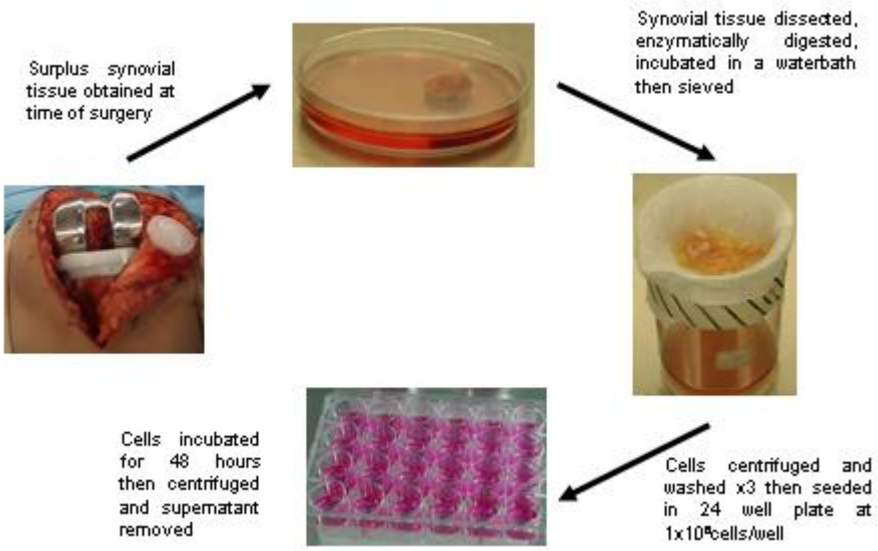


Figure 4.1 Summary of synovial supernatant preparation
Schematic diagram demonstrating extraction of synovium at the time of joint replacement surgery, through to synovial cell cultures just prior to the extraction of the supernatants by centrifugation.

4.4.4 Quantification of cytokines in synovial supernatants

Individual aliquots of each supernatant were defrosted and cytokine content estimated using commercially available ELISA kits (Quantikine®, R&D Systems, UK) for detection of IL-1β, IL-6 and TNFα as described in Section 2.6.

4.5 Experimental set up

4.5.1 Establishment of an experimental protocol

Cultures of human bone marrow stromal cells (HBMSCs) were set up following preparation of bone marrow samples according to the protocol described in Section 2.1.1. The cells were cultured initially in basal media for 6 days, washed with PBS and fresh media applied. Further media changes were carried out every 2-3 days (as detailed in Section 2.1.3) until the cells reached 95% confluence. The cells were then released from the surface of the tissue culture plastic by treatment with collagenase followed by trypsin (detailed in

Section 2.1.4). The cell suspension obtained was centrifuged (1100rpm for 4 minutes), the cell pellet re-suspended in 10mls of basal media and a cell count performed using a haemocytometer (Section 2.1.1). Cells were seeded at $1x10^5$ cells per well of a 6 well plate in either basal or osteogenic media. Single wells were labelled for used in histological analysis (ALP, type I and type II collagen) and ALP and DNA biochemistry assays, whilst 3 wells in each media were reserved for PCR analysis due to the larger cell numbers required. Cultures were run for a total of 7 days prior to analysis.

4.5.2 Final adopted protocol

Cells were seeded at $5x10^4$ cells per well of a 24 well plate in either basal or osteogenic media. Each of these two groups was then subdivided further into 10 groups: controls (no additional factors) and HBMSC + supernatant (numbered by patient of origin from 4 to 12 (see Table 4.1)). A selection of supernatants were used in each experiment to try and capture the effects of as a broad a range of concentrations of the individual cytokines as possible. For each of these 10 conditions, single wells were labelled for used in histological analysis (ALP, type I and type II collagen and cell viability staining) and duplicate wells for ALP and DNA biochemistry assays. The remaining cells were seeded at $1x10^5$ cells per T25, into 20 separate T25s (one for each of the 10 conditions being used in each of basal and osteogenic media) for use in PCR analysis due to the larger cell numbers required. Media changes were performed every 2-3 days. Cultures were run for a total of 7 days prior to analysis.

4.5.3 Statistics

Statistical calculations were performed using GraphPad Prism software version 7.04. Data were expressed as mean +/- standard deviation (SD). Comparisons for biochemistry and PCR data were carried out using one-way analysis of variance (ANOVA). If the ANOVA was significant, the Tukey-Kramer multiple comparison test was used as a post hoc test. For all analyses a *p* value less than 0.05 was considered significant. For analysis of the relationship between patients' clinical characteristics and the cytokine profile of synovial supernatants, additional analysis was performed using Stata software to generate summary statistics. Spearman correlation was used to describe the association of continuous predictors with outcome, and the Kruskal - Wallis test for categorical predictors.

4.6 Results

4.6.1 Histology

Images captured at the end of 7 day cultures confirmed typical growth and differentiation of HBMSCs according to the culture media used. Cell viability, as measured using Cell Tracker Green/Ethidium homodimer staining was excellent across all cultures. HBMSCs cultured in osteogenic media demonstrated positive staining for ALP as expected. (Figure 4.2). The degree of positive staining for ALP under osteogenic conditions varied across the different synovial supernatants used.

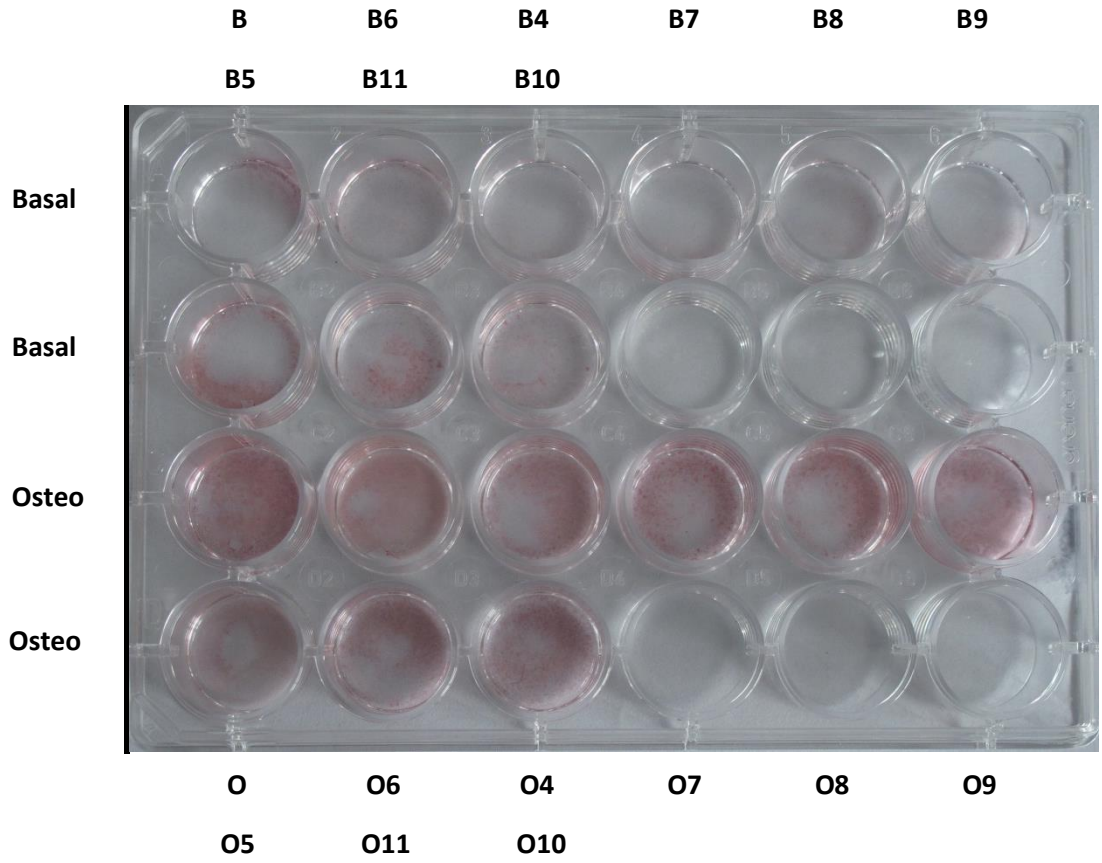


Figure 4.2 Alkaline phosphatase staining of HBMSC with or without synovial supernatants at Day 7 (F68)
B=Basal control, O=Osteogenic control, numbers 1-10 indicate different patients' supernatant samples. Osteogenic culture wells demonstrated increased pink/purple staining for ALP compared to basal cultures as predicted, with variation across the different supernatants.

4.6.2 Cytokine profiles of supernatants

The levels of IL-1β, IL-6 and TNFα in the individual synovial supernatants were determined using separate Quantikine® ELISA kits for each cytokine (as per the protocols outlined in Section 2.6) (Figures 4.3-4.5). The demographics for the first 20 patients used to derive synovium for analysis and culture are displayed in Table 4.1. Although no definitive conclusions may be drawn between the patients' individual disease pattern and their cytokine profile or supernatant effects in culture given such small patient numbers, this provides interesting preliminary data.

Table 4.1 Patient demographics for those who donated synovium for the supernatant studies
The patient ID numbers correspond to the numbers used for the ELISA cytokine profiling and those displayed on the biochemistry and molecular biology results graphs. OA = osteoarthritis, RA = rheumatoid arthritis, OP = osteoporosis, SLE = systemic lupus erythematosus, CCB = craniocleidodysostosis, KL= Kellgren-Lawrence score for radiological severity of OA

ID no	Age	Gender	Diagnosis	Duration of disease in years (acute phase))	Medications	Operation	ESR	CRP	X-ray grade (KL score)
1	68	F	OA	0.75	Co-codamol, bendrofluazide,thyroxine	R THR	N/A	N/A	KL3
2	81	F	OA	7(3)	Naproxen, lansoprazole	RTKR	N/A	N/A	KL3
3	63	F	OA	6.5	Paracetamol, DF118, omeprazole, multivitamins, glucosamine, celebrex, cod liver oil	LTHR	N/A	N/A	KL3
4	52	M	OA	4.5(0.25)	Codeine	LTHR	N/A	N/A	KL3
5	66	F	OA	0.75	Paracetamol, tramadol, amitriptyline, ramipril, simvastatin, thyroxine	LTKR	5	10	KL1
6	65	F	OA	3.5(1)	Bendrofluazide, candesartan, lansoprazole	LTHR	15	6	KL3
7	75	F	OA	10(1)	Aspirin, bendrofluazide, candesartan, diltiazem XL, simvastatin	LTKR	15	<1	KL4
8	69	M	OA	40(1.25)	Bendrofluazide, fenofibrate	LTHR	N/A	N/A	KL4
9	70	F	OA	1(0.5)	Amitriptyline, bendrofluazide, levothyroxine, lisinopril, omeprazole, rosuvastatin,	RTHR	N/A	N/A	KL3
10	61	F	OA	2.5(1.5)	Co-codamol, doxazosin, loratadine, losartan, salmeterol inhaler, simvastatin, Qvar inhaler	LTHR	N/A	N/A	KL3
11	70	M	OA	1	Paracetamol, anti-histamine, atenolol, clopidogrel, pravastatin	LTHR	N/A	N/A	KL4
12	68	M	OA	30(5)	Paracetamol, MST, oramorph, atenolol, dermol, domperidone, flixonase, furosemide, fybogel, glandosane,GTN, hylotears, latanoprost, losartan, monomax, movicol, losartan, nicorandil, tostran, vesicare, warfarin	RTHR	N/A	N/A	N/A
13	74	F	OA	3(0.5)	Aspirin, bisoprolol, lansoprazole	L THR	N/A	N/A	KL4
14	69	M	OA	1	Co-codamol, BP tablet, tetracycline	R TKR	5	11	KL3
15	54	F	OA	8(5)	Amitriptyline, DF118, co-dydramol, celecoxib, fluoxetine, omeprazole, propranolol, simvastatin	R TKR	N/A	3	KL3
16	79	M	OA	12(2)	Paracetamol, movicol, tamsulosin	L TKR	N/A	N/A	KL3
17	69	F	OA, OP, SLE, CCD	15(4)	Paracetamol, atrovent inhaler, azithromycin, cefalexin, celluvisc, detrusitol, dermovate, doxazosin, epaderm, fexofenadine, flixonase, formoterol, gaviscon, gynest cream, hydroxychloroquine, lactulose, NaCl nebs, ostron calcium, Qvar inhaler, salamol inhaler, saliveze, vit D,E,B complex	R THR	22	1	KL4
18	75	F	RA, OA	2	Naprosyn, alendronate, calcichew, cosopt eye drops, myocrsin,	L THR	N/A	<1	KL4
19	64	F	OA	N/A	Etoricoxib, duotrar eye drops, thyroxine, mometasone nasal spray, venlafaxine, viscotears	R THR	N/A	2	KL3
20	83	M	OA	6(1)	Aspirin, atenolol, avodart, ISMN, simvastatin,	R THR	15	20	KL3

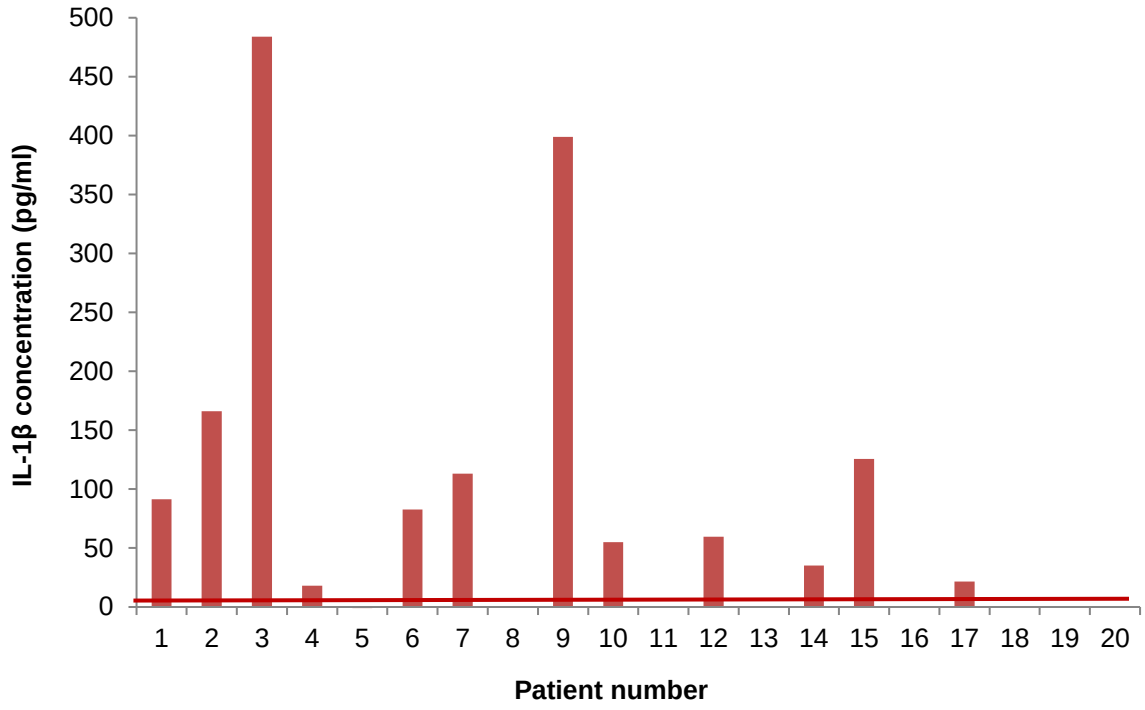


Figure 4.3 IL-1 concentrations of the patients' synovial supernatants determined by ELISA
The horizontal red line indicates the normal range for IL-1 in synovial fluid.

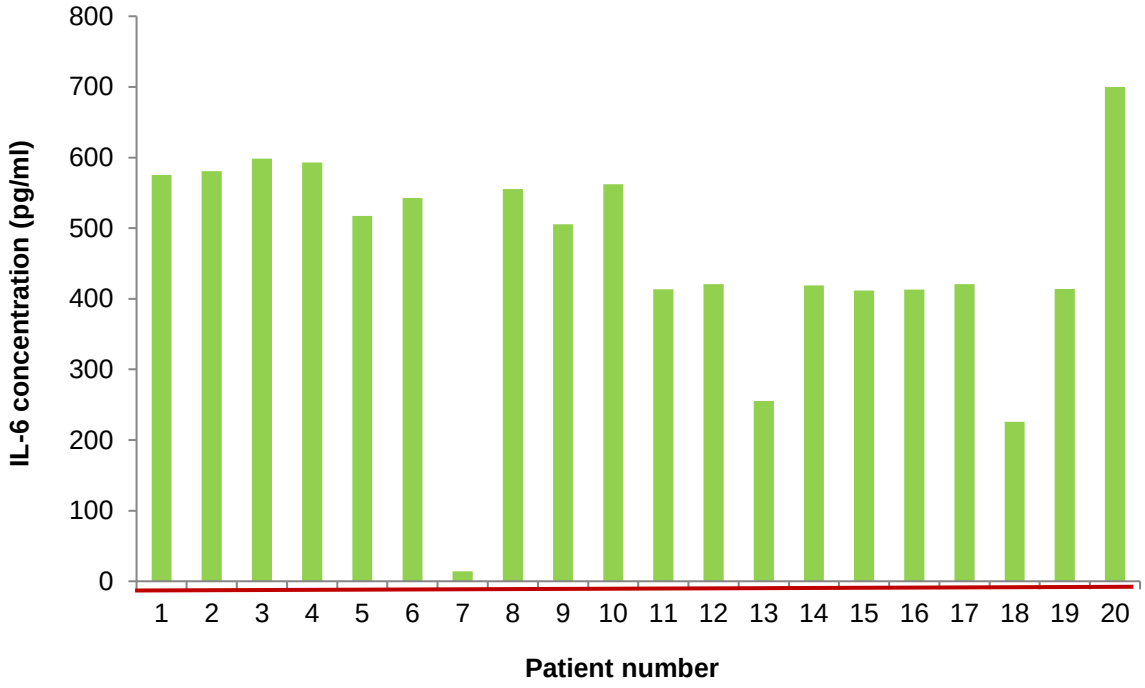


Figure 4.4 IL-6 concentrations of the patients' synovial supernatants determined by ELISA
The horizontal red line indicates the normal range for IL-6 in synovial fluid.

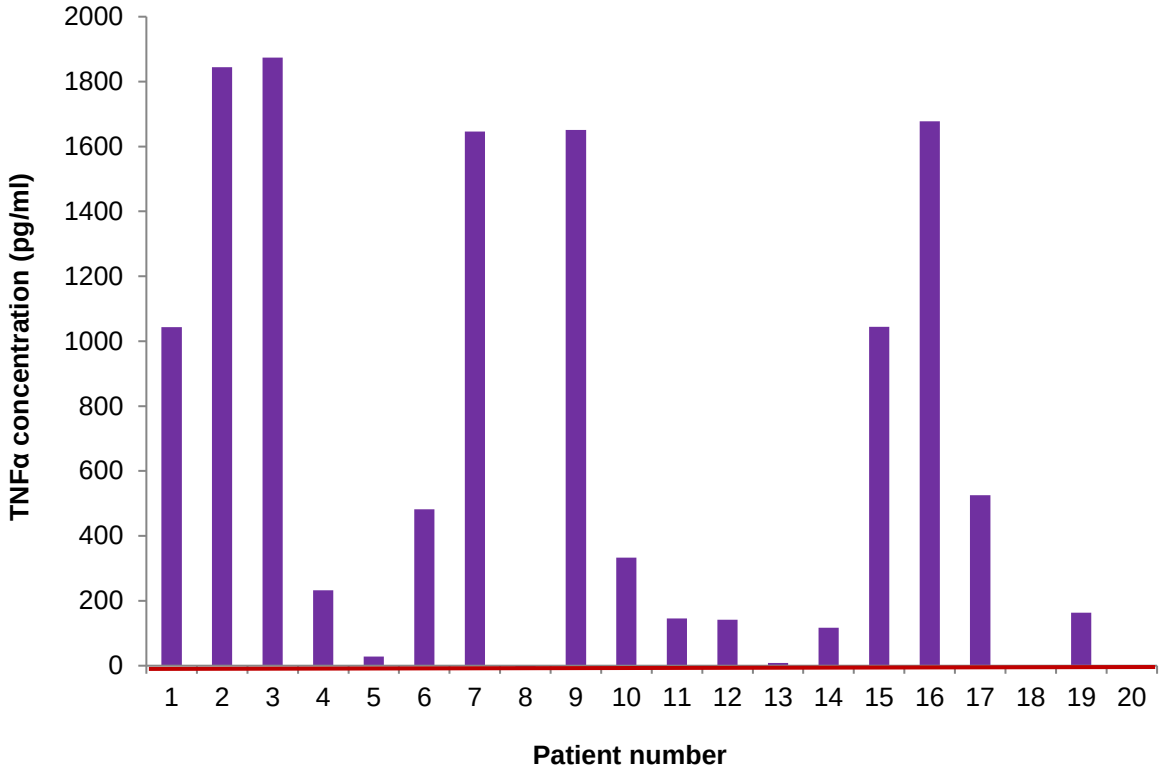


Figure 4.5 TNF alpha concentrations of the patients' synovial supernatants determined by ELISA
The horizontal red line indicates the normal range for IL-6 in synovial fluid.

IL-1 levels ranged from 0-399pg/ml, IL-6 from 14-700pg/ml and TNFα from 0-1874pg/ml. TNFα showed the greatest variability between patient samples, then IL-1β, with IL-6 concentrations remaining fairly constant. In terms of reference ranges, for IL-1β up to ~100pg was regarded as quiescent, for TNFα ~200-500pg, whereas for IL-6 levels could be much higher (personal communication from Prof Brennan). Statistical analysis of these data, together with patient demographics and disease characteristics, are summarised in Table 4.1. 2 patients had particularly high IL-1β concentrations in their synovial supernatants (Patients 3 and 9 in Table 4.3). Across the remaining patients, IL-1β concentrations increased in female patients, those undergoing total knee replacements (TKR) and with moderately severe OA, Kellgren-Lawrence (KL) grade 3. IL-6 concentrations in synovial supernatants were reduced in patients with severe OA (KL4). Concentrations of TNFα were increased in females, those undergoing TKR and patients with KL3 OA. There were insufficient samples to perform a full multivariate analysis but this would be possible with larger n numbers and forms part of the suggested further analysis.

4.6.3 Biochemical analysis

Addition of a small number of supernatants (S1 to S5), selected to provide a range of cytokine concentrations, to SSC cultures produced a marked osteogenic profile for the cultures in basal media, that was IL-1β and TNFα concentration dependent, correlating with lower supernatant dilutions on initial ELISA analysis (Figure 4.6 – *a* refers to 1:4 dilution of supernatant, *b* refers to 1:2 dilution). However, in osteogenic media there was a trend for ALP specific activity to fall relative to osteogenic control values when supernatants were added, although this did not reach statistical significance (Figure 4.6).

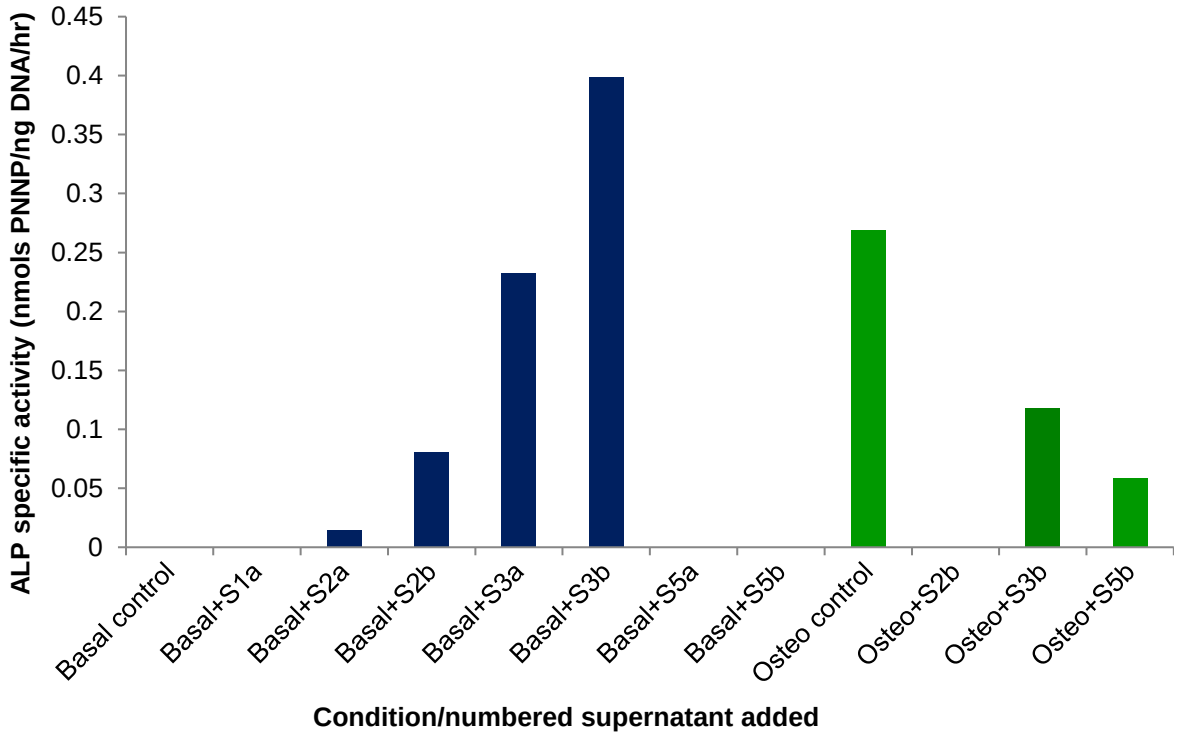


Figure 4.6 ALP specific activity of HBMSC with or without serial dilutions of synovial supernatants (M87)
Data are presented as mean ALP specific activity +/- SD for a single experiment performed in triplicate. S1=supernatant from patient 1, S2=supernatant from patient 2, S3=supernatant from patient 3, S5=supernatant from patient 3, the suffix ' a' refers to a 1:4 dilution of synovial supernatant, 'b' refers to a 1:2 dilution of synovial supernatant. Basal=basal media, Osteo=Osteogenic media. Differences between the groups did not meet statistical significance.

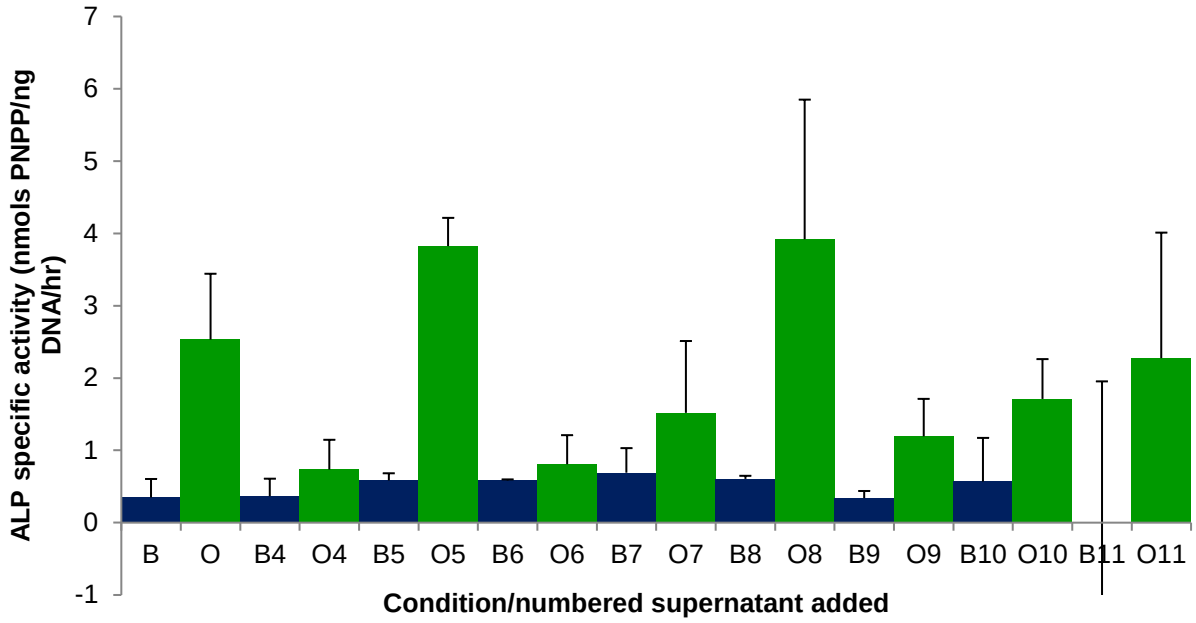


Figure 4.7 ALP specific activity of HBMSC with or without synovial supernatants (F68)
Data presented as mean +/- SD for a single experiment performed in triplicate; B=Basal media alone, O=Osteogenic media alone, numbers 4-11 refer to individual patients' synovial supernatants, which were then added such that B4=Basal media + supernatant from patient 4, O4 = Osteogenic media + supernatant from patient 4. Differences between the groups did not meet statistical significance.

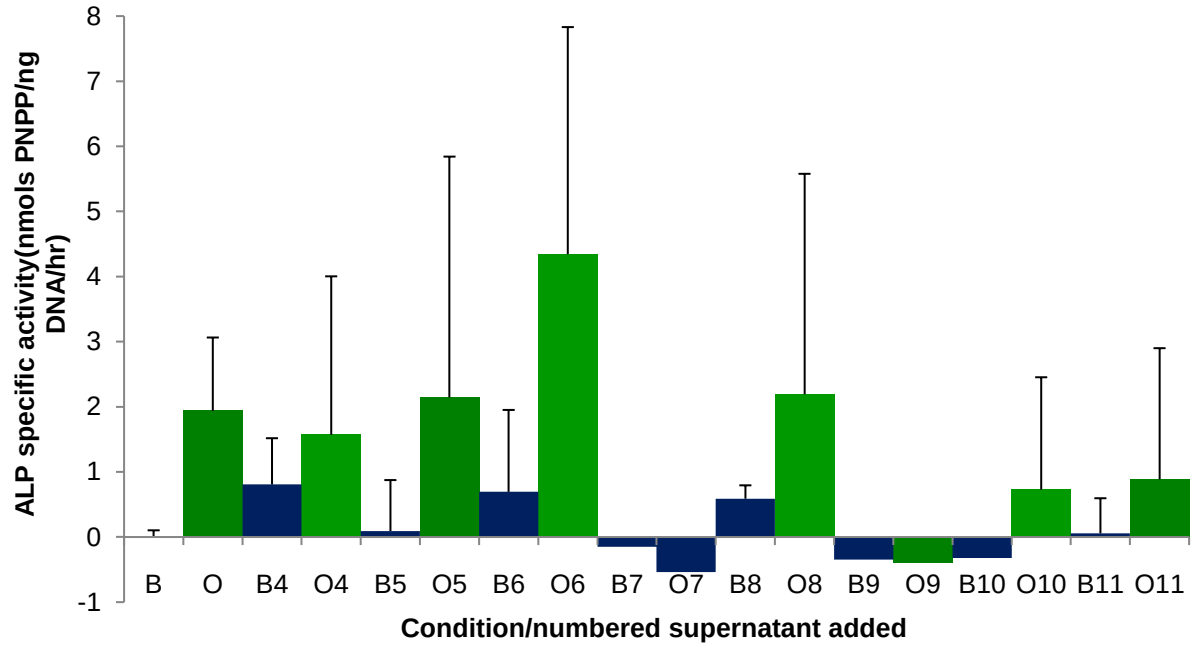


Figure 4.8 ALP specific activity of HBMSC with or without synovial supernatants (F79)
Data presented as mean +/- SD for a single experiment performed in triplicate; B=Basal media alone, O=Osteogenic media, numbers 4-11 refer to individual patient's synovial supernatants, which were then added such that B4=Basal media + supernatant from patient 4, O4=Osteogenic media + supernatant from patient 4. Differences between the groups did not meet statistical significance.

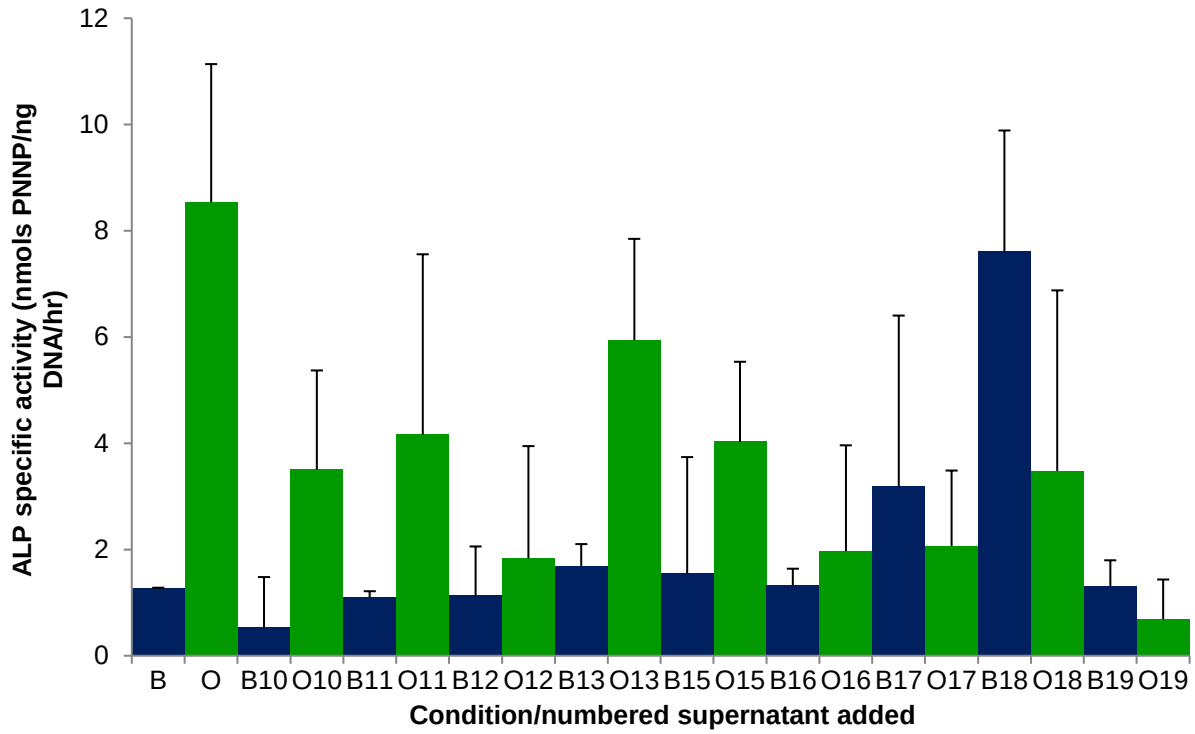


Figure 4.9 ALP specific activity of HBMSC with or without synovial supernatants (M82)
Data presented as mean +/- SD for a single experiment performed in triplicate; B=Basal media only, O=Osteogenic media only, numbers 10-19 refer to individual patient's synovial supernatants which were then added such that B10=Basal media + supernatant from patient 10, O10=Osteogenic media + supernatant from patient 10. Differences between the groups did not meet statistical significance.

In larger co-culture experiments, (Figures 4.7-4.9), the synovial supernatants produced mixed effects, with levels of ALP specific activity at or below osteogenic control values in the majority of cases, but evidence of increased osteogenic differentiation with two of the supernatants (represented by bars O5 and O8 in Figures 4.7 and 4.8). This may reflect the variation in cytokine concentrations within the supernatants, with a trend for lower IL-1β and TNFα concentrations to correlate with increased ALP specific activity (Figures 4.10 and 4.11). This is supported by the low levels of both IL-1β and TNFα present in supernatants numbers 5 and 8 on ELISA testing (Figures 4.3 and 4.5).

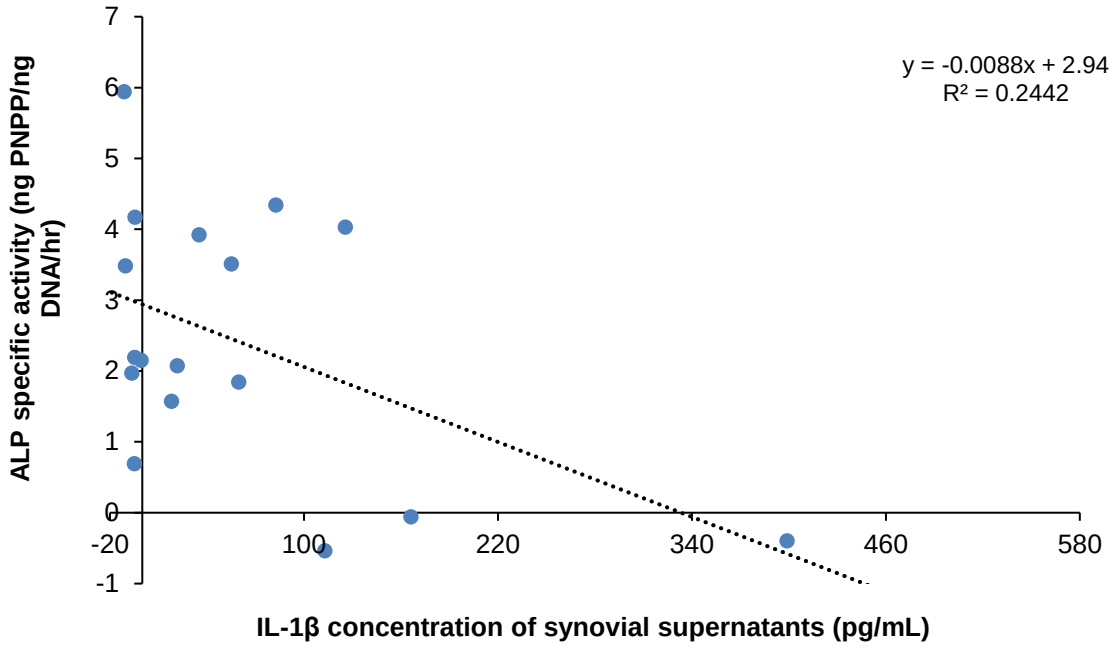


Figure 4.10 ALP specific activity of synovial supernatants plotted against concentration of IL-1beta in the supernatants
Data presented are mean ALP specific activity values for a single experiment performed in triplicate, p<0.05

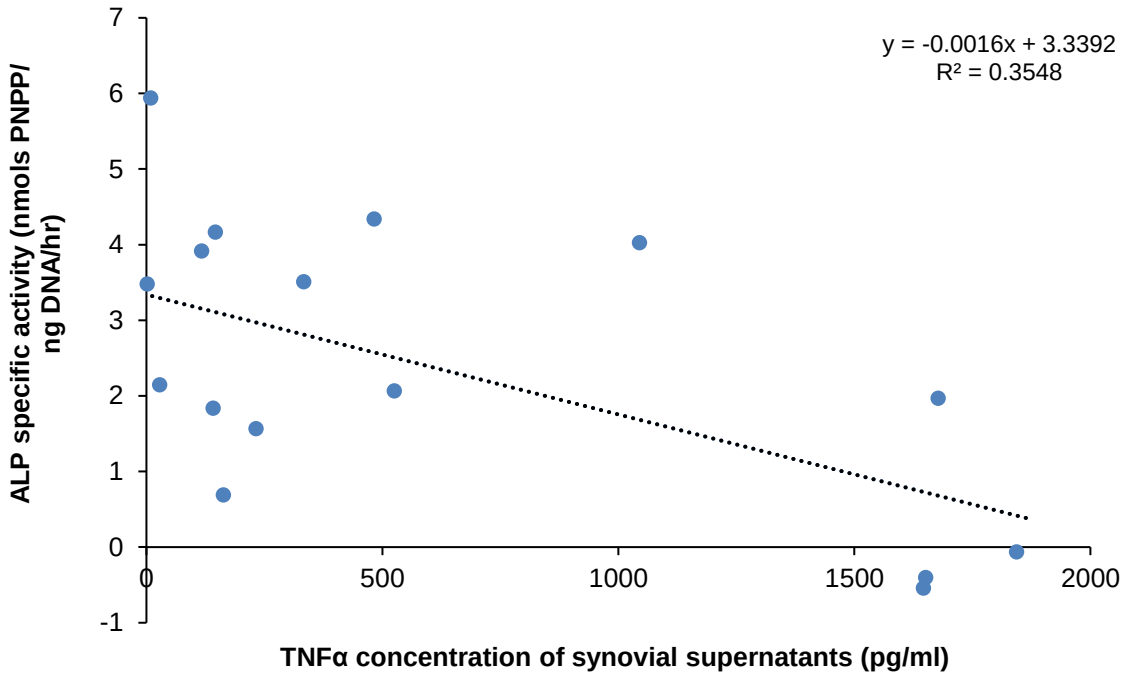


Figure 4.11 ALP specific activity of synovial supernatants plotted against concentration of TNF alpha in the supernatants
Data presented are mean ALP specific activity values for a single experiment performed in triplicate, p<0.05

A similar relationship was not seen with the IL-6 concentration present in the synovial supernatants. The cytokine profiles of larger numbers of synovial supernatants will need to be analysed using multivariate analysis to investigate this relationship further, as well as co-culture experiments.

4.6.4 Molecular biology

As observed for the biochemistry results shown above, in co-culture experiments for which the same numbered synovial supernatants were used to allow direct comparison, the expression of ALP was mainly either comparable to or lower than in the osteogenic controls (Figures 4.12-4.14), although this did not reach statistical significance. The variation in the expression of ALP across the co-cultures reflected a similar inverse relationship with the concentration of IL-1β or TNFα present in the supernatant to that seen for the biochemistry assays. The expression of both collagen 1 and Runx-2 mirrored that of ALP (Figures 4.15 and 4.16) as expected given their crucial roles in early osteogenic differentiation.

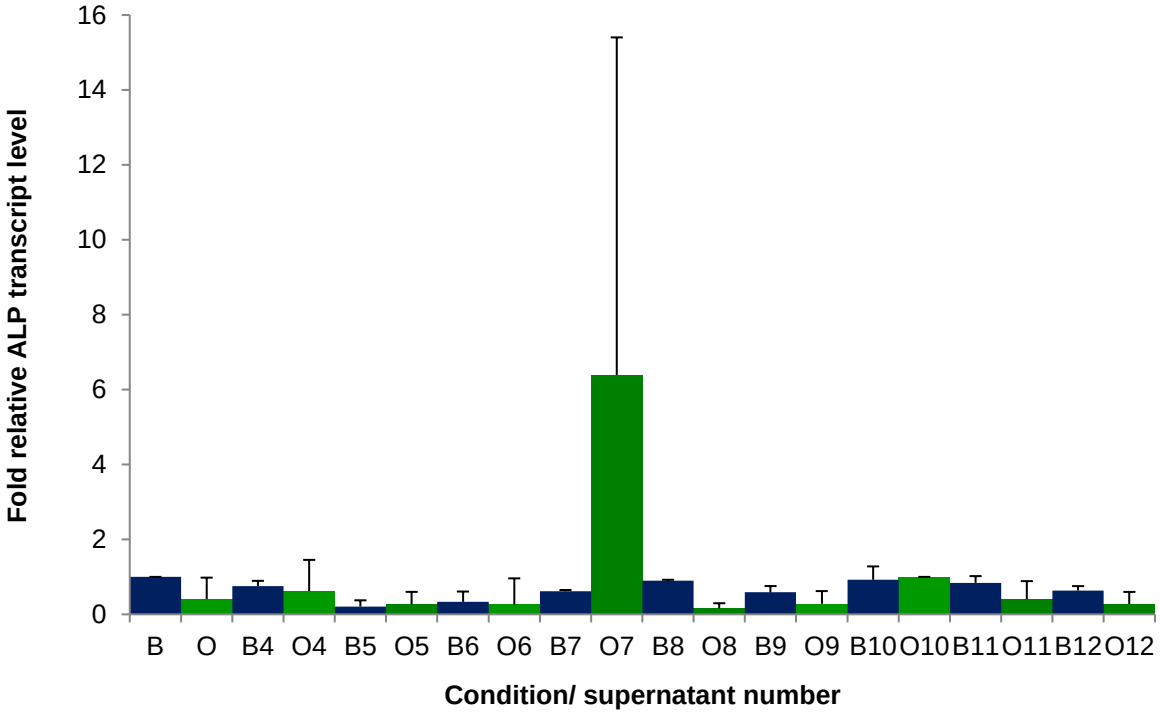


Figure 4.12 ALP expression relative to B actin expression for HBMSC at Day 7 with or without supernatants (F79)
Data are presented as mean ALP expression relative to B actin +/- SD for a single experiment performed in duplicate; B=Basal media alone, O=Osteogenic media alone, numbers 4-12 refer to individual patients' synovial supernatants, which were then added such that B4=Basal media + supernatant from patient 4, O4=Osteogenic media + supernatant from patient 4. Differences between the groups did not meet significant significance.

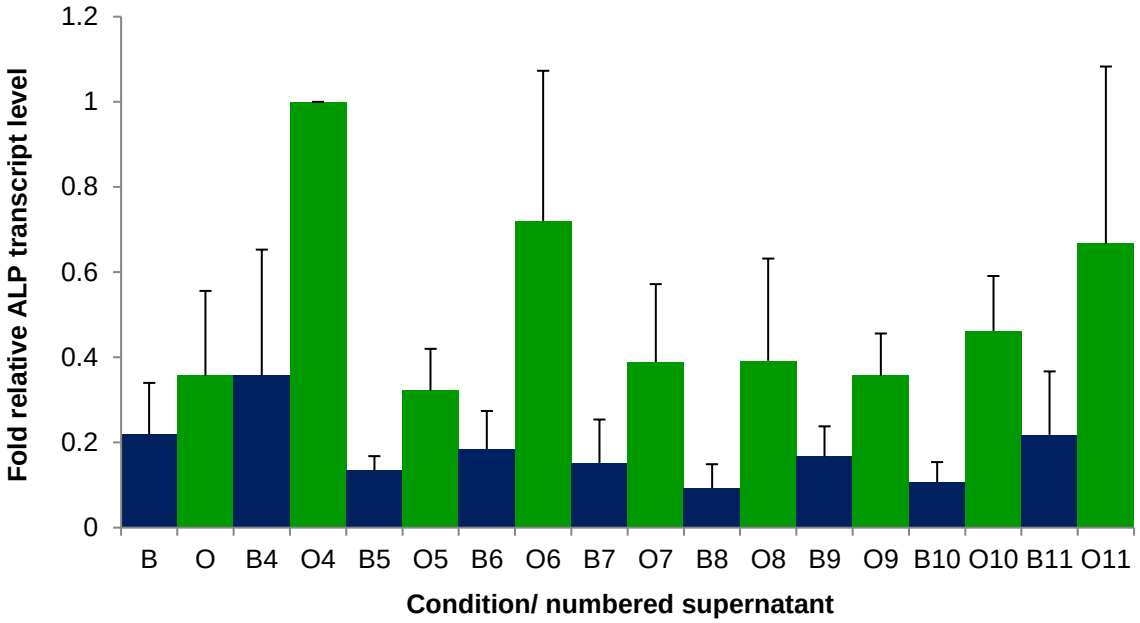


Figure 4.13 ALP expression relative to B actin expression for HBMSC with or without supernatants at Day 7 (M68)
Data are presented as mean ALP expression relative to B actin +/- SD for a single experiment performed in duplicate. B=Basal media only, O=Osteogenic media only, numbers 4-11 refer to individual patients' synovial supernatants which were then added such that B4=Basal media + supernatant from patient 4, O4=Osteogenic media + supernatant from patient 4. Differences between the groups did not reach statistical significance.

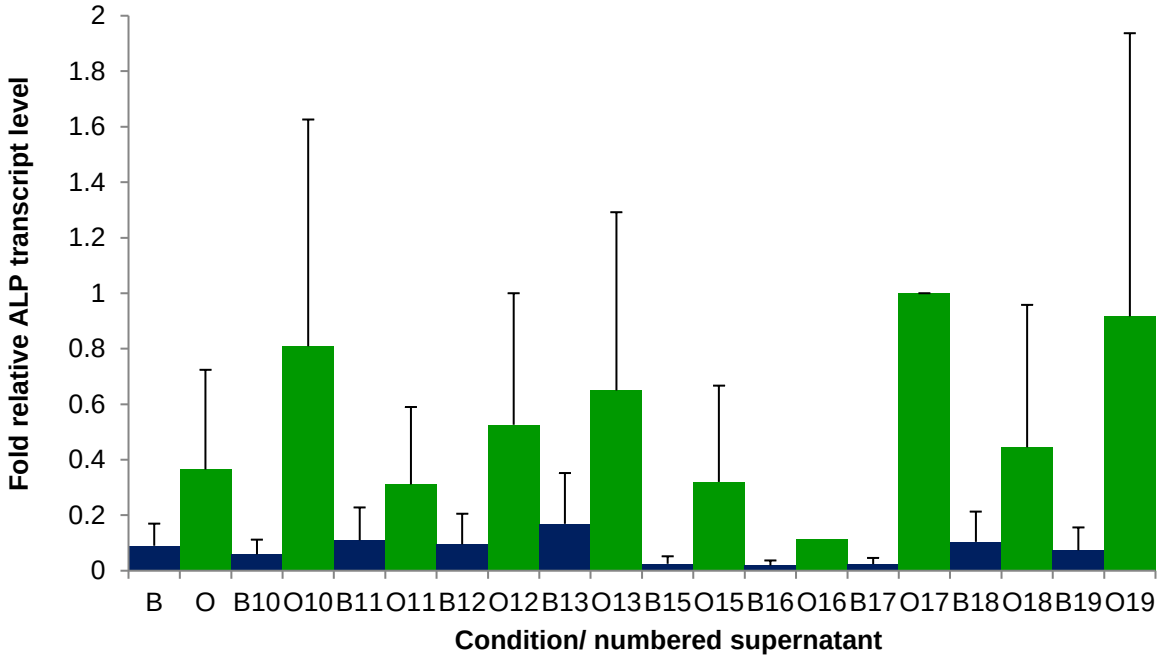


Figure 4.14 ALP expression relative to B actin for HBMSC with or without supernatants at Day 7 (M82)
Data are presented as mean ALP expression relative to B actin +/- SD for a single experiment performed in duplicate; B=Basal media only, O=Osteogenic media only, numbers 10-19 refer to individual patients' synovial supernatants, which were then added such that B10=Basal media + supernatant from patient 4, O10=Osteogenic media + supernatant from patient 10. Differences between the groups did not meet statistical significance.

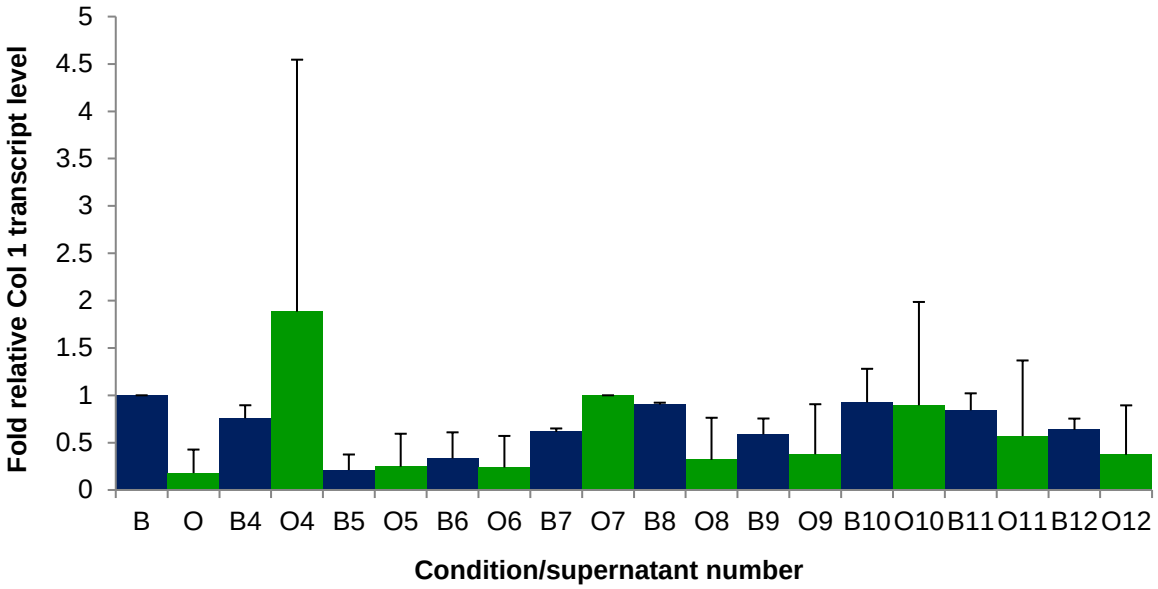


Figure 4.15 Collagen 1 expression relative to B actin expression for HBMSC at Day 7 with or without supernatants (F79)

Data are presented as mean Col-1 expression relative to B actin +/- SD for a single experiment performed in duplicate; B=Basal media only, O=Osteogenic media only, numbers 4-12 refer to individual patients' synovial supernatants, which were then added such that B4=Basal media + supernatant from patient 4, O4=Osteogenic media + supernatant from patient 4. Differences between the groups did not reach statistical significance.

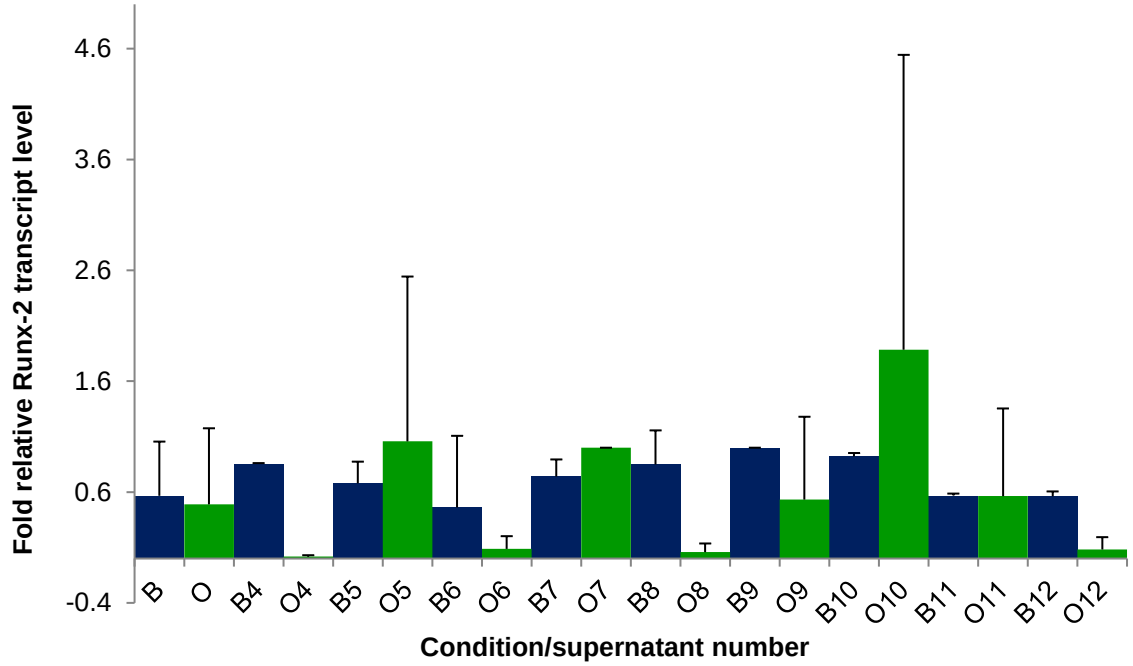


Figure 4.16 Runx-2 expression relative to B actin expression for HBMSC at Day 7 with or without supernatant

Data are presented as mean Runx-2 expression relative to B actin +/- SD for a single experiment performed in duplicate; B=Basal media only, O=Osteogenic media only, numbers 4-12 refer to individual patients' synovial supernatants, which were then added such that B4=Basal media + supernatant from patient 4, O4=Osteogenic media + supernatant from patient 4. Differences between the groups did not meet statistical significance.

	ALP specific activity	ALP specific activity	Fold relative ALP transcript level	Fold relative ALP transcript level	Fold relative Collagen 1 transcript level	Fold relative Collagen 1 transcript level	Fold relative Runx 2 transcript level	Fold relative Runx 2 transcript level
Media used	**Basal**	**Osteo**	**Basal**	**Osteo**	**Basal**	**Osteo**	**Basal**	**Osteo**
Supernatant used								
1	-							
2	++	--						
3	++++	-						
4	+	---	++	+++	-	+++++	++	--
5	++	+	-	-	--	+	++	++
6	++	--	-	++	-	+	+	--
7	++	-	-	+	-	+++	++	++
8	++	++	-	+	+	+	++	--
9	+	-	-	+	-	+	++	+
10	++	-	-	+++	++	+++	++	++++
11	-	-	+	++	++	++	+	+
12			+	++	-	++	+	-
13			++	++				
14								
15			-	-				
16			-	--				
17			-	+++				
18			+	++				
19			+	+++				

Table 4.2 Summary of biochemistry and molecular biology results for the HBMSC and supernatant experiments.

'+' = elevated compared to control conditions, '-' = decreased compared to control conditions

4.7 Discussion

The above series of experiments was designed firstly to collect synovial samples from a broad range of OA patients undergoing either hip or knee arthroplasty surgery and thereby generate a bank of synovial supernatants to use in co-cultures with HBMSCs. Then, the results from these co-cultures were compared with those from the cytokine modulated HBMSCs described in Chapter 3 to examine how the more complex mix of cytokines and

other mediators present in the synovial supernatants affected the osteogenic differentiation of HBMSCs, rather than individual cytokines alone. The assumption from our original literature review remained that the inflammatory cytokines present in OA (here recreated by use of synovial supernatants from OA patients) produce a hostile environment that limits the use of bioengineering strategies to repair damaged joints. The OA synovial supernatants collected displayed varying concentrations of IL-1β and TNFα, whilst IL-6 remained relatively constant. This variability in cytokine profile was reflected in the variation in osteogenic effect (elevated ALP specific activity) observed across 17 of these synovial supernatants (Figures 4.7-4.9) (Table 4.2). The degree of osteogenic effect was observed to correlate with the initial dilution at which the supernatant was applied to HBMSC cultures (Figure 4.6 – *a* indicates 1:4 dilution of supernatant, *b* indicates a 1:2 dilution), suggesting a dose response relationship with either IL-1β or TNF or both (Figures 4.10 and 4.11). Analysis of larger numbers of supernatants was then carried out to confirm this.

The majority of data relating cytokine profiles and SSCs so far has been derived from studies investigating the effects of synovial fluid from OA and RA patients on SSC development and growth. These studies are limited by the fact that the synovial fluid specimens used as controls are usually obtained post-mortem from non-arthritic patients, termed normal donors. The samples may therefore show non-physiological changes or have been influenced by the patients' other medical problems. Despite this, such studies provide some insight into the local cytokine profile in arthritic joints. One study comparing the effects of OA and RA synovial fluid on differentiation of subchondral mesenchymal corticospongious progenitor cells (CSP) *in vitro,* highlighted differences in chondrogenic differentiation with OA versus RA samples (Kruger, Endres et al. 2012). When RA synovial fluid was added to the progenitor cell cultures, the expression of chondrogenic genes (aggrecan, type II collagen, link-protein and type IX collagen) were all reduced at day 14 compared to cultures treated with synovial fluid from normal donors. In contrast, when OA synovial fluid was added to progenitor cell cultures, these same genes were upregulated compared to normal donor control cultures. Therefore, RA synovial fluid was shown to inhibit chondrogenic differentiation of progenitor cells, whereas OA synovial fluid was not. The authors suggest that these findings reinforce the view that cartilage regeneration would be very difficult in an RA joint, whereas in an OA joint early in the disease process, the potential remains for cartilage repair to occur, albeit with a delay in the formation of cartilage matrix.

One proposed mechanism through which synovial fluid may induce a healing response in OA is through recruitment of human subchondral bone marrow-derived skeletal progenitor cells, for example after therapeutic microfracture (Endres, Neumann et al. 2007). This hypothesis was based on chemotaxis assays on synovial fluid from normal, OA and RA patients, which demonstrated similar levels of cell recruitment for normal and OA synovial samples, but significantly reduced cell recruitment with RA samples. Another study using OA synovial fluid and tissue samples obtained during knee arthroplasty surgery demonstrated that OA synovial fluid increased the expansion of SSCs in OA synovial tissue cultures, lending support to the above hypothesis (Zhang, Muneta et al. 2008). Further work examining synovial fluid and synovial tissue fragments from patients with unexplained knee pain compared to those with early or established OA, has confirmed that SSCs are present even within normal synovial fluid (Jones, Crawford et al. 2008). In early OA there was a rise in the numbers of SSCs in the synovial fluid. When these SSCs were expanded in culture, some were grown in basal media and the comparator group had 10% OA synovial fluid added to the cultures (Jones, Crawford et al. 2008). The OA synovial fluid was found to have a stimulatory effect on proliferation of these SSCs, similarly to the findings of Hang et al.

There has only been limited work, to date, investigating the effects of IL-1β, TNFα or conditioned media from OA synovium on the growth and differentiation of human MSCs and that has focused on their effects on chondrogenic differentiation (Heldens, Blaney Davidson et al. 2012). In this study, both IL-1β and TNFα inhibited chondrogenesis in a dose-dependent manner if added either at the onset of or during differentiation, with IL-1β exerting the most potent effect. Blockade of IL-1β ameliorated this inhibition but was insufficient to overcome it altogether, suggesting other catabolic factors were present in the conditioned media and contributing to the impaired chondrogenesis. Inhibition of TNFα had minimal effect. This suggested that IL-1β and TNFα, together with other, as yet unidentified catabolic factors in OA conditioned media, were impeding chondrogenesis. Similarly, SSCs derived from equine bone marrow or synovial fluid showed no decline in cell proliferation when exposed to 100ng/mL of IFNγ and 10ng/mL of TNFα, although glucosaminoglycan production and aggrecan expression were reduced in both sets of cultures (Zayed, Schumacher et al. 2016). Cell viability was not therefore affected but there was some impairment of chondrogenic differentiation. A further study of equine bone marrow-derived SSCs demonstrated that exposure of these equine SSCs to either 20 or 50ng/mL of IFNγ and TNFα affected both cell viability and their ability to undergo osteogenic, chondrogenic or adipogenic differentiation (Barrachina, Remacha et al. 2017). Interestingly, addition of 20%

concentration of inflammatory synovial fluid to the equine SSC cultures did not appear to have any deleterious effects, though no cytokine profiling of the content of the supernatant was included to give an idea of the concentrations of any inflammatory mediators within it (Barrachina, Remacha et al. 2017). Another recent study looked at the effects of pre-treating human synovial SSCs with 25ng/mL of TNFα (Shioda, Muneta et al. 2017). Although TNFα did alter expression of some of the cell surface antigens, it also had a stimulatory effect on synovial SSC proliferation, increasing both cell numbers and colony formation, with preservation of glucosaminoglycan concentration (Shioda, Muneta et al. 2017). However, the balance of evidence so far suggests that for cartilage tissue engineering strategies future therapies will need to inhibit these catabolic factors in order to be successful for the treatment of OA patients.

As yet, there is no comparable data available in the literature on the effects of OA synovial supernatants on the growth and differentiation of human SSCs, or indeed on the osteogenic cells within the OA joint. From the data presented in this chapter, it appears that for bone tissue engineering, the majority of OA synovial supernatants impair osteogenic differentiation of SSCs, but a minority may act to promote osteogenic differentiation. It is likely that the level of IL-1β and to a lesser extent TNFα are the key determinants of this in a dose-dependent manner but further studies using larger numbers of synovial supernatants, together with concurrent inhibition of each of IL-1β, IL-6 and TNFα are required to confirm this. If proven, this would support the use of cytokine concentrations in synovial fluid as a means of stratifying individual patients into a more 'inflammatory OA' subtype versus 'pure OA' subtype. Those with the more inflammatory OA subtype, whose supernatants impaired osteogenic differentiation of SSCs, would be more likely to benefit from either systemic DMOAD treatment or tissue engineering strategies incorporating inhibitors of IL-1β +/- TNFα. Those with the 'pure OA' subtype, whose supernatants promoted osteogenic differentiation of SSCs, would conversely benefit from tissue engineering strategies that incorporated low dose IL-1β +/- TNFα. This stratified, or personalised medicine, approach is already being pursued in the STRAP (**S**tratification of **T**herapies for **RA** by **P**athobiology) study for RA, which is investigating whether the histological appearance (B cell rich or poor) of a patient's synovium from a series of wrist or knee joint synovial biopsies can be successfully used to guide which type of biologic therapy (etanercept, tocilizumab or rituximab) their RA will respond to best. Cytokine profiling of synovial fluid analysis would have the advantage of potentially achieving a similar type of stratification for OA patients, whilst being a less

invasive procedure than serial synovial biopsies, provided it could accurately distinguish the OA subtypes suggested above.

A recent study examined the secretome profile of human SSCs from a healthy donor, which were either cultured under basal conditions or with the addition of OA synovial fluid from patients with early or late stage OA (Gomez-Aristizabal, Sharma et al. 2017). Analysis of the SSC secretomes showed differential elevation of chemokine ligands 2 (CCL2) and 8 (CXCL8) and IL-6 after exposure to synovial fluid from patients with early versus late stage OA (Gomez-Aristizabal, Sharma et al. 2017). This lends further support to the proposal outlined above of using synovial fluid cytokine profiles as a means of stratifying patients with regards to the use of tissue engineering strategies for OA.

In conclusion, this work demonstrates heterogeneity in the concentrations of IL-1β and TNFα in synovial supernatants across the group of OA patients studied. IL-1β and TNFα concentrations tended to be higher in female patients, those undergoing TKRs and those with moderately severe (KL grade 3) OA. Levels of ALP specific activity and ALP expression on rt-PCR were comparable to or below those seen for osteogenic control conditions with most of the supernatants studied, suggesting an inhibitory effect on osteogenic differentiation of HBMSC, linked to higher cytokine concentrations. However, a minority of patients' supernatants showed an additive osteogenic effect in co-cultures, suggesting a division into two distinct 'inflammatory' and 'pure OA' subtypes. Those with the 'pure' or pro-osteogenic subtype may well have a more favourable environment within the synovial niche of the OA joint, which facilitates better recruitment of SSCs from the subchondral bone to repair areas of osteochondral damage. The role of additional catabolic factors within the OA conditioned media remains unclear at the present time but is a potential further area of study. Further characterisation of larger numbers of synovial samples and more detailed profiling of other cytokines and growth factors within the synovial supernatants will help to clarify these findings further. This will allow more detailed phenotyping of individual subtypes of OA, leading to stratified medicine approaches then being used to target either systemic treatments or locally applied tissue engineering strategies more effectively.

Chapter 5 Molecular mechanisms underlying the effects of inflammatory cytokines on osteogenic differentiation of HBMSCs and immunoselected SSCs

5.1 Introduction

In order to investigate the molecular mechanisms underlying the effects of inflammatory cytokines on the osteogenic differentiation of HBMSC and immunoselected SSCs, several other cell types are of vital importance. As detailed in Section 3.1.1, adult human bone-marrow derived stromal cells (HBMSCs) and the subpopulation of Stro-1+ve SSCs are multipotent, retaining the ability to differentiate into bone, cartilage and fat, given the correct conditions (Dawson and Oreffo 2008, Tare, Babister et al. 2008, Charbord, Livne et al. 2011). This is a crucial property, which enables these cells to be used in reparative strategies. However, other cell types such as embryonic stem cells (ESC) are pluripotent, with even greater regenerative potential and can give rise to any given tissue (Bongso, Fong et al. 1994, Thomson, Itskovitz-Eldor et al. 1998, Semb 2005, Trounson 2006, Albalushi, Kurek et al. 2018). ESCs provide a platform for cell differentiation and a useful testbed for testing therapeutic protocols and analysis of therapeutic agents. ESCs can also be used to study gain or loss of function mutations. These features are some of the reasons why ESCs have garnered significant interest as a cell approach to improve our understanding of both the mechanistic basis of bone development and the pathophysiology of bone diseases. There are, however, still concerns over the potential immunogenicity of any form of stem cell-based treatment and significant ethical dilemmas are associated with using ESCs as the main cell type in tissue engineering strategies.

Over recent years, as transfection techniques have improved, it has now become possible to produce induced pluripotent stem cells (iPSCs) (Shi et al, 2017; Takahashi and Yamanaka. 2016; Yamanaka, 2012; Yu et al. 2007; Takahashi et al. 2007; Takahashi and Yamanaka, 2006). This removes the ethical issues associated with using waste embryonic tissue and the ultimate goal is to produce autologous patient-derived induced pluripotent stem cells. This would negate the requirement for lifelong immunosuppression after treatment with a stem cell-based therapy, although extensive animal studies will be required to generate sufficient long-term safety data to allay fears regarding the potential risk of malignancy related to treatment. Studying the effects of pro-inflammatory cytokines in these

cell types will therefore provide valuable insights into the pathways involved in mediating cytokine effects on osteogenic differentiation and the development of OA.

Using micro-arrays to establish the patterns of microRNA (miRNA) expression in control and cytokine/supernatant treated HBMSCs and SSCs represents another exciting method of unravelling the complex effects of inflammatory cytokines on these cell types. This will also provide insights into the changes that occur in miRNA expression in OA in response to the inflammatory cytokines, through assessment of HBMSC and SSC cultures alone and then as a co-culture with OA supernatants (as described in Chapter 4). This is particularly topical given that differential expression of miR-9, miR-27b, mi-R34a, miR-98, miR-140 and miR-146 occurs between normal and OA cartilage (discussed in Chapter 1 and reviewed in (Yu, Chen et al. 2011, Barter, Bui et al. 2012, Reynard and Loughlin 2012). To recap briefly, overexpression of miR-9, miR-98 and miR-146 in OA cartilage, reduces IL-1-induced TNFα production (Jones, Watkins et al. 2009). Additionally, alterations in miR-9 expression modulate MMP-13 secretion. Overall, these miRNAs appear to have protective effects in OA.

In contrast, IL-1 stimulation leads to a reduction in miR-27b expression in chondrocytes. Levels of miR-27b are known to be reduced in OA cartilage and demonstrate an inverse correlation with their target protein MMP-13 (Akhtar, Rasheed et al. 2010). Expression of miR-34a in chondrocytes is significantly upregulated by IL-1β and inhibition of miR-34a leads to a reduction in chondrocyte apoptosis in a rat model of OA (Abouheif, Nakasa et al. 2010). This has led to the suggestion that inhibitors of miR-34a could represent novel therapies for OA. Similarly, IL-1β can reduce the expression of miRNA-140 in chondrocytes *in vitro* (Miyaki, Nakasa et al. 2009). miR-140 is expressed by differentiated human articular chondrocytes and acts to modulate the IL-1 response. When transfected into chondrocytes, ds-miR140 inhibits IL-1β induced ADAMTS-5 expression and restores IL-1β-induced suppression of aggrecan gene expression. Finally, IL-1β induces expression of miR-146 in chondrocytes *in vitro* but can also suppress IL-1-induced expression of MMP-13 though its inhibitory effects on the IL-1 receptor associated kinase (IRAK-1) and TNF associated factor-6 (TRAF-6) genes (Yamasaki, Nakasa et al. 2009). miR-146 is upregulated in early OA cartilage and down-regulated in later disease, suggesting a role in the development of inflammatory OA (Jones, Watkins et al. 2009). Therefore, an understanding of the profile of miRNAs within the co-culture systems described below would be vital to understanding and adjusting tissue engineering constructs for use within the inflammatory milieu of an OA joint.

The experiments in this chapter were therefore designed to investigate the effects of the inflammatory cytokines IL-1β, IL-6 and TNFα on both embryonic stem cells and induced pluripotent stem cells as alternative cell types currently being used in bone and cartilage tissue engineering strategies for the reasons described above. This will then provide information on whether the levels of these cytokines would have to be differentially modulated for these cell types or not. It will thereby provide further insights into the mechanisms through which these cytokines might exert their effects on osteogenic differentiation of HBMSCs. As miRNAs have been an area of increasing study in OA and other bone disorders, it was decided to examine the up or downregulation of some of the miRNAs recently found to have a role in OA in order to ascertain whether or not they were a potential mechanism through which each of these individual inflammatory cytokines might produce their effects on osteogenic differentiation of SSCs. Finally, the addition of synovial supernatants was included in the experimental design to allow investigation of how the combination of cytokines present in OA joints would affect these same miRNAs.

5.1.1 Overall aim

- To investigate whether the inflammatory cytokines present in OA produce a hostile environment that limits the use of bioengineering strategies to repair damaged joints.

5.1.2 Objectives:

- To investigate whether the inflammatory cytokines IL-1β, IL-6 and TNFα have the same effects on other cell types, namely embryonic stem cells and induced pluripotent stem cells, currently being used in tissue engineering strategies for repairing bone and cartilage.

- To understand the molecular mechanisms involved in mediating the effects of inflammatory cytokines on the osteogenic differentiation of HBMSCs and immunoselected SSCs.

- To establish which of these mechanisms are important in synovial joints through the study of OA synovial supernatants.

5.2 Human embryonic stem cells (hESC)

5.2.1 Experimental set up

5.2.1.1 Baseline experiments

(Protocol courtesy of Dr Franchesca Houghton, Associate Professor in Stem Cells and Developmental Biology, University of Southampton)

Hues7 (D. Melton, Howard Hughes Medical Institute/ Harvard University) hESC were cultured at 20% oxygen in Knockout Dulbecco's Modified Eagle's Medium (DMEM)(Invitrogen) supplemented with 15% knockout serum replacement (Invitrogen), 100µg/mL penicillin streptomycin (Invitrogen), 1mM L-glutamine (Invitrogen), 1x non-essential amino acids (Invitrogen), 0.1mM 2-mercaptoethanol and 10ng/mL fibroblast-derived growth-factor 2 (FGF-2)(Invitrogen) on gamma-irradiated mouse embryonic fibroblasts (MEFs; a primary source derived in institutional facilities following University of Southampton ethical review committee approval and in accordance with UK Home Office regulations). hESC were then transferred to Matrigel (BD Biosciences) coated 6 well plates and cultured in MEF-conditioned media at 20% oxygen.

hESCs from 3 wells of a 6 well plate were passaged to set up a baseline hESC experiment. The media from the chosen wells was pipetted off and 1ml of collagenase was added per well, trickling it carefully down the side of the well. The plate of hESCs was then returned to the incubator for 4 minutes. Meanwhile a 6 well plate containing Matrigel, that had been plated out 24 hours earlier to allow it to settle, was removed from the incubator. The media was removed and 0.5ml of MEF conditioned media added to each well. 2mls of hESC media was then added to each well of the MEF plate, which was returned to the incubator to replenish the media. The hESC plate was removed from the incubator and the cells in each well were pipetted up and down once. 0.5ml of the cell suspension was added to each well of the 6 well plate, covering the base of each well evenly, using a 5ml pipette in order to avoid breaking up the cells too much. The hESCs were returned to the incubator, tapping them lightly once on the shelf to ensure an even spread across the wells. Media changes were performed on the hESCs every day as described above. At day 7 the cells were fixed in 4% w/v paraformaldehyde in PBS (PFA, Sigma-Aldrich, UK). Immunostaining was then performed for the hESC markers OCT-4 and TRA 1-60, the osteogenic marker Stro-1 and the chondrogenic marker Runx-2.

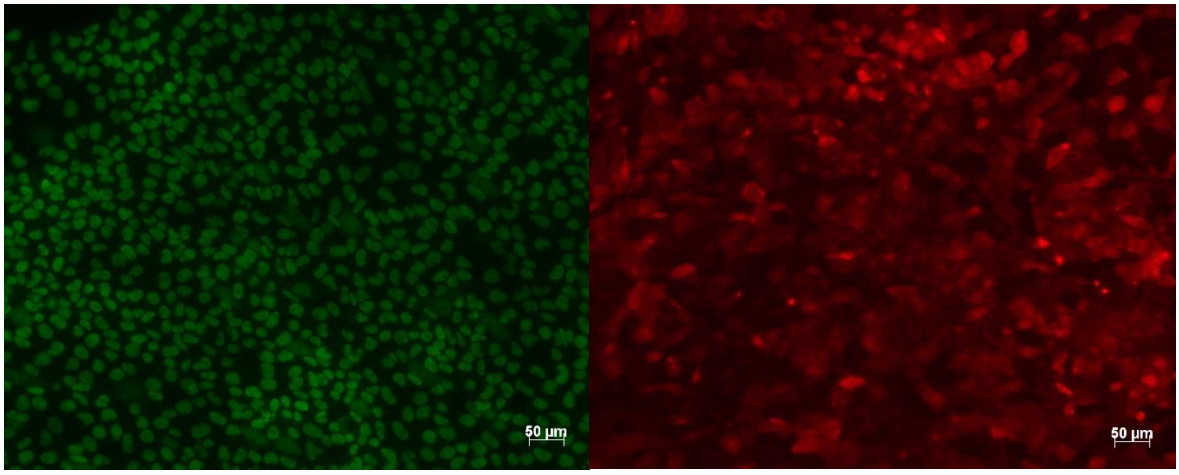
For immunostaining, first the required reagents were made up. These included the blocking buffer, PBS Tween and an antibody dilution buffer. Blocking buffer was made using phosphate-buffered saline without calcium or magnesium (PBS) (PAA, UK), 0.3% Triton (diluted from 100x concentrate) (Sigma-Aldrich) and 5% goat serum. PBS Tween was made using PBS and 0.5% Tween 20 (Sigma-Aldrich). The antibody dilution buffer consisted of PBS with 1% bovine serum albumin (BSA Fraction V, pH7) (PAA,UK) and 0.3% Triton. The ES cells were washed with PBS and covered with blocking buffer for 30 minutes. Wells were labelled for primary antibody, isotype control and negative controls. The primary antibodies were diluted in the antibody dilution buffer to give a total volume of 400µl per well: red anti-rabbit IgG for OCT-4 was added at a 1:200 dilution; red anti-mouse IgM for TRA 1-60 was added at a 1:200 dilution; green anti-mouse IgM for Stro-1 was added neat and green anti-rabbit IgG for Runx-2 was added at 1:100 dilution. The blocking buffer was removed from the wells and the diluted primary antibodies added as per the stated dilutions. The cells were covered and incubated on a rocking bed mixer for at least 30 minutes or overnight. The cells were washed three times with PBS Tween. The secondary antibody was added at a 1:100 dilution (4µl of secondary antibody and 400µl of antibody dilution buffer) to each well. For OCT 4 and Runx-2 the secondary antibody was goat anti-rabbit IgG. For TRA 1-60 and Stro-1 the secondary antibody used was goat anti-mouse IgM. The cells were incubated, covered with foil on a rocking bed mixer for 1 hour at room temperature before washing the cells again three times with PBS Tween. Finally, the cells were covered with PBS and stored at 4ºC wrapped in foil until they could be imaged. Images were taken using an AxioCam HR fluorescence microscope with AxioVision Version 4.1 software.

5.2.1.2 Human embryonic stem cells and cytokine experiments

hESCs were passaged from cultures in a 6 well plate as described above. Matrigel plates were used as in baseline experiments, with 0.5ml of cell suspension transferred to each well, together with 0.5ml of conditioned media, then 1ml of either basal or osteogenic media. 4 wells were set up for each of 8 conditions: Basal; Basal/IL-1β; Basal/IL-6; Basal/TNFα; Osteo; Osteo/IL-1β; Osteo/IL-6 and Osteo/TNFα. The cytokines were added at the same concentrations as in the HBMSC cultures described in Chapter 3; IL-1β 10ng/mL, IL-6 100ng/mL and TNFα 10ng/mL. 4 control wells without additional media or cytokines were set up alongside these. Media changes were performed every day as for the baseline experiments and cells were fixed in 4% PFA on D7 for immunohistochemical analysis as described above.

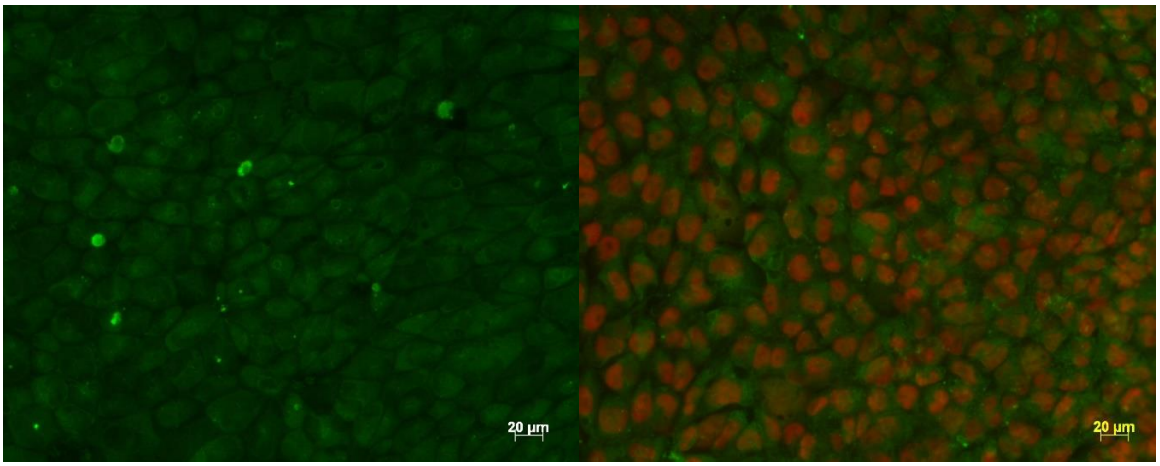
Results

The HUES 7 hESCs showed positive staining for both nuclear (OCT-4) and cell surface (TRA 1-60) ESC markers (Figure 5.1a and b). Similarly, hESCs also produced positive staining for markers of osteogenic differentiation (Stro-1 and Runx-2) (Figures 5.3a and 5.3b). Taken together, the immunostaining shown in Figures 5.1 to 5.3 demonstrated that these baseline hESC cultures retained their pluripotency at the end of 7 days in culture.



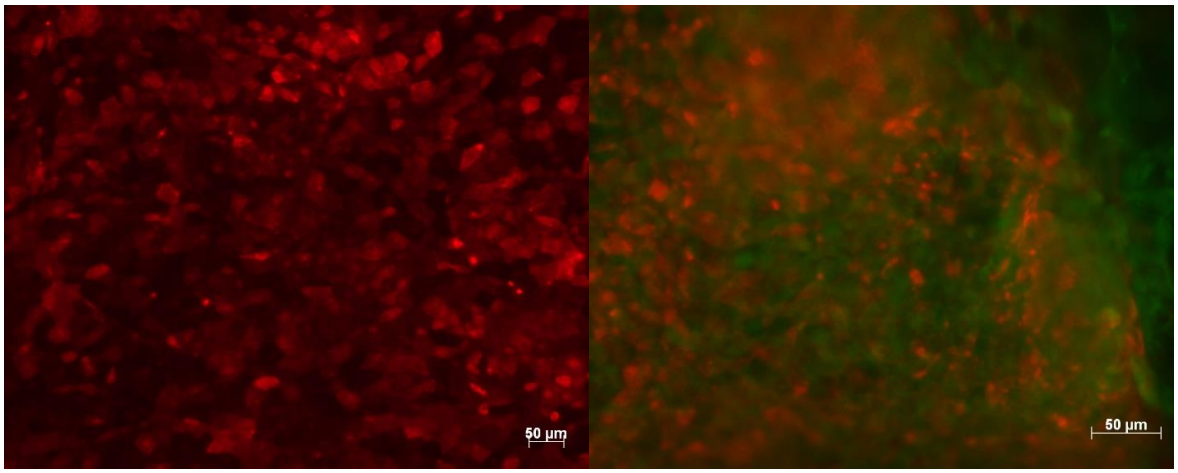
a **b**

Figure 5.1 Immunostaining for hESC markers
a – positive staining for the nuclear-based hESC marker OCT-4, viewed at 10x magnification; b – positive staining for the surface hESC marker TRA 1-60, viewed at 10x magnification; scale bar 50um.



a **b**

Figure 5.2 Immunostaining for hESC and osteogenic markers
a - positive staining for the osteogenic cell surface marker Stro-1 at 20x magnification; b – combined staining for the hESC marker OCT-4 (red nuclear-based staining) and the osteogenic cell surface marker Stro-1 (green) at 20x magnification; scale bar 20um.



a b

Figure 5.3 Immunostaining for hESC and chondrogenic markers
a – positive staining for the surface hESC marker TRA 1-60 at 10x magnification; b – combined staining for the hESC cell marker TRA 1-60 (red cell surface based) and the osteogenic marker Runx-2 (green nuclear-based staining) at 10x magnification; scale bar 50um

Additional hESC and cytokine experiments were planned as described above but problems with the Matrigel supplies and in passaging and maintaining the hESCs in culture precluded cytokine experiments.

5.3 Induced pluripotent stem cells (iPSCs)

5.3.1 Experimental set up

(Protocol courtesy of Dr Patrick Stumpf, Bone and Joint Group, University of Southampton, who also generated the iPSCs for use in the experiments described below)

The non-viral generation of induced pluripotent stem cells (iPSCs) from somatic cells using episomal plasmids, first described and further refined by Yu et al. in 2009, was closely followed (Yu, Hu et al. 2009, Yu, Chau et al. 2011). Primary somatic cells were derived and cultured for several passages to obtain sufficient numbers. For each transfection, $1x10^6$ or $0.5x10^6$ cells were resuspended in the pre-mixed nucleofection solution provided with the Human Mesenchymal Stem Cell Nucleofector kit (Lonza, Slough, UK, VPE-1001) or Nucleofector kits for Human Dermal Fibroblasts (Lonza, Slough, UK, VPD-1001) and the episomal plasmids shown in Figure 2.1. Cells were transfected using the Amaxa Nucleofector II device and cell specific nucleofection programs. Immediately after completed transfection, $1x10^6$ cells were plated onto a total surface area of $230cm^2$, which was

additionally pre-seeded with mouse epidermal fibroblasts (MEFs) at a density of $5x10^4$cells/cm^2 or alternatively coated with Matrigel (Becton-Dickinson, Oxford, UK). The medium was renewed every other day. The nutrient composition was based on pluripotent stem cell medium, which was conditioned previously with MEFs for 24h and supplemented with 10ng/ml fibroblast growth factor-2 (FGF-2) and combinations of small molecules. Between day 14 and day 45 dense cell clusters, henceforth named colonies, formed. Colonies were passaged manually using a 21gauge needle to fragment the colonies and a specifically truncated 1000ml pipette tip served as a spatula to detach the fragments. The small colony fragments (~100-500 cells) were plated onto freshly prepared cell culture plates pre-seeded with MEFs and denominated as passage 0. Over the course of 5 to 10 days the colonies repopulated the surface and grew to a diameter of approximately 0.5cm at which point they were passaged again. In further passages, sub-colonies were picked from individual colonies and screened for phenotypic similarities with other pluripotent stem cells and loss of plasmids.

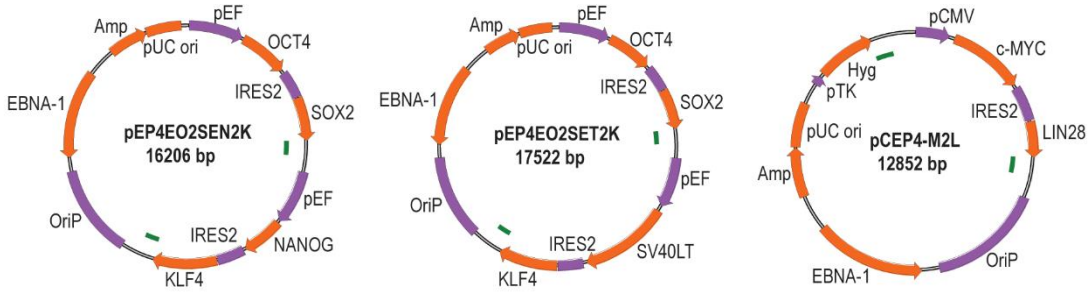


Figure 5.4 Vector maps of the episomal plasmids used for reprogramming somatic cells
Original seven factor combination with highest reprogramming efficiency for human neonatal foreskin fibroblast cells. (Yu, Chau et al. 2011).

For the experiments described herein, fetal femur cells from a female with normal karyotype, were transfected using three plasmids incorporating OCT4, SOX2, NANOG, KLF4, SV4OLT, cMYC and LP28 (Figure 5.4). The cells were repeatedly passaged after transfection, as described above. They were proven to be pluripotent at passage 12 (P12) by immunostaining for Tra180 and Tra160. At each passage, colonies were disrupted and plated out onto MEFs and gelatin/fibronectin in order to maintain pluripotency. The cells used for these experiments were at passage 21 (P21). To overcome variation in cultures at the start of an experiment, biologic replicates were set up for each condition using cells with the same passage number.

Colonies of iPSCs from 3 wells of a 6 well plate were passaged manually using a 21gauge needle to fragment the colonies and a specifically truncated 1000ml pipette tip served as a spatula to detach the fragments. The small colony fragments (~100-500 cells) were plated onto freshly prepared cell culture plates pre-seeded with MEFs. Individual wells of a 24 well plate containing either basal or osteogenic media were used. Each of these two groups were then subdivided further into 4 groups: controls (no additional factors); IL-1β (10ng/ml); IL-6 (100ng/ml); TNFα (10ng/ml). For each of these 8 conditions, wells were labelled in duplicate for use in histological analysis (2 for each of ALP, type I and type II collagen) and ALP and DNA biochemistry assays (as described in Chapter 2). The remaining small colony fragments were seeded into individual wells of a 6 well plate (duplicates for each of the 8 conditions being used) for use in PCR analysis. Media changes were performed at days 3 and 6. Cultures were run for a total of 7 days prior to analysis.

5.3.2 Results

5.3.2.1 Histology

Colonies of iPSCs of the same passage number as those used to set up these experiments were stained for ALP. This staining was positive, confirming the iPSCs retained the ability to differentiate towards the osteogenic lineage (Figure 5.5).

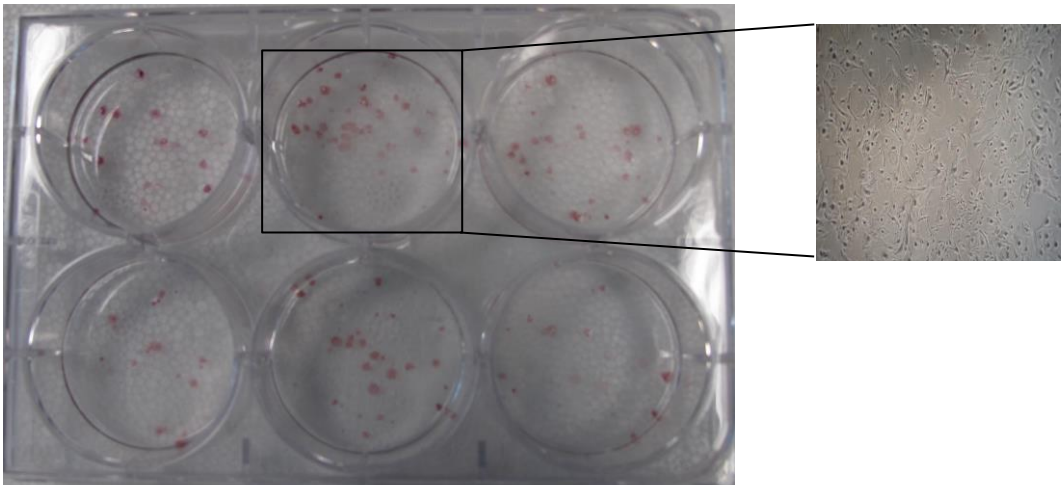


Figure 5.5 ALP staining of iPSC colonies passaged at the time of experimental set up
Typical purplish pink staining for ALP seen in colonies in all wells. **Insert** - high power magnification showing typical fibroblastic morphology of cells and purplish pink staining.

Colony growth in the iPSC and cytokine cultures was inconsistent, making staining for all three planned markers (ALP, collagen I and II) difficult. However, positive staining for ALP was observed in the wells containing osteogenic media, most prominently in those also containing IL-6. No additive osteogenic effect was observed with IL-1β in these iPSC cultures. This pattern was in keeping with findings in earlier cultures of fetal femur cells with cytokines, where IL-6 had once again shown an additive osteogenic effect on ALP production (Chapter 3).

5.3.2.2 Biochemistry

As with the histology results described above, in terms of ALP production, IL-1β did not produced an additive osteogenic effect in the IPS cell and cytokine cultures, in contrast to IL-6, which did. TNFα did however produce a reduction in ALP production, as observed in HBMSC and cytokine cultures described in Chapter 3. None of these effects reached statistical significance though so further experiments with increased group numbers would be required to evaluate these results further.

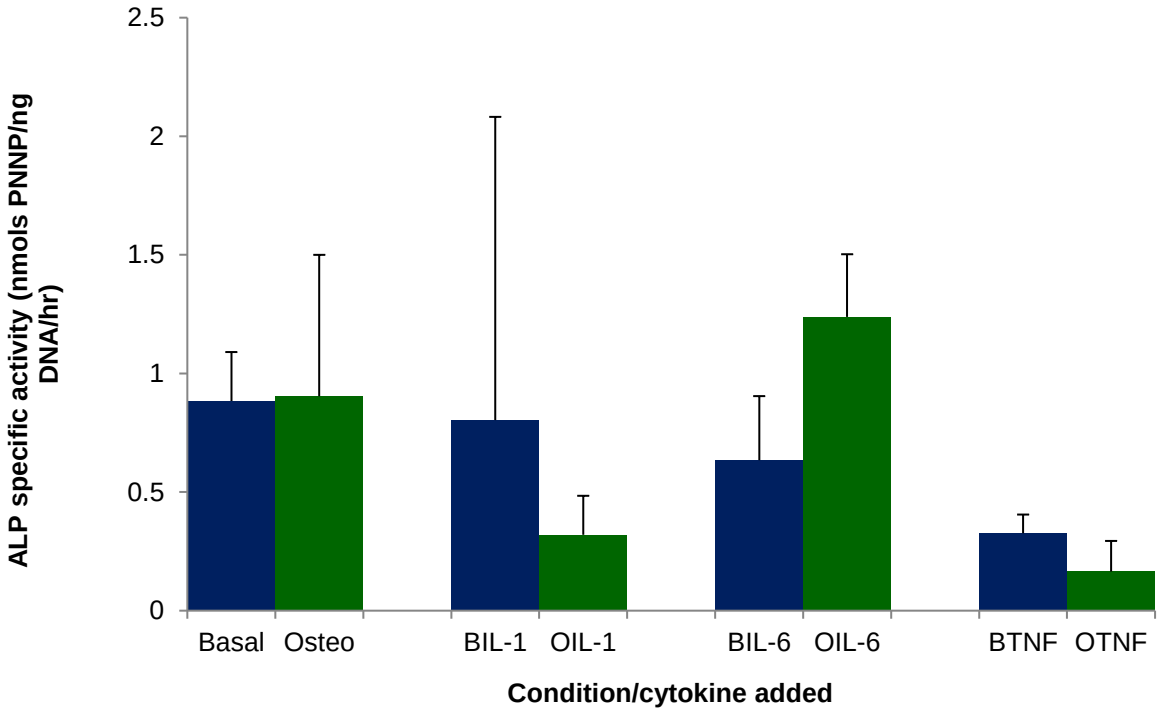


Figure 5.6 ALP specific activity of iPSCs with or without cytokines at Day 7
Data are presented as average ALP specific activity + SD for a single experiment performed in duplicate. B=Basal media alone, O=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media+IL-1, BIL-6=Basal media+IL-6, IL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. Differences between the groups did not reach statistical significance.

5.3.2.3 Molecular biology

ALP gene expression on rt-PCR mirrored the findings observed, within the histological and biochemical analyses, in terms of IL-6 producing an additive osteogenic effect, which here did attain statistical significance (Figure 5.4). IL-1β exerted only a modest stimulatory effect that did not reach statistical significance. TNFα had a similarly modest inhibitory effect on ALP expression.

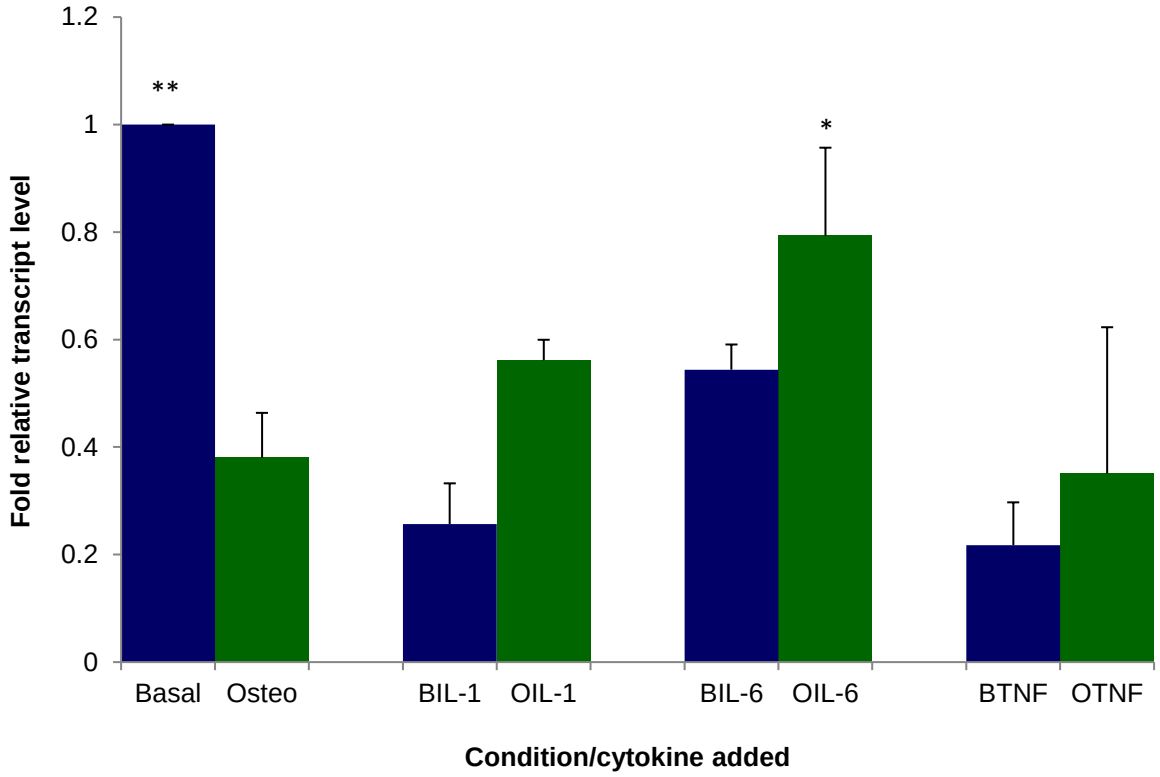


Figure 5.7 ALP expression relative to β actin for iPSCs at Day 7 with or without cytokines
Data are presented as average ALP expression relative to β actin + SD for a single experiment performed in duplicate. B=Basal media alone, O=Osteogenic media alone, BIL-1=Basal media+IL-1, OIL-1=Osteogenic media+IL-1, BIL-6=Basal media+IL-6, IL-6=Osteogenic media+IL-6, BTNF=Basal media+TNF, OTNF=Osteogenic media+TNF. *p<0.05 compared to osteogenic control sample, **p<0.01 compared to osteogenic control sample.

Summary

- iPSCs in culture maintained their osteogenic potential in the presence of inflammatory cytokines, showing positive staining for ALP.
- IL-1β did not exert an additive osteogenic effect on ALP production but had a modest stimulatory effect on ALP expression by iPSCs in culture.
- IL-6 had an additive osteogenic effect, as seen previously for fetal femur cultures.
- TNFα exerted a modest inhibitory effect.

5.3.3 Discussion

The effects of IL-1β on osteogenic differentiation in iPSC cultures were largely stimulatory, whilst those of TNFα were inhibitory, in keeping with results from HBMSC cultures (Figures 5.6 and 5.7). IL-6, however, exerted an additive osteogenic effect in iPSC cultures, both in terms of ALP production and ALP gene expression. This was in contrast to the results in HBMSC cultures discussed in Chapter 3, where IL-6 had minimal impact on osteogenic differentiation, except in the Stro-1 +ve HBMSC experiments (Figures 3.18). These results were in keeping with changes in ALP production and gene expression seen in fetal femur and cytokine cultures (Figure 3.19).

These studies of proinflammatory cytokines and iPSCs give important insights into how these bioactive factors might be beneficial in future tissue engineering strategies for OA based on iPSCs. With their capacity for almost unlimited self-renewal and the ability to differentiate along any lineage, iPSCs have already been recognised as a promising option for high-throughput screening for novel medications. Specifically in OA, murine iPSCs have already been used to generate engineering cartilage that, after exposure to IL-1α for 3 days to mimic the inflammatory environment in OA, has been shown to represent a valid model for testing potential disease-modifying drugs for OA (DMOADs) (Willard, Diekman et al. 2014). Disease -specific iPSCs have been generated successfully from synovial tissue taken from

OA patients at the time of hip arthroplasty surgery (Kim, Son et al. 2011). Another group have successfully generated disease specific iPSCs from fibroblast-like synoviocytes (FLS) derived from the synovium of patients with OA and RA obtained during arthroscopic synovectomy or total knee replacement (Lee, Kim et al. 2014). These iPSCs demonstrated all the cell markers associated with pluripotency. It is hoped that these disease-specific iPSCs can be used to develop disease specific platforms in the future to facilitate testing of novel treatments for RA and OA, as well as furthering our understanding of the complex pathophysiology of these conditions (Lee, Kim et al. 2014). There has also been considerable interest in utilizing iPSCs to repair focal cartilage defects and to obtain a greater understanding of the basis of rare skeletal dysplasias and evaluate potential treatments for these conditions (Liu, Yang et al. 2017). The models and platforms discussed above provide potential systems for testing the stimulatory effects of IL-1β and IL-6 on osteogenic differentiation of SSCs in the future to help devise optimal tissue engineering strategies for treating OA.

Recent studies have demonstrated that exosomes secreted by iPSC-derived MSCs (iMSCs) have superior efficacy to those secreted by synovial membrane derived SSCs (SMMSCs) for attenuating OA in a murine model of collagenase-induced OA (Zhu, Wang et al. 2017). The cartilage generated using these iMSC- secreted exosomes more closely resembled normal hyaline cartilage than that produced by the SMMSC exosomes (Zhu, Wang et al. 2017). iMSCs-derived exosomes have several advantages over those from SMMSCs in that iPSCs can be derived from patient-specific adult somatic cells. These can be isolated from many tissues, including peripheral blood, so harvesting them does not require invasive surgical procedures such as those for obtaining synovial tissue. As iPSCs are patient-specific, implantation of the iMSC-secreted exosomes may not require the recipient to remain on potent lifelong immunosuppression. As iPSCs are autologous, this also circumvents many of the ethical issues associated with the usage of alternative cell types such as embryonic stem cells, as mentioned above. Finally, autologous iMSCs are a continually renewable source of MSCs for use in tissue engineering strategies (Zhu, Wang et al. 2017). However, safety concerns centred around risks of teratoma formation and immunogenicity with iPSCs still remain and are a significant impediment to clinical translation at the present time. Work is ongoing to find alternatives to the oncogenes c-myc and klf4 in the reprogramming of somatic cells to generate iPSCs, which would overcome one of these barriers to using iPSC-based therapies in clinical practice (Lee, Kim et al. 2014). A further issue requiring future research is how patients' previous therapies,

particularly disease-modifying OA drugs (DMOADs), might affect the behavior of somatic cells used for transformation to iPSCs (Lee, Kim et al. 2014). This will help clarify if there is a certain point in the development of OA at which iPSC-based strategies will be most effective and if previous exposure to certain drug treatments might be a barrier to their use.

5.4 Analysis of microRNA expression within HBMSC and cytokine or supernatant cultures

5.4.1 Experimental set up

Cultures of HBMSCs were set up following preparation of bone marrow samples according to the protocol described in Section 2.1.1. HBMSCs were cultured initially in basal media for 6 days, washed with PBS and fresh media applied. Further media changes were carried out every 2-3 days (as detailed in Section 2.1.3) until the cells reached 95% confluence. The cells were then released from the surface of the tissue culture plastic by treatment with collagenase followed by trypsin (detailed in Section 2.1.4). The cell suspension obtained was centrifuged (1100rpm for 4 minutes), the cell pellet re-suspended in 10mls of basal media and a cell count performed using a haemocytometer (Section 2.1.1). Cells were seeded at 2.5 x 10^4 cells per well of a 12 well plate in either basal or osteogenic media. Each of these two groups were subdivided further into 3 groups: controls (no additional factors); IL-1β (10ng/mL); synovial supernatants in 250µl aliquots (selected for high IL-1β concentration). For each of these 6 conditions, wells were labelled singly for use in histological analysis (ALP, type 1 and type II collagen), in duplicate for ALP and DNA biochemistry assays and in triplicate for PCR analysis. Media changes were performed every 2-3 days. Cultures were run for a total of 7 days prior to analysis. In these experiments, in addition to qPCR for the osteogenic genes ALP, Runx-2, osteonectin and osteocalcin and the chondrogenic genes Sox-9, Collagen II and Collagen X performed in the cytokine experiments described in Chapter 3, miRNA PCR was performed for miRNA 146a, 34a, 140 and 138 using a specific miRNA isolation kit then the standard VILO method described in Section 2.5.2.

5.4.2 Results

5.4.2.1 Biochemistry

IL-1β produced an additive stimulatory effect on ALP production in HBMSC cultures, consistent with findings using HBMSC and cytokine experiments detailed in Chapter 3 (Figure 5.8). The addition of synovial supernatants from OA patients produced an additive osteogenic effect on ALP production in two experiments (Figures 5.8 and 5.9) but an inhibitory effect in the third one (Figure 5.10). This osteogenic effect correlated with the concentration of IL-1β in the synovial supernatants used.

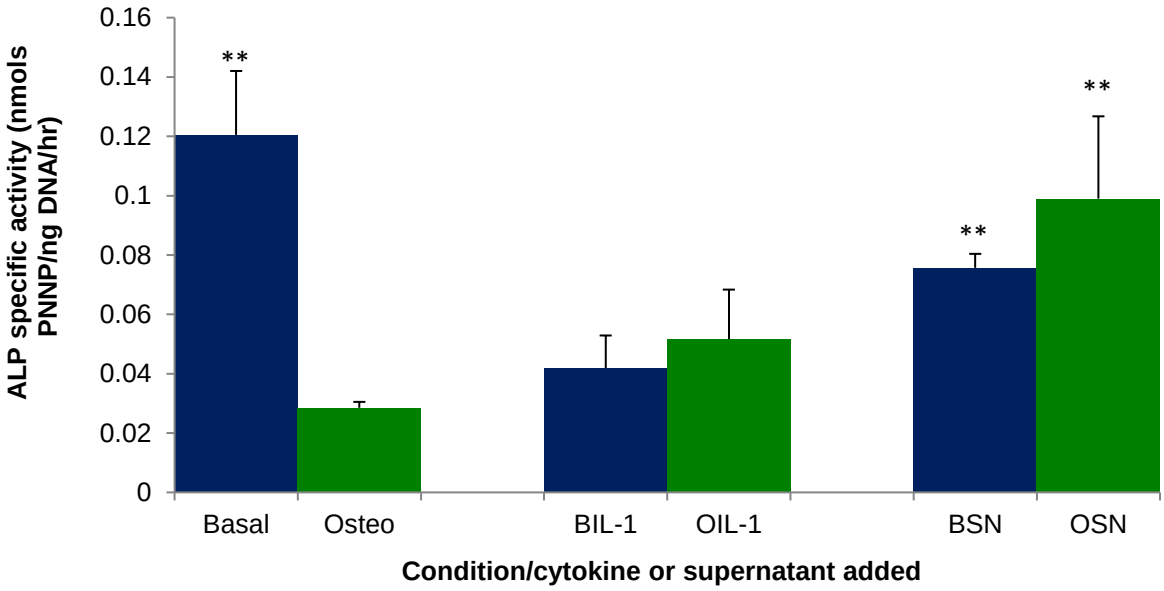


Figure 5.8 ALP Specific activity of HBMSC with or without cytokine or synovial supernatants (F91)
Data presented as average ALP specific activity + SD for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+ IL-1, OIL-1=Osteogenic media+IL-1, BSN=Basal media + supernatant, OSN=Osteogenic media + supernatant. ** p<0.01 vs osteogenic control sample.

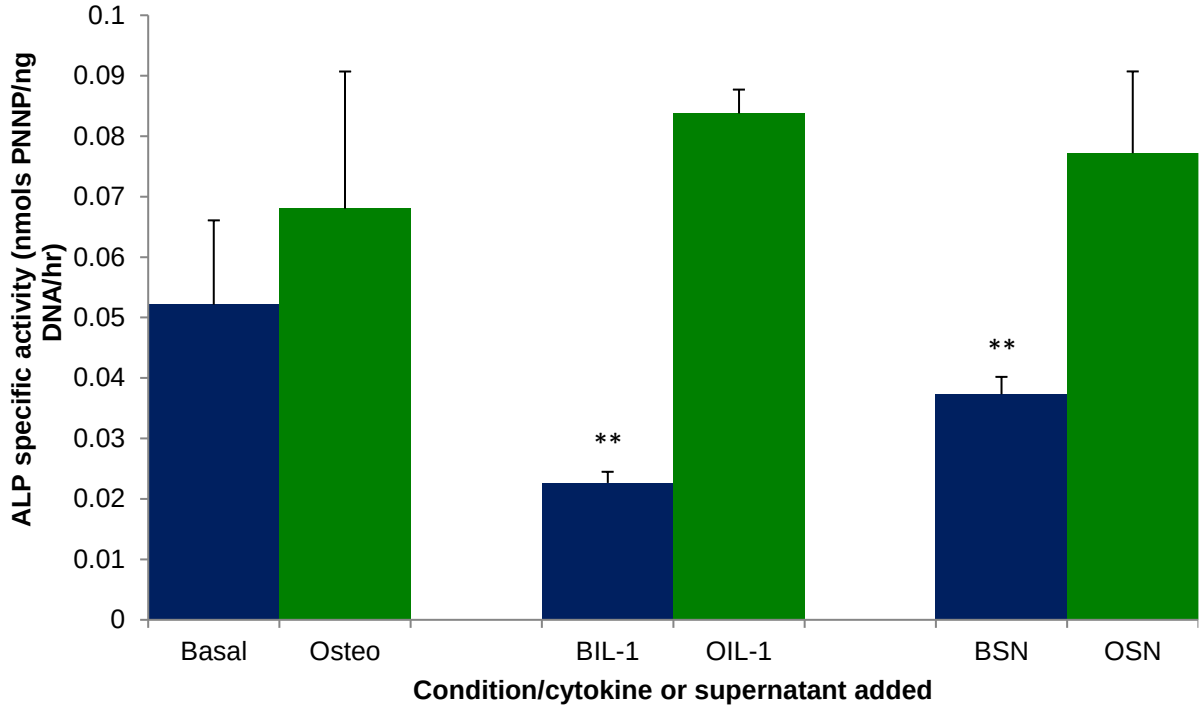


Figure 5.9 ALP specific activity of HBMSC with or without cytokine or synovial supernatants (F80)

Data presented as average ALP specific activity + SD for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+ IL-1, OIL-1=Osteogenic media+IL-1, BSN=Basal media + supernatant, OSN=Osteogenic media + supernatant. **p<0.01 versus osteogenic control sample.

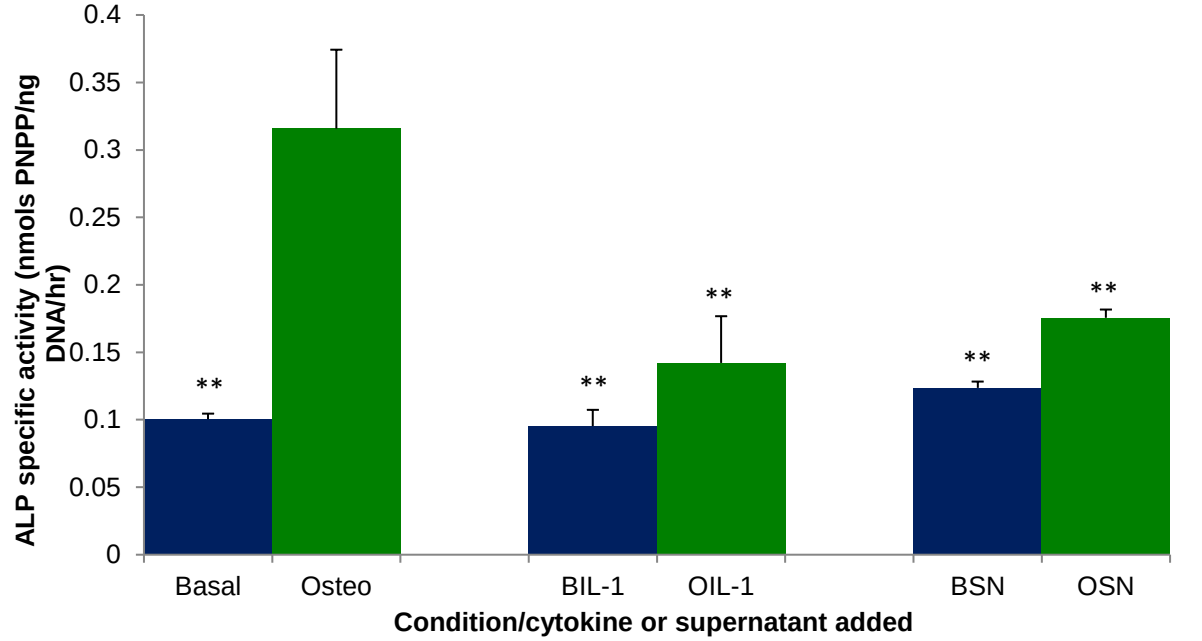


Figure 5.10 ALP Specific activity of HBMSC with or without cytokine or synovial supernatants (F81)

Data presented as average ALP specific activity+SD for a single experiment performed in duplicate. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+ IL-1, OIL-1=Osteogenic media+IL-1, BSN=Basal media + supernatant, OSN=Osteogenic media + supernatant. ** p<0.01 vs osteogenic control sample.

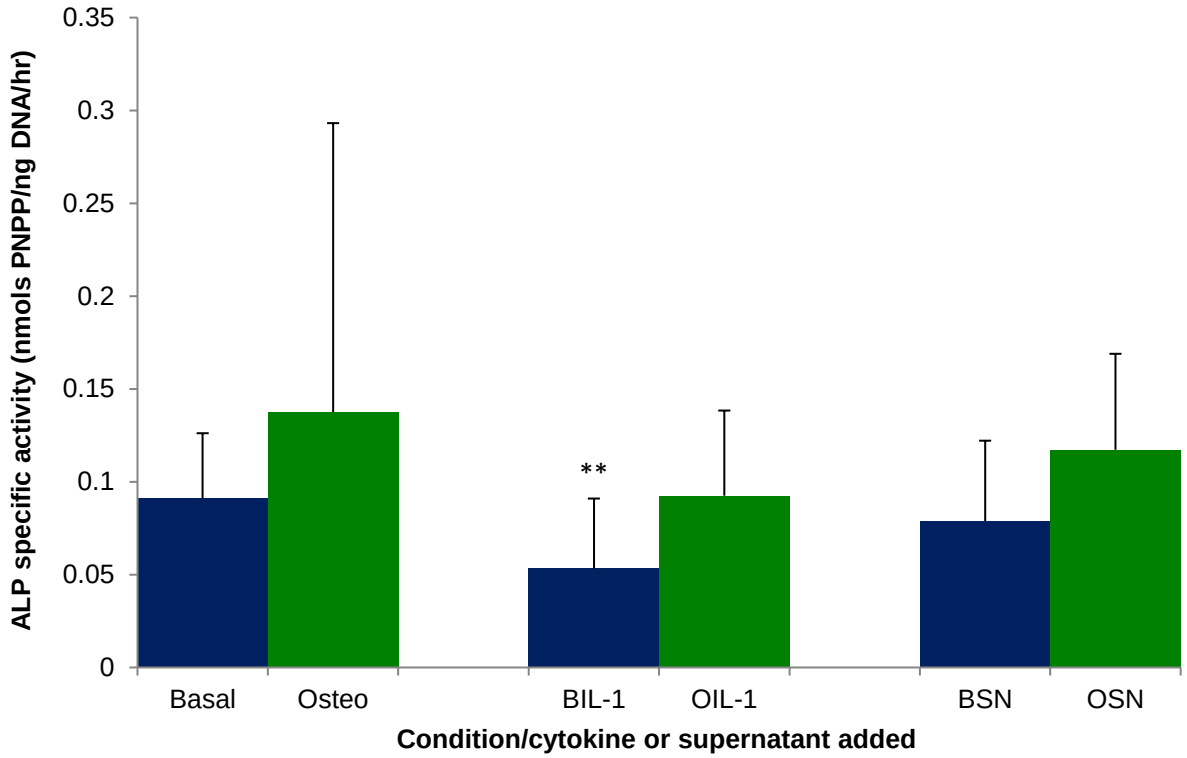


Figure 5.11 ALP Specific activity of HBMSC with or without cytokine or synovial supernatants
Data presented as mean ALP specific activity + SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+ IL-1, OIL-1=Osteogenic media+IL-1, BSN=Basal media + supernatant, OSN=Osteogenic media + supernatant.**p<0.01 versus osteogenic control sample.

5.4.2.2 Molecular biology

Addition of IL-1β to HBMSC cultures resulted in upregulation of miR146a in both basal and osteogenic culture conditions (Figure 5.12). This upregulation reached statistical significance in the HBMSC and cytokine cultures but not in the co-cultures with synovial supernatants. miR138 expression was also upregulated in HBMSC cultures when IL-1β was added to osteogenic cultures, although this effect did not reach statistical significance (Figure 5.13). However, miR140 expression was downregulated by the addition of IL-1β to HBMSC in either basal or osteogenic media (Figure 5.14).

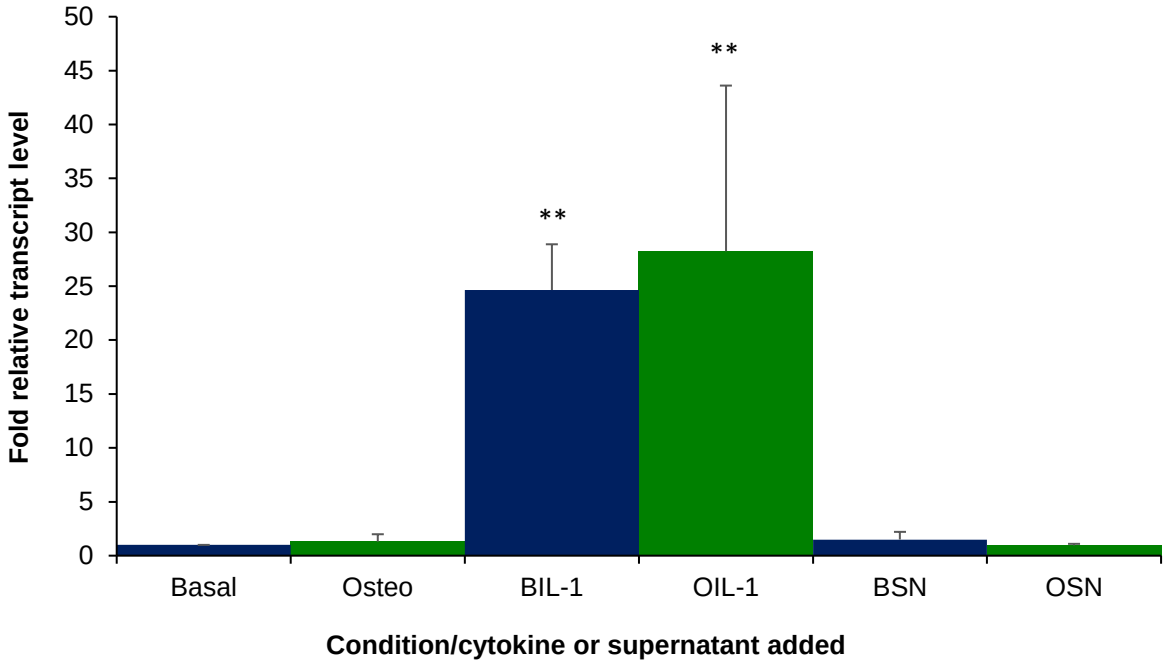


Figure 5.12 miR-146a expression of HBMSC with or without cytokine or synovial supernatants
Data presented as mean miRNA expression + SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+ IL-1, OIL-1=Osteogenic media+IL-1, BSN=Basal media + supernatant, OSN=Osteogenic media + supernatant.**p<0.01 versus osteogenic control sample.

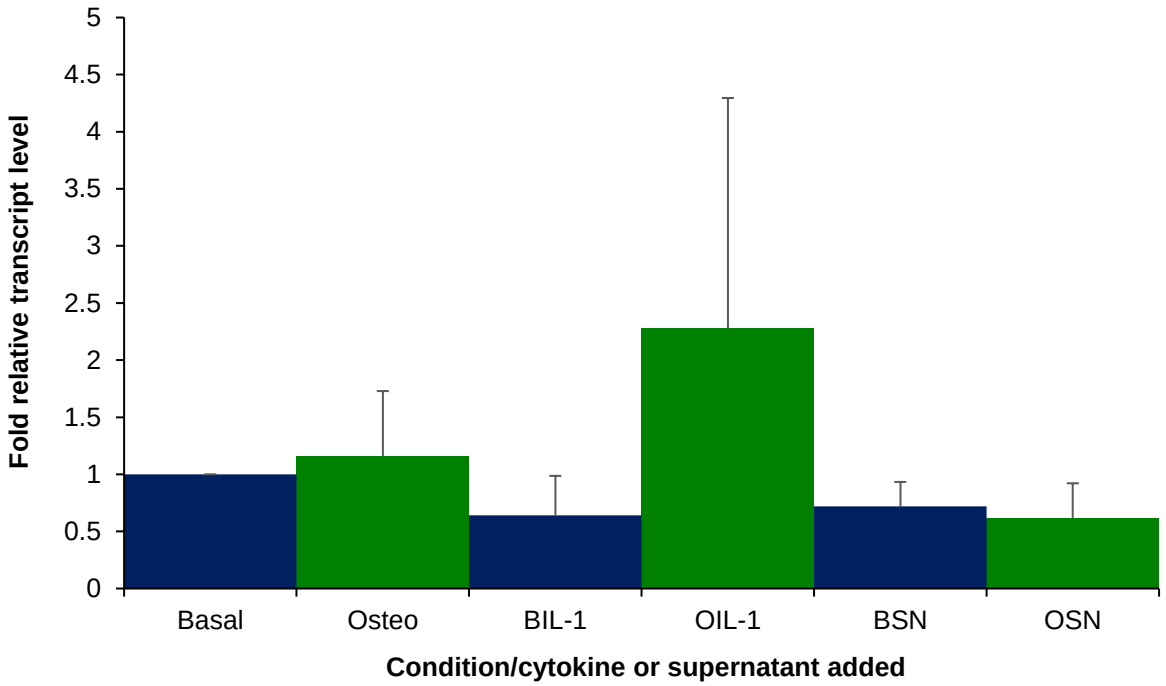


Figure 5.13 miR-138 expression of HBMSC with or without cytokine or synovial supernatants
Data presented as mean miRNA expression + SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+ IL-1, OIL-1=Osteogenic media+IL-1, BSN=Basal media + supernatant, OSN=Osteogenic media + supernatant. Differences between the groups were not statistically significant.

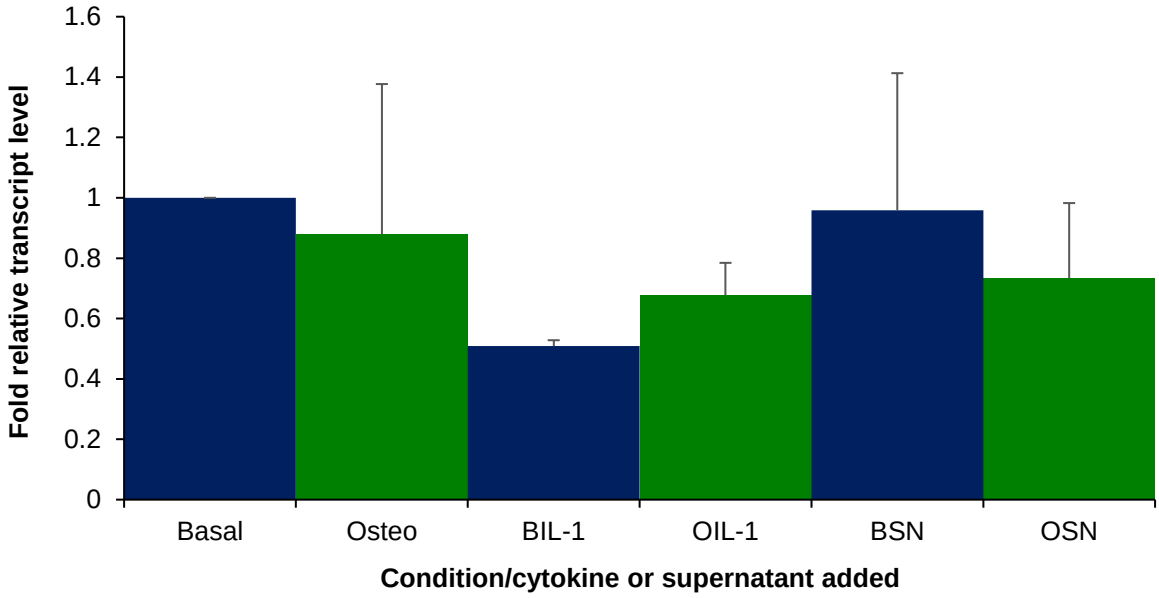


Figure 5.14 miR-140 expression of HBMSC with or without cytokine or synovial supernatants
Data presented as mean miRNA expression + SD, n=3. Basal=Basal media alone, Osteo=Osteogenic media alone, BIL-1=Basal media+ IL-1, OIL-1=Osteogenic media+IL-1, BSN=Basal media + supernatant, OSN=Osteogenic media + supernatant. Differences between the groups did not reach statistical significance.

Summary

- IL-1β exerted an additive osteogenic effect on HBMSCs in culture.
- The majority of OA synovial supernatants exerted an additive osteogenic effect on HBMSCs, related to their concentration of IL-1β.
- Expression of miR-146a and miR-138 by HBMSCs were upregulated by IL-1β, whilst that of miR-140 was downregulated.
- Expression of all three miRNAs was downregulated in co-cultures of HBMSCs with OA synovial supernatants.

5.4.3 Discussion

Several factors could account for why the effects seen with IL-1β in HBMSC cultures were not replicated in the co-cultures with synovial supernatants. The synovial supernatants contained reduced concentrations of IL-1β, which may have been insufficient to produce the

upregulation of miR146a and miR138 seen with the 10ng/ml concentration of IL-1β used in the cytokine experiments. Furthermore, synovial supernatants contain many other cytokines and bioactive factors, not least TNFα, which may exert an opposing downregulation of these microRNAs. Additional experiments using other pro and anti-inflammatory cytokines are required to investigate this further.

The role of miRNAs in the pathogenesis of OA is a rapidly expanding area of research, which has delivered new insights into the many pathways involved and their complex interactions (reviewed in (Tsezou 2014, Wu, Tian et al. 2014, Li YP 2015, Yu, Meng et al. 2015, Nugent 2016, Portal-Nunez, Esbrit et al. 2016, Xu, Li et al. 2016, Budd, Waddell et al. 2017). However, most of these studies, to date, have focused on the pathogenesis of cartilage damage in OA. miR-146a, miR-155, miR-181a and miR-223 are all upregulated in peripheral blood mononuclear cells from OA patients compared to healthy controls (Okuhara, Nakasa et al. 2012). In contrast, miR-16, miR-132, miR-146a and miR-223 are downregulated in synovial fluid from OA patients compared to that from non-OA controls (Murata, Yoshitomi et al. 2010). This is in keeping with the relative downregulation of miR-146a seen in the above HBMSC/ OA synovial supernatant co-cultures (Figure 5.12). In early stage OA, miR-146a and miR-223 are upregulated compared to late stage OA. Other groups have also confirmed that miR-146a is upregulated in cartilage with a low Mankin grade (Taganov, Boldin et al. 2006). miR-146a levels have been shown to decline in late stage OA cartilage (Jones, Watkins et al. 2009), which would also fit with downregulation of miR146a levels in the above co-cultures, given that the supernatants were derived from synovial samples taken from patients with late stage OA severe enough to require joint replacement surgery.

The expression of miRNA-146a was significantly upregulated in HBMSC cultures by the addition of IL-1β, either under basal or osteogenic conditions (Figure 5.12). This is in keeping with the known upregulation of miR-146a in chondrocytes by IL-1β, mediated via activation of the NF-κB pathway (Taganov, Boldin et al. 2006). Subsequent work has demonstrated that increased miR-146 expression in isolated human chondrocytes suppresses IL-1β induced TNFα production (Jones, Watkins et al. 2009). This reduced cytokine signalling is due to downregulation of TRAF6 and IRAK1 (Nakasa, Nagata et al. 2011). Inhibition of TRAF6 expression also suppresses activation of the NFκB signalling pathway, increasing proliferation of OA chondrocytes and reducing apoptosis, with a resultant positive impact on joint repair (Zhong, Li et al. 2017). Transfection of human articular chondrocytes with synthetic miR-146a reduces IL-1β-induced expression of MMP-

13 and ADAMTS-5 and inhibits production of the inflammatory cytokines IL-6 and IL-8 in synovial cells (Li X 2011). Together, this demonstrates that miR-146a antagonises IL-1β-induced expression of MMP-13 and ADAMTS-5 via its inhibitory effects on IRAK-1 and TRAF6, as well as reducing the release of other inflammatory cytokines. miR-146a levels are therefore negatively correlated with the expression of MMP-13 in OA cartilage (Li J 2012).

Studies in human fetal femur derived SSCs have provided new data on the targets of miR-146a and helped elucidate some of the pathways through which miR-146a modulates both chondrogenic and, to a lesser extent, osteogenic differentiation of SSCs (Cheung, Sposito et al. 2014). When miR-146a is overexpressed in epiphyseal cell cultures, a significant downregulation of Smad 3 mRNA and downregulation of both Smad 2 and Smad 3 at a protein level has been observed. This is accompanied by downregulation of the chondrogenic marker SOX-9 and upregulation of the osteogenic marker Runx-2, suggesting that miR-146a is a negative regulator of chondrogenic differentiation, yet also has an indirect effect in promoting osteogenic differentiation (Cheung, Sposito et al. 2014). This is confirmed by the overexpression of miR-146a in SSCs stimulated with TGFβ3, which produces a marked attenuation of TGFβ-induced upregulation of collagen X. The addition of TGFβ3 to SSC cultures also leads to downregulation of miR-146a, suggesting that there is an autoregulatory mechanism operating between TGFβ stimulation and miR-146a expression (Cheung, Sposito et al. 2014).

miR-9, miR-98 and mi-146 have all been shown to be involved in the regulation of TNFα expression, thereby exerting a protective role in OA pathogenesis (Iliopoulos, Malizos et al. 2008). When peripheral blood mononuclear cells (PBMC) from healthy volunteers are transfected with miR-146a, this inhibits osteoclastogenesis (Nakasa, Nagata et al. 2011). Intravenous injection of ds-miR-146a twice daily prevents joint destruction in mice with collagen-induced arthritis (CIA), confirming these protective osteochondral effects *in vivo* as well as *in vitro* (Nakasa, Nagata et al. 2011). miR-146a is also involved in knee joint homeostasis and OA-associated pain, balancing the inflammatory responses in OA cartilage and synovium with pain related factors in glial cells (Li X 2011). In a medial meniscal transection model of OA in rats, downregulation of miR-146a and or the miR-183 cluster in the dorsal root ganglion and spinal cord is associated with upregulation of inflammatory pain mediators (Li X 2011). Therefore, maintaining or upregulating levels of miR-146a has the potential not only to provide a target for preventing or repairing the cartilage damage present in OA but also for influencing the mechanisms driving chronic pain in OA.

Similar effects of miR-146a have also been reported in OA synovial cells. HDAC inhibitors have been shown to increase expression of miR146a in OA fibroblast-like synoviocytes (FLS), augmenting the downregulation of IL-1β signalling (Wang, Shih et al. 2013). However, miR-146a also induces the expression of VEGF and inhibits the expression of Smad 4, reducing responsiveness to TGFβ (Li J 2012). It is therefore importance to harness the beneficial effects of miR-146a on articular tissues whilst minimising any less advantageous ones.

The related miRNA, miR146b, has recently been studied by our group and found to be downregulated during chondrogenic differentiation of HBMSSCs and upregulated in OA (Budd E 2017). Overexpression of miR-146b via a gain of function mutation results in downregulation of SOX-5 (Budd E 2017). This suggests that miR-146b may also have a future role, combined with stem cell therapy, in providing a tissue engineering strategy that can improve cartilage regeneration and represent a future treatment for OA.

miR-138 was also upregulated by the addition of IL-1β in our HBMSC cultures, although only under osteogenic conditions (Figure 5.13), and downregulated in the co-cultures with OA supernatants. MiR-138 has been shown inhibit osteogenic differentiation of hMSCs by reducing the expression of Protein Tyrosine Kinase 2 (PKT2) or Focal Adhesion Kinase (FAK) (Kim, Bae et al. 2009). MiR-138 is markedly reduced in OA cartilage tissues compared to normal cartilage (Wei, Liu et al. 2017). Stimulation of chondrocytes with IL-1β significantly increases expression of miR-138-5p (Yuan, Zhang et al. 2016). Current results suggest that certainly the higher concentrations of IL-1β in the HBMSC and cytokine cultures were sufficient to produce upregulation of miR138. miR-138-5p overexpression significantly increases IL-1β-induced downregulation of COL2A1, aggrecan and glucosaminoglycans and increases expression of MMP-13, resulting in cartilage damage via its effects on Forkhead BoxC1 (FOCX1) (Yuan, Zhang et al. 2016). Exposure to TNFα reduces miR-138 expression in normal and OA chondrocytes (Wei, Liu et al. 2017). miR-138 overexpression reduces p65, COX-2 and IL-6 in human OA chondrocytes, attenuating the effects of TNFα on cartilage damage. The reduction in miR-138 expression in the HBMSC/ OA supernatant co-cultures could therefore have either been due to the lower concentrations of IL-1β present, or to the coexistence of sufficient TNFα in the synovial supernatant added to produce downregulation of miR-138 as reported in articular chondrocytes in culture.

miR-138 has already been explored as a potential therapeutic target for treating OA. A series of experiments with hMSCs transfected with control miR, miR-138 or anti-MiR-138 on hydroxyapatite/tricalcium phosphate scaffolds and implanted into SCID mice, revealed that

inhibiting miR-138 significantly increased ectopic bone formation *in vivo* (Eskildsen, Taipaleenmaki et al. 2011). miR-140 was, in contrast, downregulated by the addition of IL-1β to HBMSC cultures, more so in basal than osteogenic conditions (Figure 5.14). In co-culture with OA synovial supernatant, miR-140 was also downregulated under osteogenic conditions. miR-140 shows the greatest differential expression of all miRNAs between chondrocytes and MSCs (Miyaki, Nakasa et al. 2009). Its expression significantly reduces with increasing severity of OA (Miyaki, Nakasa et al. 2009). This may well explain the reduced miR-140 expression seen in the above HBMSC / OA supernatant co-cultures, which reflect the inflammatory milieu present within the joint of patients with advanced OA. In keeping with this hypothesis, miR-140 expression is reduced in synovial fluid taken from patients with knee OA and negatively correlates with the severity of their OA (Wu, Tian et al. 2014).

When normal human articular chondrocytes are exposed to IL-1β, their expression of miR140 falls (Miyaki, Nakasa et al. 2009), similar to the reduction in miR-140 seen in IL-1β treated HBMSC cultures above. This effect is NFκB-dependent (Liang, Zhuang et al. 2012). Overexpression of miR-140 reduces IL-1β induced expression of ADAMTS5 and IL-1β dependent inhibition of aggrecan gene expression in human articular chondrocytes (Miyaki, Nakasa et al. 2009). Knockout mice for miR-140 develop spontaneous age-related OA type changes in cartilage with reduction in proteoglycan content and fibrillation (Miyaki, Sato et al. 2010). Conversely, overexpression of miR-140 in chondrocytes is protective against either surgically-induced or antigen-induced arthritis (AIA) *in vivo* (Miyaki, Sato et al. 2010). Transfection of OA chondrocytes with either pre miR-140 or its inhibitor confirm that miR-140 directly targets insulin-like growth factor binding protein-5 (IGFBP-5) (Tardif, Hum et al. 2009). HDAC-4 and IGFBP5 are both down-regulated by miR-140. It acts as a negative feedback regulator of MMP-13 (Wu, Tian et al. 2014) and has been shown to directly mediate MMP-13 expression *in vitro* (Nakamura, Inloes et al. 2011). Combination treatment with miR-140 and miR-29 have been shown to reverse IL-1β-stimulated MMP-13 and TIMP-1 levels and rescue type 2 collagen levels in an *in vitro* model of OA, supporting upregulation of miR-140, either alone or in combination, as part of a tissue engineering based treatment for OA.

miR-140 also targets DNPEP, a downstream BMP signalling antagonist, increasing BMP signalling, which promotes chondrogenic differentiation (Xu, Li et al. 2016). It also acts synergistically with miR-455-3p to regulate TGFβ signalling through inhibition of the Smad 2/3 pathway (Tuddenham, Wheeler et al. 2006, Swingler, Wheeler et al. 2012). Therefore,

strategies involving upregulation of both miR-140 and miR-455-3p may represent a further potential target for future OA treatments.

The potential for harnessing this miRNA-driven modulation of osteogenic or chondrogenic differentiation of HBMSCs to devise a tissue-engineering based treatment for the osteochondral lesions seen in OA has recently been reviewed (Budd, Waddell et al. 2017). miR-138 was one of the potential targets identified where downregulation optimised osteogenic differentiation of hMSCs as mentioned above (Eskildsen, Taipaleenmaki et al. 2011). Upregulation of miR-34a also had beneficial effects on osteogenic differentiation of hMSCs in a similar *in vivo* murine model of bone formation (Chen, Holmstrom et al. 2014). Silencing of miR-221 has been shown to promote chondrogenic differentiation of hMSCs *in vitro* (Lolli, Lambertini et al. 2014). More recent studies have confirmed that silencing of miR-221 produces improving healing of a cartilage defect *in vivo* (Lolli, Narcisi et al. 2016). The work summarised here, in HBMSCs cultured with cytokine or synovial supernatant, demonstrates that miR-138, miR-140 and miR-146a behave similarly under these conditions to what has been reported in chondrogenic cultures. This supports the selection of upregulation of miR-140 or miR-146a/b, either alone or in combination with the synergistic miRNAs mentioned above, as suitable adjuncts to a tissue engineering strategy for prevention or treatment of the osteochondral lesions seen in early OA. It remains unclear at the present time as to whether using single miRNAs or combinations of miRNAs is the most effective strategy, although it is thought that a combination approach is likely to be the preferable option (Miyaki and Asahara 2012). miRNAs are difficult to deliver *in vivo* and concerns remain around potential carcinogenicity given that miRNAs frequently have multiple targets, including in some instances oncogenes such as c-myc (Nakasa, Nagata et al. 2011). Therefore, minimising any off target effects of miRNAs will be key to incorporating them into future tissue-engineering strategies for the prevention or treatment of early OA.

Chapter 6 Cytokine modulation of SSCs in *ex vivo* and *in vivo* models of bone formation

6.1 Introduction

An organotypic culture system can be used as an intermediate step in evaluating the effects of cytokine modulated SSCs on scaffolds in a surrogate *in vivo* model of bone formation. This type of *ex vivo* model allows manipulation of multiple factors involved in the integration and differentiation of cells implanted as part of a tissue engineering construct, while maintaining the three-dimensional tissue environment. Thus, an *ex vivo* model represents an important alternative approach to evaluate the effects of cytokines and OA synovial supernatants within a three-dimensional system with the potential to inform *in vivo* studies. The model selected was the *ex vivo* organotypic culture system of embryonic chick femora. The details of the model setup have already been described in Section 2.1.6. The advantages of this model are that the organotypic model provides a rapid, high-throughput screening of the effects of different culture conditions, bioactive factors and medications, on skeletal development. The model is reliable, reproducible, cost-effective and highly sensitive to applied external stimuli (Kanczler, Smith et al. 2012). This type of *ex vivo* model also lacks the majority of the systemic influences present when using an *in vivo* model, which have the potential to act as confounders when assessing the influence of applied bioactive factors. The chick femur organotypic culture model has been previously validated by our group for studying factors involved in the modulation of skeletal development, repair and tissue responses (Kanczler, Smith et al. 2012). The effects of basal, osteogenic and chondrogenic media on embryonic skeletal development are already fully documented (Kanczler, Smith et al. 2012), as are the influences of parathyroid hormone (PTH) and parathyroid-related hormone (PTHrP) (Smith, Kanczler et al. 2012). A 10 day culture period was chosen as although chick femurs can be maintained in culture for up to 18 days, tissue necrosis and cell death have been reported to occur, at an increased rate, beyond 10 days (Smith, Kanczler et al. 2013).

This organotypic culture system has since been adapted, within the Bone and Joint Research Group, University of Southampton, to create a femoral defect model, which has been used to examine the effects of dual growth factor releasing hydrogels on skeletal tissue repair (Smith, Kanczler et al. 2014). The growth factors studied to date include BMP-2, TGFβ3 and VEGF (Smith, Kanczler et al. 2014). The combination of Stro-1 positive SSCs,

BMP-2 and TGFβ3-containing microparticles with an extracellular matrix hydrogel scaffold demonstrates significantly increased production of structured bone matrix (Smith, Kanczler et al. 2014). However, the effects of inflammatory cytokines on this model remain, to date, unknown.

The findings from studies in this *ex vivo* model were used to set up an *in vivo* model of bone formation, the murine subcutaneous implant model. To date, the subcutaneous implant model has been widely used to assess the efficacy of osteo-inductive agents and cell populations in the initiation of bone formation (Black C.R.; Goriainov V 2015). This type of ectopic implantation of SSCs has been recognised as a suitable method of assessing their *in vivo* differentiation potential, by creating an ectopic niche within which the behaviour of human or murine SSCs can be examined. The subcutaneous implant model was used here to demonstrate the ability of HBMSCs cultured with appropriate combinations of growth factors and antibodies to promote cartilage and bone formation in severely compromised immunodeficient (SCID) mice, to validate the applicability of our novel approach. This model remains the industry standard for the assessment of skeletal tissue formation and our research group have published extensively on the use of this model for the assessment of skeletal tissue engineering approaches (Howard, Partridge et al. 2002, Partridge, Yang et al. 2002, Yang, Green et al. 2003).

The advantage of the subcutaneous implant model is that it can be used to determine the functionality of a desired population, such as the SSC in a short time frame, allowing high-throughput testing of multiple bioactive factors. The subcutaneous implant model has the additional benefit of shorter surgical times for implantation and harvesting of the implants compared to other more invasive models where the implant is placed directly into a bony defect. However, the approach still necessitates a general anaesthetic and a significant transplantation period (4 to 12 weeks). An alternative strategy would be percutaneous injection of HBMSC and hydroxyapatite /tricalcium phosphate (HA/TCP) suspensions, which in preliminary studies have been shown to allow delineation of the osteogenic potential of cell populations as injections of the cell suspension were capable of developing into mature bone (Mankani, Kuznetsov et al. 2008). However, it can be difficult with this type of subcutaneous implant model to differentiate host and implanted tissue, whereas the diffusion chamber model used by our group has the advantage of allowing easier distinction of the two tissue types (Gundle, Joyner et al. 1995, Oreffo and Triffitt 1999, Tare, Mitchell et al. 2012).

Rapid improvements in microCT (µCT) over recent years, resulting in smaller machines with greater resolution, have provided a practical, non-invasive means of assessing bone formation, which can be coupled with traditional histological analysis to provide greater information on changes in bone composition under different culture conditions (Mankani, Kuznetsov et al. 2004). µCT provides additional quantitative detail on bone microarchitecture, improving our understanding of three-dimensional skeletal morphology. Thus, µCT was incorporated in the current work, to evaluate bone formation in e*x vivo* and *in vivo* models. Alternative imaging modalities such as MRI, with or without gadolinium enhancement, are also now being used to evaluate bone formation *in vivo* but are currently constrained both by cost and by the size of the scanners themselves.

The experiments presented in this chapter were designed to build upon the data generated on the effects of the selected inflammatory cytokines in *in vitro* HBMSC cultures described in Chapters 3 and 4. An *ex vivo* model, the organotypic culture system of embryonic chick femora, will be used, for the reasons explained above, to assess the effects of individual cytokines and of the synovial supernatants, in a three-dimensional system. Finally, the results from both the *in vitro* and *ex vivo* work will be used to set up an *in vivo* experiment, using the murine subcutaneous implant model of bone development. The latter will come closest to assessing how the individual cytokines and synovial supernatants might modulate osteogenic differentiation in an OA patient. The overall results will be used to provide information on how the individual cytokines or synovial supernatants affect osteogenic differentiation of SSCs under a variety of culture conditions, with the aim of using this information to advise how future tissue engineering strategies for repairing bone defects, including those seen in OA, should be adapted to overcome these effects of inflammation.

6.2 Overall aim

- To investigate whether the inflammatory cytokines present in OA produce a hostile environment that limits the use of bioengineering strategies to repair damaged joints.

6.3 Objectives

- To use a previously validated *ex vivo* organotypic culture system to establish the effects of individual cytokines or synovial supernatants on *ex vivo* bone formation.

- To use a previously validated small animal model of bone formation to assess the effects of cytokines and their inhibitors on the osteogenic potential of MSCs on calcium phosphate scaffolds *in vivo*.

6.4 *Ex vivo* model of bone formation

6.4.1 Experimental methods

(protocol devised by Southampton group, published as (Smith, Kanczler et al. 2012))

The establishment of this model has already been briefly described in Section 2.1.6. In brief, Day 1 fertilised chick eggs were obtained from P.D. Hook Hatcheries (Oxford, UK) and incubated at 38°C in an automatic Hatchmaster A incubator for 11 (E11) days. After this incubation time, embryos were removed from within the eggs and sacrificed by decapitation. Femurs were dissected out of each embryo, soft tissue and muscle removed, and washed in sterile PBS. Control non-cultured femurs were placed onto Millicell inserts (0.4µm pore size, 30mm diameter, Millipore, UK) (two femurs paired from the same chick per insert) and placed into individual wells of 6 well tissue culture plates containing 1ml of media per well, at the liquid/gas interface (Figure 6.1). Culture media was basal (α-MEM containing 100 units penicillin, 100µg/ml streptomycin and 100µM L-ascorbic acid 2-phosphate), alone or supplemented with IL-1β (10ng/ml), IL-6 (100ng/ml) or TNFα (10ng/ml) (all from Sigma-Aldrich, UK) or aliquots of human synovial supernatants (prepared as described in Section 2.1.5 above).

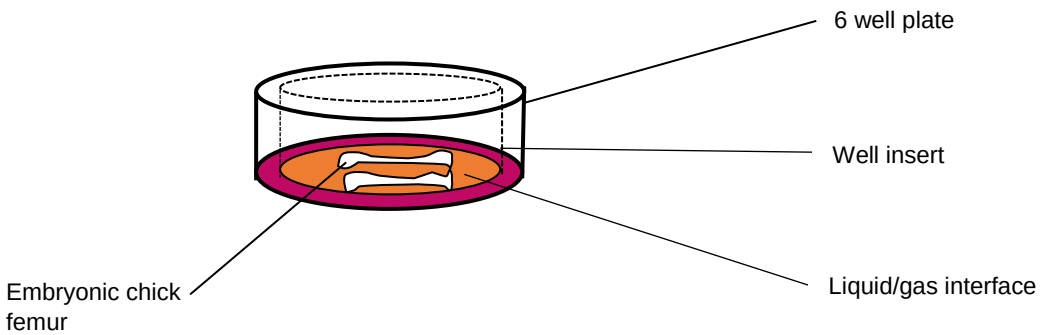


Figure 6.1 Schematic diagram of the *ex vivo* organotypic culture system of embryonic chick femora

Twelve femurs were cultured per condition at 37°C, supplemented with 5% CO_2 in air, for 10 days, with media changed every 24 hours. Prior to culture, each femur (except the control one) was micro-injected at the distal portion around the epiphysis/diaphysis junction with HBMSCs, which were previously removed from tissue culture flasks using collagenase, centrifuged and resuspended in 10mls αMEM +1% penicillin/streptomycin. The concentration of the cell suspension in each case was 40 cells/µL. After 10 days in culture, selected non-cultured and cultured femurs (n=4 for each condition) were used to assess glucosaminoglycan (GAG) content. The remaining samples were fixed in 4% w/v paraformaldehyde in PBS (PFA, Sigma-Aldrich, UK) for at least 24 hours then imaged radiographically using a Faxitron® Specimen Radiography System (MX-20) (Qados Ltd, Sandhurst, UK) and their lengths measured. Quantitative 3D analysis was then performed using a Xtek BenchTop 160Xi CT scanning system for micro-computed tomography (X-TEK Systems Ltd, Tring, Hertfordshire, UK) (n=6 for each condition). Following Faxitron and microCT analysis, the femurs were dehydrated through a series of ethanols (50%, 90%, 100%-1, 100%-2), cleared in chloroform and embedded in paraffin wax using an automated Shandon Citadel 2000. 6µm sections were cut from each femur for staining with Alcian blue/Sirius Red or von Kossa to assess proteoglycan and collagen production or mineralisation respectively. The remaining femurs were immunohistochemically stained for type 1 and type 2 collagen to assess for bone and cartilage formation respectively, STRO-1 as a skeletal stem cell marker and finally for the proliferation marker proliferating cell nuclear antigen (PCNA).

Whole slide images of the histological and immunohistochemical stained sections were analysed using an Olympus BX-51/22 dotSlide microscope and images created using OlyVIA 2.1 software (Olympus Soft Imaging Solutions, GmBH), and by a Carl Zeiss Axiovert 200 microscope with Carl Zeiss Axiovision 3.1 software package used to capture images.

6.4.2 Results

6.4.2.1 Histology

The control non-cultured femurs showed positive staining for proteoglycan around the periosteal collar following A/S staining (Figure 6.2a), with evidence of mineralisation in the same region with von Kossa staining (Figure 6.2b). The periosteal collar also stained positive for collagen 1, indicating early osteogenic differentiation (Figure 6.2c). There was only limited staining for Stro-1 (Figure 6.2d).

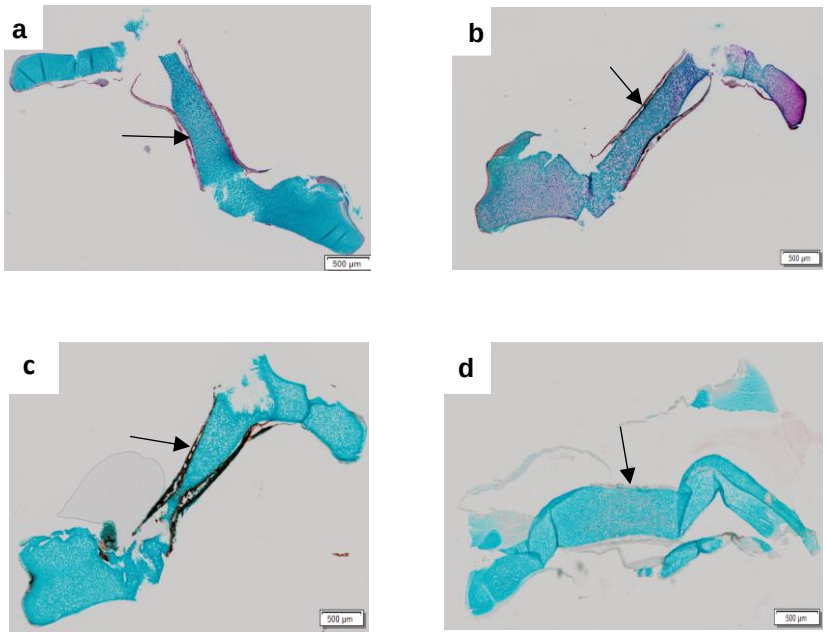


Figure 6.2 Control (non-cultured) chick femurs
a – A/S staining pinkish-purple for proteoglycan; b- von Kossa staining - linear black staining indicating mineralisation; c - Type 1 collagen staining - brownish colour- demonstrating osteogenic differentiation; d - Stro-1 staining for SSC markers- very faint brownish staining indicating low levels of expression. Images all taken at 20x magnification, scale bars indicated (500µm). Black arrows indicate the relevant areas of staining.

Under basal conditions, A/S staining was reduced (Figure 6.3a), reflecting lower levels of proteoglycan and collagen expression. This persisted even when IL-1β, IL-6 or TNFα were added to the cultures (Figures 6.3 b and c). However, increased mineralisation, with positive purplish-pink colouration around the periosteal collar, was apparent on von Kossa staining of femurs cultured in basal media with IL-1β (Figure 6.3d).

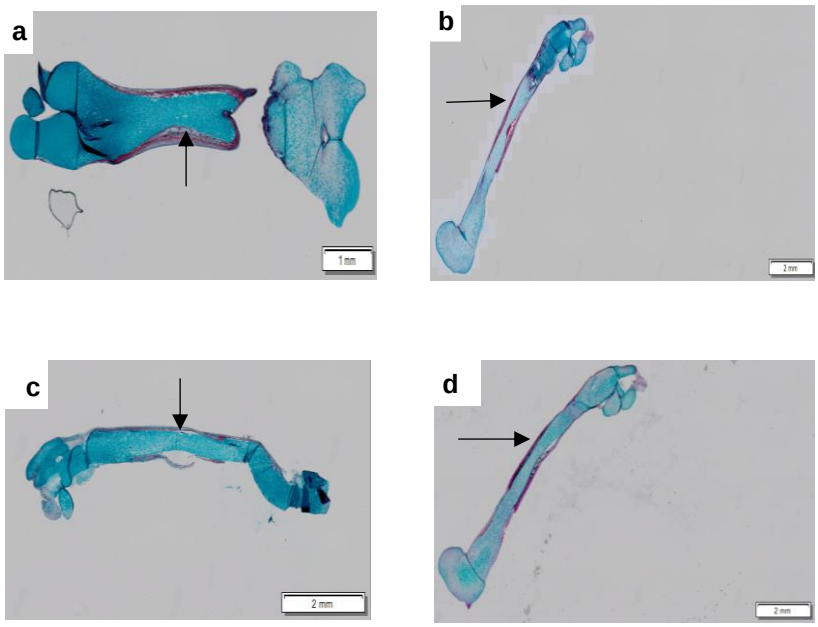


Figure 6.3 Chick femurs cultured under basal conditions
a – A/S staining basal control femurs; b – A/S staining for basal culture conditions with IL-1β added at a concentration of 10ng/mL; c – A/S staining for basal culture conditions with IL-6 added at a concentration of 100ng/mL; d - von Kossa staining for basal culture conditions with IL-1 β at a concentration of 10ng/mL, showing linear black colouration indicative of mineralisation, in the region of the periosteal collar. Images all taken at 20x magnification, scale bars indicated (2mm). Black arrows indicate the relevant areas of staining.

In contrast, femurs cultured in osteogenic media showed a marked increase in mineralisation on von Kossa staining (Figure 6.4). This was most marked for femurs cultured in osteogenic media supplemented with IL-1β (Figure 6.4b), with TNFα supplementation exerting an intermediate effect (Figure 6.4c). In all osteogenic conditions, ectopic new bone formation was evident around the site of microinjection of HBMSCs (Figure 6.4). There was modest limited expression of collagen 1, suggestive of early bone differentiation, in the femurs cultured in osteogenic media with IL-1β (Figure 6.4d).

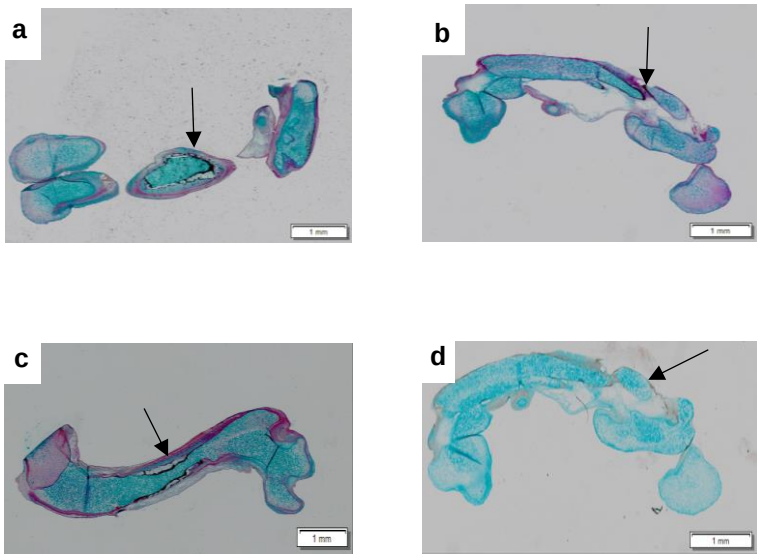


Figure 6.4 Chick femurs cultured under osteogenic conditions
a – von Kossa staining of osteogenic control femurs - linear black staining for mineralisation; b – von Kossa staining of femurs from osteogenic culture conditions with IL-1β added at a concentration of 10ng/mL - linear black staining for mineralisation; c – von Kossa staining of femurs from osteogenic culture conditions with TNFα added at a concentration of 10ng/mL – linear black staining indicating mineralisation; d – Collagen 1 immunostaining of femurs from osteogenic culture conditions with IL-1 β at a concentration of 10ng/mL- brownish linear staining around periosteal collar and region of microinjection of HBMSCs. Images all taken at 20x magnification, scale bars indicated (1mm). Black arrows indicate the relevant areas of staining.

In the co-cultures with OA synovial supernatants, effects on osteogenic differentiation of the chick femurs varied dependent on the supernatant used (Figures 6.5 - 6.8). However, there were similarities across the 4 experiments with all chick femurs displaying modest levels, mainly around the periosteal collar, of expression of proteoglycans (Figures 6.5- 6.8 a). Around the same area there was increased mineralisation evident on von Kossa staining (Figures 6.5- 6.8 b). This was most marked in the femurs cultured with M69 (Figure 6.7b) and M68 OA synovial supernatants (Figure 6.8b). Immunostaining for Collagen 1 mirrored that for mineralisation, as expected (Figures 6.5-6.8c), whilst that for Stro-1 was less prominent (Figures 6.5-6.8d).

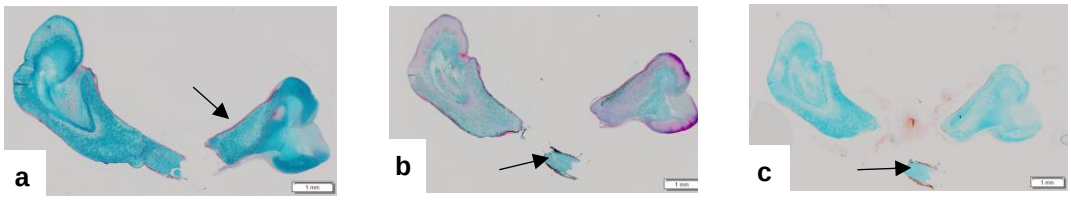


Figure 6.5 Chick femurs co-cultured with F61 OA synovial supernatant
a- A/S staining with typical purplish-pink staining for proteoglycan; b- von Kossa staining – black linear staining for mineralisation; c- collagen 1 immunostaining with faint red/brown staining in the region of the periosteal collar. Images all taken at 20x magnification, scale bars indicated (1mm). Black arrows indicate the relevant areas of staining.

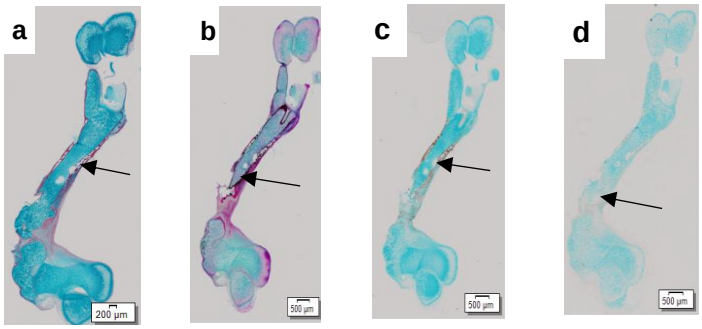


Figure 6.6 Chick femurs co-cultured with M70 OA synovial supernatant
a- A/S staining with typical purplish-pink staining for proteoglycan; b - von Kossa staining- back linear staining for mineralisation; c - collagen 1 immunostaining with more marked red/brown staining in the region of the periosteal collar; d- Stro-1 immunostaining showing faint brownish staining consistent with low levels of expression. Images all taken at 20x magnification, scale bars indicated (200-500µm). Black arrows indicate the relevant areas of staining.

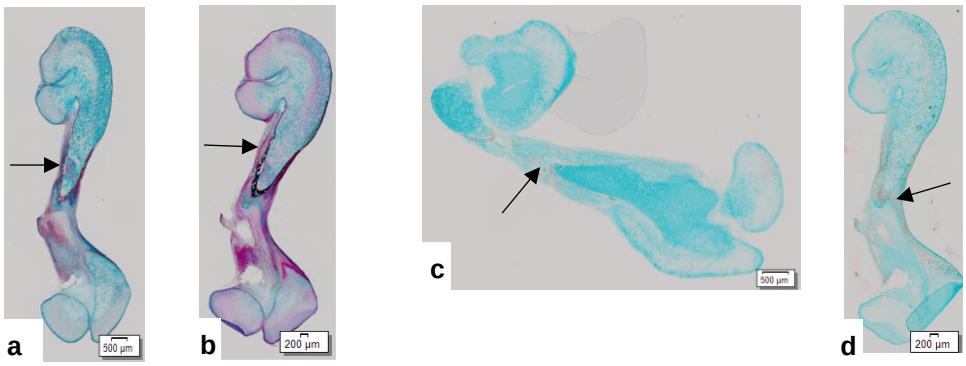


Figure 6.7 Chick femurs co-cultured with M69 OA synovial supernatant
a- A/S staining with typical purplish-pink staining for proteoglycan; b - von Kossa staining black for mineralisation around the periosteal collar; c - collagen 1 immunostaining – very faint red/brown staining in the region of the periosteal collar indicative of early osteogenic differentiation; d - Stro-1 immunostaining showing brownish staining consistent with low levels of expression. Images all taken at 20x magnification, scale bars indicated (200-500µm). Black arrows indicate the relevant areas of staining.

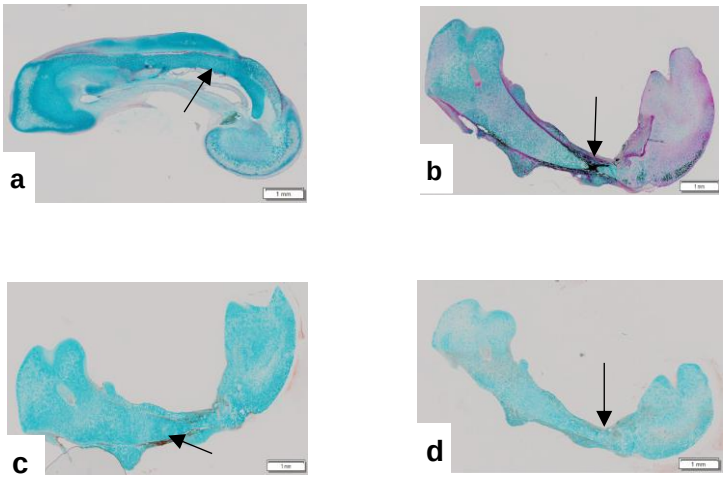


Figure 6.8 Chick femurs co-cultured with M68 OA synovial supernatant
a- A/S staining with typical purplish-pink staining for proteoglycan; b - von Kossa staining – black linear staining (positive) around periosteal collar; c - collagen 1 immunostaining – faint red/brown staining in the region of the periosteal collar indicative of very early osteogenic differentiation; d- Stro-1 immunostaining showing brownish staining consistent with low levels of expression. Images all taken at 20x magnification, scale bars indicated (1mm). Black arrows indicate the relevant areas of staining.

6.4.3 Discussion

Macroscopically, all femurs, whether exposed to basal or osteogenic conditions, increased in size over the 10 day culture period. This was in keeping with findings in the original baseline experiments in the chick femur organotypic model carried out by our group. Thus, significant increases in femur length were seen under all three organotypic culture conditions (basal, osteogenic, chondrogenic) over the 10 day culture period, compared with non-cultured controls (Kanczler, Smith et al. 2012). Femurs cultured in osteogenic conditions showed increased mineralisation (positive von Kossa staining - Figure 6.4a-c) compared to those cultured in basal conditions, as well as increased immunostaining for collagen 1 (Figure 6.4d). This was consistent with experiments in basal, osteogenic and chondrogenic culture conditions previously published by our group (Kanczler, Smith et al. 2012). In terms of immunostaining for the SSC enrichment marker Stro-1, this was increased in osteogenic compared to basal cultured femurs, in keeping once again with published studies (Kanczler, Smith et al. 2012).

The co-culture experiments with pro-inflammatory cytokines IL-1β, IL-6 and TNFα demonstrated that these cytokines had little effect on HBMSC growth and differentiation

under basal conditions. Proteoglycan content, indicated by A/S staining, was reduced under basal conditions (Figure 6.3) and even IL-1β produced only minimal mineralisation, shown on von Kossa staining (Figure 6.3d). However, under osteogenic culture conditions, the addition of IL-1β produced increased mineralisation, with markedly positive von Kossa staining (Figure 6.4b), supported by increased expression of collagen 1 on immunostaining (Figure 6.4d). IL-6 had negligible effect (data not shown) and TNFα produced a modest increase in mineralisation alone (Figure 6.4c). The effects of IL-1β in stimulating osteogenic differentiation are in keeping with the effects of this cytokine on HBMSC growth and differentiation demonstrated in our cytokine experiments discussed in Chapter 3, suggesting that stimulation of osteogenic differentiation is maintained in this *ex vivo* culture system, as well as in monolayer cultures. A modest stimulatory effect of TNFα was also been seen in our HBMSC and cytokine experiments, in terms of increased ALP specific activity (Section 3.2.2.1), although this did not attain statistical significance and with larger n numbers and in Stro-1+ve cultures, TNFα exerted an inhibitory effect (Section 3.2.2.2).

The co-cultures with OA synovial supernatants produced a modest increase in proteoglycan on A/S staining, predominantly around the periosteal collar (Figures 6.5- 6.8 a). There was increased mineralisation evident on von Kossa staining (Figures 6.5- 6.8 b). This was most marked in the femurs cultured with M70 (Figure 6.7b) and M68 OA synovial supernatants (Figure 6.8b). Similarly, immunostaining for collagen was most marked in the femurs from co-cultures with M69 and M68 OA synovial supernatants (Figures 6.5-6.8c). Expression of Stro-1 in the co-cultures with OA synovial supernatants showed considerable variation between supernatants, with higher Stro-1 expression correlating with greater mineralisation on von Kossa staining, suggesting a link with the degree of development of the periosteal cuff (Figures 6.6-6.8d).

On the basis of the cytokine profiling of synovial supernatants carried out previously and described in Chapter 4, the M69 OA synovial supernatant contained intermediate concentrations of IL-1β and IL-6 and the lowest concentration of TNFα out of the four supernatants used. The M68 OA synovial supernatant had the highest concentration of IL-1β, coupled with an intermediate concentration of TNFα. Higher concentrations of IL-1β and lower concentrations of TNFα in the synovial supernatant therefore appeared to correlate with greater mineralisation. This supports the findings from our cytokine and HBMSC cultures described in Section 3.2.2, as if IL-1β has a stimulatory effect on osteogenic differentiation and TNFα an inhibitory one, then synovial supernatants with high IL-1β and low TNFα should indeed exert the most favourable effect on osteogenic differentiation. This

was in keeping with the observation that the F61 synovial supernatant had only a slightly lower IL-1β concentration than the M68 one but a much higher TNFα concentration and mineralisation and collagen 1 expression appeared considerably reduced in co-cultures with the M68 OA synovial supernatant (Figure 6.5b,c). It is, however, at odds with how the OA synovial supernatants behaved in co-cultures with HBMSCs, where the majority of synovial supernatants (including the 4 used here) had a comparable or lesser effect on osteogenic differentiation to that seen in the osteogenic control cultures (Sections 4.6.3 and 4.6.4). This suggests that additional factors present within the synovial supernatants, such as other cytokines or growth factors, have different effects in organotypic versus monolayer cultures and act to modulate these cytokine effects. Given the current dearth of evidence in the published literature for the effects of OA synovial supernatants on osteogenic differentiation and conflicting evidence for the effects of IL-1β, TNFα or OA synovial supernatants on chondrogenic differentiation (Heldens, Blaney Davidson et al. 2012, Zayed, Schumacher et al. 2016, Barrachina, Remacha et al. 2017, Shioda, Muneta et al. 2017), further research in this area is still needed. Novel means of analysing synovial fluid, including using nuclear magnetic resonance (NMR) to construct metabolomic profiles of RA and OA from very small volumes of synovial fluid, have already proved useful in preliminary studies for distinguishing between OA and RA and thus improving diagnosis but may also yield future insights into some of the mechanisms involved in the pathogenesis of these conditions (Anderson et al. BSR Conference abstract, 2018).

In summary, the *ex vivo* organotypic chick femur model presents a reliable, rapid and cost-effective model for studying the effects of applied external factors, including the proinflammatory cytokines as described here. The inflammatory cytokines IL-1β, IL-6 and TNFα, when applied individually, exerted the same effects as seen in HBMSC and cytokine cultures (Section 3.2.2). However, co-cultures with OA synovial supernatants produced effects directly related to the concentrations of IL-1β and TNFα*,* rather than being largely comparable to osteogenic controls, as observed for the HBMSC and OA synovial supernatant co-cultures (Section 4.6.3 and 4.6.4). As this *ex vivo* model is more in keeping with the 3D influences governing normal skeletal development, this raises the question as to whether these results are closer to the true *in vivo* effects of the proinflammatory cytokines on osteogenic differentiation of HBMSC or not. This *ex vivo* organotypic chick femur model still holds inherent limitations separating it from the more costly, lengthy and invasive *in vivo* models of skeletal development. These limitations are based around the observation the model does not account for either the vascularisation crucial to skeletal development or the influences of

mechanical forces on bone development (Kanczler, Smith et al. 2012). Modifications of the organotypic model including the chick chorioallantoic membrane (CAM) model (Moreno-Jimenez, Hulsart-Billstrom et al. 2016, Moreno-Jimenez, Kanczler et al. 2017) or a rotating bioreactor have been proposed to address these additional factors (Kanczler, Smith et al. 2012). Additional studies in these extended models, or indeed in the critical femoral defect version of the organotypic culture system, would provide valuable further insights into how proinflammatory cytokines and OA synovial supernatants modulate skeletal development and repair. This will then allow optimisation of current tissue engineering strategies for the inflammatory environment present within OA joints.

Summary

- The first use of the chick femur organotypic culture model for evaluation of the effects of proinflammatory cytokines on skeletal development was described.
- Increased mineralisation and collagen 1 expression were seen in chick femurs cultured in osteogenic compared to basal conditions, together with increased Stro-1 expression.
- IL-1β produced a stimulatory effect on osteogenic differentiation, demonstrated by increased mineralisation and collagen 1 immunostaining.
- TNFα exerted a mild stimulatory effect on osteogenic differentiation.
- OA synovial supernatants produced increased osteogenic differentiation in terms of both mineralisation and collagen 1 expression, that was both IL-1β and TNFα concentration dependent.
- The importance of further research in extended *ex vivo* and *in vivo* models was discussed, in order to inform future tissue engineering strategies for the treatment of OA.

6.5 *In vivo* model of bone formation

6.5.1 Experimental set up

The setup of this model was summarised in Section 2.1.7 and is presented diagrammatically in Figure 6.9. Further details of the protocol are discussed below.

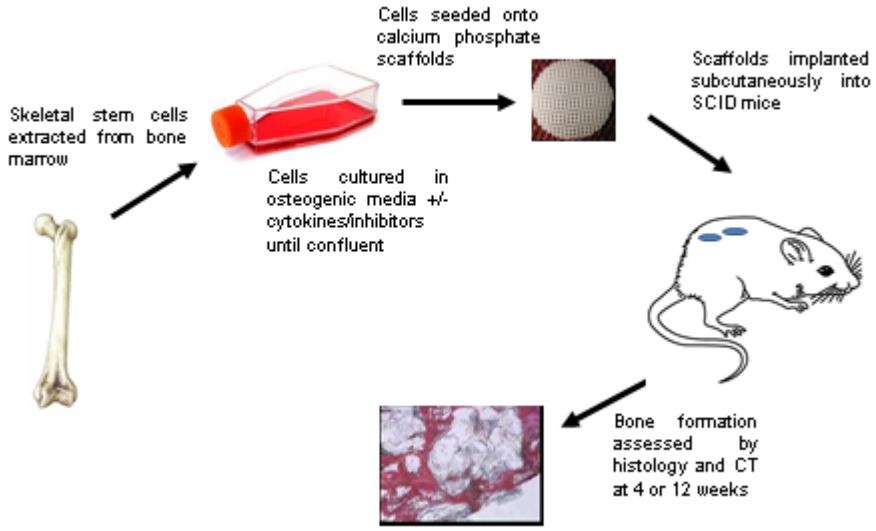


Figure 6.9 Schematic diagram showing the processes involved in the subcutaneous mouse model of bone formation from cell extraction through to histological analysis of the excised scaffold

HBMSC were obtained from a bone marrow sample and expanded in culture as previously described in Section 2.1.1. At confluency, cells were treated with trypsin to release them from the tissue culture plastic and centrifuged at 1100rpm for 4 minutes (see section 2.1.4 for detailed protocol). The cell pellet was resuspended in basal media. Aliquots of the cell suspension were placed in individual tubes, at a concentration of 0.5 x10^6cells per tube and pelleted by centrifugation at 1100rpm for 4 minutes. 30 cell pellets were generated in total and media added carefully to the tubes, without disrupting the pellet, for a total of 10 different conditions: Basal; Basal/IL-1β; Basal/IL-6; Basal/TNFα; Basal/TNF inhibitor; Osteo; Osteo/IL-1β; Osteo/IL-6; Osteo/TNFα; Osteo/TNF inhibitor. The concentrations of cytokines/inhibitor were identical to those validated in the HBMSC and cytokine experiments described in Chapter 3, IL-1β 10ng/mL, IL-6 100ng/mL, TNFα10ng/mL and TNF inhibitor 10ng/mL. The number of pellets generated allowed for each condition to be carried out in triplicate. The cell

pellets were incubated in these different media/cytokine combinations for 48 hours before adding them to the diffusion chambers ready for implantation.

Meanwhile, the sterile diffusion chambers were created using plastic surrounds with outer diameter 14 mm, inner diameter of 10 × 2 mm (Millipore), onto which discs of mixed cellulose ester membranes, 13 mm diameter with 0.45 µm pore size (Millipore) were attached with cement fixative as shown in Figure 6.10. The base disc was attached to each surround and left in a sterile hood under UV light overnight to sterilise the components. The pre-prepared calcium phosphate was added to the scaffold and the top disc secured in place with cement fixative (Millipore) before once again being placed in a sterile hood under UV light overnight to sterilise it.

a **b** **c**

Figure 6.10 Assembly of diffusion chambers
a - surround and base membrane layer ready for sterilisation; b – surround with both top and base membrane layers with calcium phosphate scaffold inside ready for sterilisation; c – diffusion chambers complete with scaffold, cell suspension and cytokine/inhibitor ready for implantation

On the day of the *in vivo* experiment set up, each cell pellet was carefully resuspended in a 150µl volume and pipetted via a small port on the side of the diffusion chamber onto the calcium phosphate scaffold inside. A small sterile plastic plug was inserted into the port on the side of the diffusion chamber to prevent the cell suspension leaking out. The individual diffusion chambers were implanted sub-cutaneously by Dr Kanczler into severely compromised immunodeficient (SCID) mice, 4 into each mouse, for 4 weeks (Project licence to Prof Oreffo). Male MF-1 *nu/nu* immunodeficient mice (Harlan, Loughborough, UK) were acclimatised for at least a week prior to the start of the experiment. All animals had *ad libitum* access to standard mouse chow and water at all times. All procedures carried out were done with prior ethical approval and in accordance with the regulations laid down in the Animals (Scientific Procedures) Act 1986, UK. Mice were anaesthetised via the intraperitoneal route using fentanyl-fluanisone (Hypnorm) (Jansson-Cilag Ltd) and

midazolam (Hypnovel) (Roche) in sterile water in a ratio of 1:1 and a dose of 10ml/kg. The mice were placed on a warming pad, the back of each mouse swabbed with an alcohol wipe and a longitudinal 1–2 cm incision on one side of the vertebral column made using a N°10 scalpel blade. A subcutaneous pouch large enough to contain the diffusion chambers was created lateral to the vertebral column using blunt dissection. Perforated capsules containing either scaffold alone (n=3), scaffold plus HBMSC (n=3) or scaffold + HBMSC + chosen cytokine (IL-1β, IL-6 or TNFα) or cytokine inhibitor (n=3 for each condition) were inserted into the subcutaneous pocket using sterile forceps. A total of 4 diffusion chambers were implanted into each mouse. The incision was closed using Michel staple clips. The mice were transferred back to the recovery incubator until they recovered from the anaesthetic and then placed back in appropriate cages. At the end of the 4 week period, the mice were euthanased and the subcutaneous implants removed. Thereafter, skeletal tissue formation was assessed using a combination of histological staining (A/S, von Kossa), immunofluorescence for type 1 and type 2 collagen and microCT, as previously published (Bolland, Kanczler et al. 2008).

6.5.2 Statistics

MicroCT data was analysed using GraphPad Prism software version 7.06. Data were expressed as mean +/- standard deviation, followed by the number of experiments performed, usually n=3 unless specified otherwise. Comparisons were carried out using one-way analysis of variance (ANOVA). If the ANOVA was significant, the Tukey-Kramer multiple comparison test was used as a post hoc test. For all analyses, a *p* value less than 0.05 was considered significant.

6.5.3 Results

6.5.3.1 MicroCT

Micro CT analysis of the individual diffusion chambers generated data on the total volume of tissue within each diffusion chamber (TV), bone volume (BV), tissue surface area (TS), bone surface area (BS), tissue surface to volume ratio (TS/TV), bone surface area to volume ratio (BS/BV) and trabecular pattern factor (TBPF). IL-1β produced an additive osteogenic effect in terms of increased bone volume, when combined with osteogenic culture conditions (Figure 6.11). However, this was a trend rather than a statistically significant increase in bone volume.

TNFα exerted an inhibitory effect on bone volume, only under osteogenic culture conditions, which again was a trend rather than a statistically significant decrease (Figure 6.11). IL-6 also had an inhibitory effect on bone volume. The addition of a TNFα inhibitor attenuated the reduction in bone volume seen with TNFα in basal culture conditions, though this effect was much less marked in osteogenic culture conditions (Figure 6.11). The same pattern was seen when comparing bone surface area to tissue volume ratio across conditions, which adjusted for the total tissue volume within a given diffusion chamber. (Figure 6.12). Further studies with higher n numbers are required to determine whether these trends would attain statistical significance.

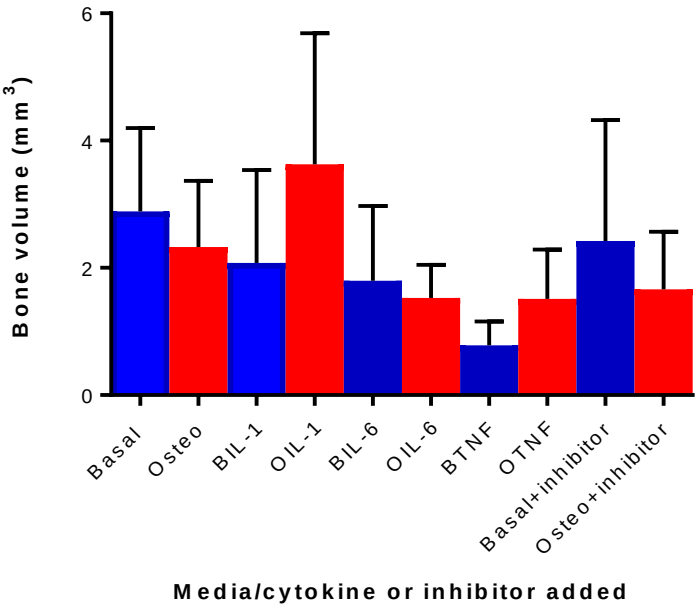


Figure 6.11 Bone volume (mm3) formed within the diffusion chambers, measured by µCT.
Values expressed as mean bone volume +/- SD, n=3. BIL-1=Basal media+IL-1; OIL-1=Osteogenic media+IL-1; BIL-6=Basal media+IL-6; OIL-6=Osteogenic media+IL-6; BTNF=Basal media+TNF; OTNF=Osteogenic media+TNF; Basal + inhibitor=Basal media+anti-TNF; Osteo + inhibitor=Osteogenic media+anti-TNF. Differences between the groups did not reach statistical significance.

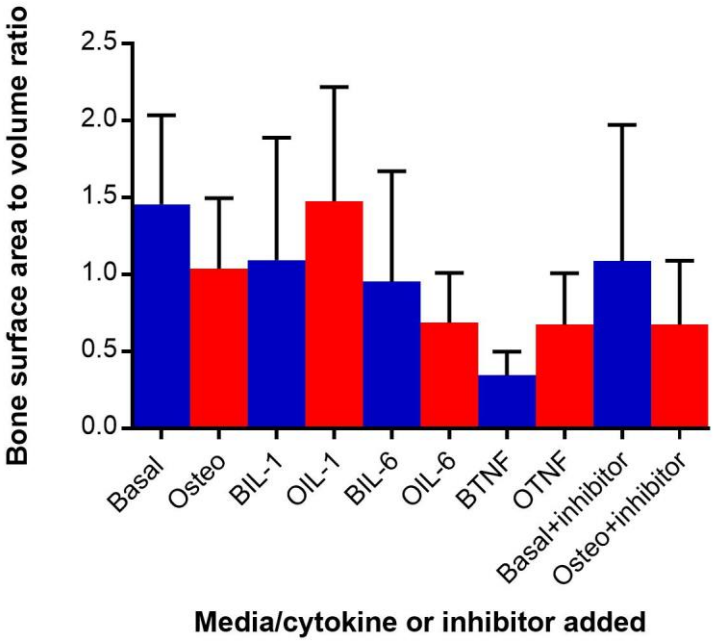


Figure 6.12 Bone surface area to volume ratio within diffusion chambers, measured by µCT. Values expressed as mean bone surface area to volume ratio +/- SD, n=3. BIL-1=Basal media+IL-1; OIL-1=Osteogenic media+IL-1; BIL-6=Basal media+IL-6; OIL-6=Osteogenic media+IL-6; BTNF=Basal media+TNF; OTNF=Osteogenic media+TNF; Basal + inhibitor=Basal media+anti-TNF; Osteo+ inhibitor=Osteogenic media+anti-TNF. Differences between the groups did not reach statistical significance.

Unfortunately, there was insufficient material retrieved from within the individual diffusion chambers to allow the planned histological analysis to be completed to determine the above trends in bone volume further.

6.5.4 Discussion

The addition of IL-1β to osteogenic cultures produced an increase in bone volume within the diffusion chambers on µCT, indicative of a stimulatory effect on the osteogenic differentiation of HBMSCs on calcium phosphate scaffolds *in vivo* (Figure 6.11). In contrast, the presence of TNFα in osteogenic cultures resulted in reduced bone volume within the diffusion chambers, in keeping with an inhibitory effect on osteogenic differentiation of HBMSCs (Figure 6.11). This inhibitory effect of TNFα was attenuated by the inclusion of a TNFα inhibitor in the co-cultures, more markedly under basal than osteogenic culture conditions.

The effects of inflammatory cytokines on *in vivo* models of bone formation have not been previously studied in the literature. However, both the subcutaneous implant model of bone

formation and the diffusion model variant of it described here, have been previously validated for the study of different exogenous factors on bone formation (Gundle, Joyner et al. 1995, Oreffo and Triffitt 1999, Howard, Partridge et al. 2002, Partridge, Yang et al. 2002, Yang, Green et al. 2003, Tare, Mitchell et al. 2012). The data presented here are in keeping with findings from the *in vitro* cytokine and HBMSC experiments described in Section 3.2.2. In these studies, IL-1β produced an additive osteogenic effect on the differentiation of unselected HBMSCs. In some unselected HBMSC cultures, TNFα had a mildly stimulatory effect on osteogenic differentiation but with larger n numbers or in Stro-1+ve SSC cultures it exerted an inhibitory effect. As discussed in Section 3.2.3, data from the literature also supports the role of IL-1β as the most potent cytokine for enhancing osteogenic differentiation in cells exposed to osteogenic media, mediated via the mWnt5a-Ror2 pathway (Sonomoto, Yamaoka et al. 2012). Similar pathways therefore appear to be stimulated by IL-1β and TNFα in both *in vitro* and *in vivo* models.

The addition of IL-6 to osteogenic cultures resulted in reduced bone volume on μCT compared to osteogenic control cultures (Figure 6.11). This was in contrast to our earlier *in vitro* studies (Section 3.2.2) in which IL-6 had a negligible effect on osteogenic differentiation in unselected cultures, although in Stro-1 +ve SSC and fetal femur cultures IL-6 had an additive osteogenic effect. This suggests that some additional factors are present in the *in vivo* culture system that modulate the effects of IL-6 on osteogenic differentiation.

Further work with larger n numbers, both with the individual proinflammatory cytokines and exploring the inhibition of IL-1β and TNFα in greater detail are required to confirm the significance of the above findings. These results can then be used to optimise tissue engineering strategies for the treatment of OA.

Summary

- The first use of the subcutaneous implant model for evaluation of the effects of proinflammatory cytokines on skeletal development was described.
- The addition of IL-1β to osteogenic conditions within the diffusion chambers produced an increase in bone volume measured by microCT, consistent with a stimulatory effect on osteogenic differentiation.
- In contrast, inclusion of TNFα within the diffusion chambers led to a decrease in bone volume due to its inhibitory effects on osteogenic differentiation, which was attenuated by the presence of a TNFα inhibitor.
- A decrease in bone volume was seen with the addition of IL-6 to the diffusion chambers, suggestive of an inhibitory effect on osteogenic differentiation that had not been reported in previous *in vitro* studies.
- The importance of further research, using cytokine inhibitors in co-cultures to validate the above findings, are required and will inform future tissue engineering strategies for the treatment of OA.

Chapter 7 Discussion, conclusions and future research

7.1 Introduction

The experiments described in this thesis were designed to investigate the effects of inflammatory cytokines on bioengineering strategies to repair arthritic joints, beginning with *in vitro* studies and progressing to *in vivo* work. The aim of this chapter is to consolidate the data generated from this body of experimental work and to discuss how far the data generated meet our overall aim of evaluating whether the inflammatory cytokines present in OA produce a hostile environment that limits the use of bioengineering strategies to repair damaged joints. The implications of these findings for tissue engineering strategies for OA are discussed, together with suggestions for future research.

7.2 The effects of inflammatory cytokines on bioengineering strategies to repair arthritic joints

Whilst OA has less of an inflammatory component than RA and other inflammatory arthropathies, both animal and human studies have reinforced the importance of inflammation in its pathogenesis (Pelletier, Martel-Pelletier et al. 2001, Goldring and Otero 2011). Women with early OA of the knees have been shown to have elevated levels of C-reactive protein (CRP) and higher levels increase the likelihood of disease progression (Goldring , Goldring 2000). The same inflammatory cytokines known to drive RA, namely interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα), are now known to be involved in this inflammatory component of OA (Pelletier, Roughley et al. 1991, Goldring 1999, Martel-Pelletier, Alaaeddine et al. 1999, Fernandes, Martel-Pelletier et al. 2002). Further recent studies have demonstrated the importance of cytokines in the pathophysiology of OA (reviewed by (Malemud 2010, Kapoor, Martel-Pelletier et al. 2011)). Evidence for IL-1β and TNFα occupying a pivotal role in the development of OA continues to accrue, with elevated levels of these cytokines or their gene expression reported in synovial tissues, synovial fluid and/or plasma in various animal models (Huebner, Seifer et al. 2007, Ley, Ekman et al. 2007, Maccoux, Salway et al. 2007). In baseline cytokine experiments, IL-1β produced an additive osteogenic effect on HBMSCs in culture. IL-6 had a negligible effect, whilst TNFα exerted an inhibitory effect on HBMSCs in culture. Strontium itself produced a mild stimulatory effect on osteogenic

differentiation of HBMSC. IL-1β, both in the current work and in the literature, is the key cytokine implicated in enhancing osteogenic differentiation in OA. The effects of IL-1β on osteogenic differentiation are thought to be mediated through multiple mechanisms including: BMP-2/Smad signalling; stimulation of BMP-7; RAS/MAPK signalling and the Wnt/β catenin pathway. IL-1β and strontium have a role as potential bioactive factors that could be harnessed to improve the success of future targeted tissue engineering strategies for the treatment of OA. Thus, our original aim of evaluating whether the inflammatory cytokines present in OA produce a hostile environment that might limit the use of bioengineering strategies to repair damaged joints was achieved.

7.3 Implications for clinical practice

OA is the most common form of arthritis worldwide and is associated with substantial morbidity, disability and high economic costs. Currently, treatments for OA centre around physiotherapy, analgesia, minimally invasive surgical approaches and, once mechanical joint failure occurs, joint replacement surgery. Tissue engineering approaches have proved successful in repairing bone and cartilage defects both in animal models and, increasingly, in a clinical setting. As techniques and biomaterials develop, the goal of a tissue engineering construct, complete with SSCs and bioactive factors, that can be administered via either minimally invasive surgery or an intra-articular injection, is not only attractive but becomes ever more attainable. The experiments summarised here provide vital information on how such strategies will need to be modified to account for the inflammatory environment present within osteoarthritic joints. The data generated indicate that, in the majority of OA patients, the synovial fluid inhibits osteogenic differentiation, mainly through the effects of IL-1β and, to a lesser extent TNFα. *In vitro* and *in vivo* studies described in this thesis (Sections 3.2, 3.3, 6.4 and 6.5) confirm that IL-1β and strontium both have a stimulatory effect on osteogenic differentiation, whereas TNFα has an inhibitory effect. Using this information, adding IL-1β and strontium to tissue engineering constructs, with or without the addition of a TNF inhibitor, offers the potential to improve the success of tissue engineering strategies in treating the osteochondral defects present in mild to moderate OA. Ultimately these strategies offer the prospect to prevent progression to severe OA and obviate the need for joint replacement surgery.

The work presented with OA synovial supernatants suggests that synovial fluid analysis could be used as a means of stratifying patients into ‘pure OA’ and ‘inflammatory OA’ subtypes, in order to determine the optimal balance of cytokines/inhibitors/other growth factors that offers an individual patient the greatest chance of repairing their osteoarthritic joint. In the sample of patients studied, the synovial supernatants from the

majority of patients (80%) exerted an inhibitory effect on osteogenic differentiation of HBMSCs, which was demonstrated by increased ALP specific activity (Section 4.6). This group of patients were viewed as having an 'inflammatory OA' subtype, likely to benefit from either systemic DMOAD treatment or incorporation of IL-1β or TNFα inhibitors into tissue engineering strategies. In contrast, those patients whose synovial supernatants had a stimulatory effect on osteogenic differentiation of HBMSCs were likely to benefit from the addition of low dose IL-1β or TNFα into tissue engineering constructs and were designated as the 'pure OA' subtype (20%). Further cytokine profiling of larger numbers of OA synovial supernatants is required to evaluate the utility of this stratification process more fully. Combining this cytokine profiling with miRNA analysis or metabolomics (Anderson et al. 2018) on synovial fluid samples could facilitate even more accurate phenotyping of patients and better tailoring of OA treatments for a given individual in the future.

7.4 Limitations

Specific limitations within individual experiments have been highlighted in the relevant experimental chapters. There are, however, some limitations that are applicable to several sets of experiments. These centre mainly around small n numbers, due to the constraints in the availability of human bone marrow samples for generation of HBMSC cultures, together with the time required to generate sufficient cells to set up individual experiments. These low sample sizes limited statistical analyses and data interpretation. The other factor that was problematic was the development of unanticipated problems with the culture plates (Matrigel) for the human embryonic stem cell cultures (hESCs) and the stability of induced pluripotent stem cell (iPSC) colonies, which limited the number of experiments that could be undertaken in the time available.

With the synovial supernatant experiments, there was a large pool of elective Orthopaedic patients undergoing hip or knee replacement surgery for recruitment. However, changes in admissions procedures for elective surgery, due to pressures on bed occupancy, resulted in increasing numbers of patients being admitted on the day of surgery instead of the day before. The Ethics Committee requirement for patients to be given a minimum of 24 hours to consider whether they wished to participate in the study, therefore meant the patients could not be consented on the ward on the same day they were due for surgery. Instead, patients were recruited from consecutive preadmission clinics and asked to confirm with the research team once they had a date for surgery. This ensured that the surgical team could be reminded to collect the synovium in a universal, which was collected by a member of the research team to allow the synovium to be

processed the same day. However, increasing use of waiting list initiatives to meet targets for elective surgery meant that patients' dates for surgery were frequently changed and samples often missed. Alternatively, patients were operated on at one of the local private hospitals as part of the waiting list initiative, which precluded samples being collected without a member of the research team having an honorary contract there, as well requiring an amendment to the ethics approval to add an additional site. As these changes in terms of waiting list initiatives occurred part way through the study, leaving insufficient time for reapplications to the Ethics Committee, this had a significant impact in terms of reducing the planned number of synovial samples collected for analysis. As a result, detailed multivariate analysis of patient factors affecting the cytokine profile of synovial supernatants was not possible, nevertheless, trends were identified from the data generated.

7.5 Implications for future research

The work described within this thesis has identified a number of potential areas for future research:

HBMSC cultures

- Further studies of the effects of inhibiting individual inflammatory cytokines on both unselected HBMSC and Stro-1+ve cell differentiation, to confirm the stimulatory effect of IL-1β, and the inhibitory effect of TNFα in HBMSC cultures.
- Establishment of co-cultures with synovial supernatants to determine the effects of inhibiting the individual inflammatory cytokines on HBMSC and Stro-1+ve cell differentiation.
- Investigation of the effects of strontium on HBMSCs and Stro-1 +ve cells in co-cultures with synovial supernatants.

Co-culture experiments

- Co-cultures of human OA synoviocytes and MSCs, facilitating analysis of the effects of the inflammatory milieu present within the synovial supernatant on the synoviocytes themselves as well as on the MSCs.

Further insights into mechanisms

- Co-cultures of embryonic stem cells (ES) and OA synoviocytes, initially alone, then with the individual inflammatory cytokines (IL-1β, IL-6 and TNFα) and finally with synovial supernatants. This will allow assessment of both individual cytokines and the synovial supernatants on both cell types and comparison with the effect on HBMSCs and SSCs alone.

- Co-cultures of induced pluripotent stem cells (iPSCs) and synoviocytes, initially alone, then with the individual inflammatory cytokines (IL-1β, IL-6 and TNFα) and finally with synovial supernatants. This will allow assessment of both individual cytokines and the synovial supernatants on both cell types and comparison with the effect on HBMSCs and SSCs alone.
- Expanded analysis of microRNA (miRNA) expression initially in HBMSC+ cytokine and HBMSC/supernatant cultures and subsequently within the HBMSC/synoviocyte co-cultures described above. Findings from these experiments will help to resolve the issue of whether over expression or silencing of single or multiple miRNAs is the most effective adjunct to a cell-based tissue engineering strategy for prevention of OA.
- More detailed profiling of synovial supernatants, including a wider panel of cytokines, as well as growth factors will provide enhanced characterisation of the inflammatory environment within an OA joint. This will help to determine the optimum combination of bioactive factors for use in tissue engineering strategies for OA.
- To use the *ex vivo* and *in vivo* models described here to assess the effects of individual synovial supernatants, alone or with cytokine inhibitors, on the differentiation of SSCs.

7.6 Conclusions and summary

The body of research presented in this thesis, provides novel insights into the mechanisms behind the effects of the inflammatory cytokines IL-1β, IL-6 and TNFα on the osteogenic differentiation of HBMSCs and Stro-1 +ve SSCs. These experiments demonstrate that IL-1β, at the low concentrations used here, is a key cytokine for promoting osteogenic differentiation of HBMSCs and Stro-1 +ve SSCs. In contrast, IL-6 had a minimal effect and TNFα in these studies was predominantly inhibitory. This suggests that the inflammatory cytokines present in OA do not all produce a hostile environment that limits the use of bioengineering strategies to repair damaged joints, given that at least IL-1β has beneficial effects on osteogenic differentiation.

Expanding this work into two pluripotent cell types, hESCs and iPSCs, provided novel data on how the same three inflammatory cytokines, IL-1β, IL-6 and TNFα affected osteogenic differentiation of these cell types and hence may influence future tissue engineering strategies for generation or repair of bone and cartilage in OA. The effects of IL-1β and TNFα on osteogenic differentiation were similar in iPSCs, being stimulatory and inhibitory respectively. IL-6 displayed a much more marked stimulatory effect on

osteogenic differentiation of iPSCs compared to the limited impact on osteogenic differentiation in HBMSCs or Stro-1 +ve SSCs cultures. The current challenges of using hESCs and iPSCs were discussed. With hESCs, these centre around the ethical issues of using cells derived from embryonic tissue, as well as the potential immunogenicity of this form of stem cell-based treatment. iPSCs in contrast, overcome some of these ethical issues by being autologous. However, as several of the factors used to reprogramme somatic cells to iPSCs are oncogenes, concerns remain about the potential for immunogenicity or teratoma formation, which at present still represent a significant barrier to clinical translation of strategies using iPSCs. Novel uses of iPSCs to devise platforms for testing future drug treatments for OA and RA were also explored as a possible extension of these strategies (Lee et al. 2014; Willard et al. 2014; Liu et al. 2017).

The discovery of miRNAs has provided a further exciting avenue for exploring potential pathways and their complex interactions in OA (Xu et al. 2016; Portal-Nunez et al. 2016; Nugent. 2016; Li et al. 2015, Wu et al, 2014; Tsezou et al. 2014). While miRNAs have been evaluated in synovial fluid from OA patients (Murata et al. 2010) and in the context of investigating cartilage damage in OA previously (Okuhara et al. 2012), this was the first work to examine miRNA profiles in co-cultures of HBMSCs and OA synovial supernatants. Evaluating the expression of miR-138, miR-140 and miR-146a showed that all three miRNAs were downregulated in co-cultures of HBMSCs and OA synovial supernatants. The expression of miR-138 and miR-146a were upregulated by IL-1β, whereas miR-140 was downregulated. Using the existing literature on miRNAs, together with these findings, resulted in the proposal of several pathways through which IL-1β might control osteogenic differentiation, including BMP-2/Smad signalling; stimulation of BMP-7; RAS/MAPK signalling and the Wnt/β catenin pathway (Miyaki et al. 2009; Tardif et al. 2009; Miyaki et al. 2010; Nakamura et al. 2011; Liang et al. 2012; Huang et al. 2012; Wang et al. 2013; Cheung et al. 2014; Wu et al. 2014; Yuan et al. 2016; Zhong et al. 2017).

Finally, *ex vivo* and *in vivo* models of bone formation were used for the first time to determine whether the abovementioned effects of inflammatory cytokines on HBMSCs and other cell types *in vitro* would be replicated in these models. The studies presented herein showed that both IL-1β and TNFα exhibited similar effects on osteogenic differentiation in these models, confirming the utility of focusing on modifying these two cytokines in order to devise successful tissue engineering strategies for treating the osteochondral defects present in mild to moderate OA. This work also validated both the organotypic chick femur and subcutaneous implant models for the testing of other future bioactive factors or miRNAs being considered for incorporation into tissue engineering approaches for the management of OA.

Summary

- IL-1β, both in the current work and in the literature, is the key cytokine for enhancing osteogenic differentiation in OA.
- IL-6 had a negligible effect, whilst TNFα exerted an inhibitory effect on HBMSCs in culture.
- Strontium displayed a mild stimulatory effect on osteogenic differentiation of HBMSCs.
- Effects on osteogenic differentiation are likely to be mediated through multiple mechanisms including: BMP-2/Smad signalling; stimulation of BMP-7; RAS/MAPK signalling and the Wnt/β catenin pathway.
- IL-1β and strontium have a role as potential bioactive factors that could be harnessed to improve the success of future targeted tissue engineering strategies for the treatment of OA.
- There was heterogeneity in the concentrations of IL-1β and TNFα in synovial supernatants across the group of OA patients studied. IL-1β and TNFα concentrations tended to be higher in female patients, those undergoing TKRs and patients with moderately severe (KL grade 3) OA.
- IL-1β had a modest stimulatory effect on ALP expression by iPSCs in culture. IL-6 had an additive osteogenic effect, as seen previously for fetal femur cultures. TNFα exerted a modest inhibitory effect.
- Most OA synovial supernatants studied, produced an inhibitory effect on osteogenic differentiation of HBMSCs. A minority of patients' supernatants showed an additive osteogenic effect in co-cultures, suggesting a division into two distinct 'inflammatory' and 'pure OA' subtypes.
- In the chick femur organotypic culture model, IL-1β and TNFα had similar effects to in HBMSC cultures. OA synovial supernatants produced increased osteogenic differentiation in terms of both mineralisation and collagen 1. expression, that was both IL-1β and TNFα concentration dependent.
- The first use of the subcutaneous implant model for evaluation of the effects of proinflammatory cytokines on skeletal development was described. The addition of IL-1β to osteogenic conditions within the diffusion chambers produced an increase in bone volume as measured by microCT, consistent with a stimulatory effect on osteogenic differentiation. In contrast, TNFα exerted an inhibitory effect on osteogenic differentiation, which was attenuated by the presence of a TNFα inhibitor (adalimumab).

Appendix A Study approvals and documents

A1 Study approvals - Ethics

MFK

8 July 2008

Emma Williams

National Research Ethics Service

ISLE OF WIGHT, PORTSMOUTH & SOUTH EAST HAMPSHIRE RESEARCH ETHICS COMMITTEE

1ST Floor, Regents Park Surgery
Park Street, Shirley
Southampton
Hampshire
S016 4RJ

Tel: 023 8036 2863
Fax: 023 8036 4110

Email: scsha.SI=HREC@nhsnet

Academic Clinical Fellow in Rheumatology
Rheumatology Research Unit
Southampton General Hospital
Southampton
S016 6YD

Dear Dr Williams

Full title of study: **Healing in Osteoarthritis and Rheumatoid Arthritis; the Effects of Inflammatory Cytokines on bioengineering Strategies to Replace Arthritic Joints**

REC reference number: **08/H0501/58**

Thank you for your letter of 30 June 2008, responding to the Committee's request for further information on the above research and submitting revised documentation, subject to the conditions specified below.

The further information has been considered on behalf of the Committee by the Vice-Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Manaaement Permission or approval must be obtained from each host organisation prior to the start of the studv at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum:nhs.uk.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	5.6	20 May 2008
Investigator CV		01 April 2008
Protocol		01 April 2008
Covering Letter		22 May 2008
Summary/Synopsis	1	01 April 2008
Peer Review		14 May 2008
Interview Schedules/Topic Guides	1	01 April 2008
Participant Information Sheet	2	30 June 2008
Participant Information Sheet	1	01 April 2008
Participant Consent Form	1	01 April 2008
Response to Request for Further Information		30 June 2008
Letter from Funder - Southampton Rheumatology Trust		15 April 2008
Supervisor's CV		29 April 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review - guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referenceorouo@nres.npsa.nhs.uk.

[**08/H0501/58** . **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

on Dabbs
***Vice* Chair**

Email: scsha.SEHREC@nhs.net

Enclosures: "After ethical *review*- guidance for researchers" SL- AR2

Site approval form

Copy to: Mrs Angela Jackson, R&D Unit, Duthie Building, Ground Floor, SUHT.

A1 Study approvals - R and D

Southampton University Hospitals

Please reply to: Research and Development
Duthie Building (Trust) MP138
Southampton General Hospital Tremona
Road Southampton SO166YD

Telephone: 023 80794752
Fax: 023 80798678
E-mail: christine.mcgrath@suht swest.nhs.uk

Dr Emma Williams
Rheumatology Research Unit
Mailpoint 63,
G Level , West Wing
Southampton General Hospital
Tremona Road
S016Y 6YD

A d O 0

15 August 2008

Dear Dr Williams

ID: RHM MED0831 Healing in osteoarthritis and rheumatoid arthritis: the effects of inflammatory cytokines on bioengineering strategies to replace arthritic joints.

EudraCT: AJ/A

Thank you for submitting all the required documentation for Trust R&D approval. I write to inform you that your study has full SUHT R&D approval. Please find attached the Conditions of Trust R&D approval which you are obliged to adhere to.

You are required to keep copies of all your essential documents relating to this study. Please download a copy of the relevant Investigator Site File template from the R&D website: http://www.suht.nhs.uk/index.cfm?articleid=2548.

Your project is subject to R&D monitoring and you will be contacted by our office to arrange this.

Please note: A condition of approval is that any changes need to be timeously notified to the R&D office. This includes providing copies of:
. All NRES substantial amendments and favourable opinions;
. All Serious Adverse Events (SAEs);
. NRES Annual Progress Reports;
. Annual MHRA Safety Reports;
. NRES End of Study Declaration;
. Significant breach of GCP or protocol

Please quote the above RHM No. on any correspondence with our office.

Should you, or any of your team, require training in any of the policies and procedures required to ensure compliance with the conditions of approval, please refer to the ROSU (Research & Development Support Unit) website: www.rdsu.soton.ac.uk for an up-to-date calendar of training events.

Yours sincerely

Christine McGrath

Director of Research & Development

A2 Patient information leaflet

Southampton NHS
University Hospitals NHS Trust

Rheumatology Research Unit,
Mailpoint 63, G level, West Wing,
Southampton General Hospital,
Tremona Road,
Southampton, SO16 6YD

Tel: 02380 795279
Fax: 02380 796711

RESEARCH PARTICIPANT INFORMATION SHEET

Study Title

The Effects of Inflammation on Bone and Cartilage Healing in Patients With Arthritis

You are being invited to take part in a research study. Before you decide whether or not to participate, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like further information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

This is a study to look and see how inflammation in patients with rheumatoid arthritis (RA) and osteoarthritis (OA) affects the normal growth of cartilage and bone. In particular, we are interested to see if chemicals called cytokines that control inflammation may stop bone and cartilage healing.

Why have I been chosen?

You have been chosen because you have rheumatoid arthritis or osteoarthritis and are due to have a hip or knee replacement or synovectomy (removal of joint lining) in the near future. We need samples of bone and joint lining from patients having this type of surgery. These samples are always removed as part of these types of surgery but are called surplus (waste) tissue as they are usually thrown away after the operation. We would be asking to collect these samples instead to use in the study. No extra samples at all would be taken.

Do I have to take part?

It is up to you whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you do decide to take part, you are still free to withdraw from the study at any time and do not have to give a reason for doing so. If you decide not to take part, or withdraw at any time, this will not affect the standard of care you receive.

What will happen to me if I take part?

If you agree to take part in the study, there will be one extra appointment in addition to your usual routine appointments. We will pay travel expenses for attending this appointment. It will take about an hour. You will be seen in the Wellcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital by a rheumatologist who will explain about the study and answer your questions, and then if you are happy to take part in the study you will sign a consent form. We will then check your diagnosis and examine you to see how active your rheumatoid arthritis or osteoarthritis is. The samples that we need will be taken from your hip or knee at the time of your planned surgery.

What do I have to do?

Taking part in the study does not alter any of your normal care. The samples we take will be from tissue around your hip or knee that is removed as part of hip or knee replacement surgery and is usually discarded. You do not need to alter your diet or lifestyle in anyway.

What are the possible benefits of taking part?

It is unlikely that taking part in this study will directly benefit your medical treatment. However, the information from this study may help us to treat future patients with RA and OA more effectively.

What happens when the research study stops?

After the study you will continue with your normal treatment in the Rheumatology/Orthopaedic departments.

What if something goes wrong?

It is extremely unlikely that taking part in this research project will harm you. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way in which you have been approached or treated during the course of the study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital will have your name and address removed so that you cannot be recognised from it.

What happens to the results of the research study?

We hope that the results are useful and we intend to publish them in a rheumatology journal. You will not be identified in any report or publication. You will receive a copy of the study results if you wish to do so.

Who is organising and funding the research?

Rheumatologists at Southampton General Hospital and Southampton University are carrying out the study, together with their Orthopaedic colleagues at the same sites.

Who has reviewed the study?

The study has been peer reviewed by the Southampton University Hospitals NHS Trust Research and Development Department and Southampton University. The Isle of Wight, Portsmouth and South East Hampshire Local Research Ethics Committee have also reviewed this study.

If you would like to take part in the study or would like further information, please contact Dr E. Williams on ext 5279, or return the reply slip below.

The Effects of Inflammation on Bone and Cartilage Healing in Patients With Arthritis

Please return to:-

Dr E. Williams
Rheumatology Research Unit,
Mailpoint 63,
Level G, West Wing,
Southampton General Hospital.
SO16 6YD.

I would like to take part in this study and am happy to be contacted by a member of the study team to receive further information.

A3 Consent Form

Southampton NHS
University Hospitals NHS Trust

Rheumatology Research Unit
Mailpoint 63, Level G, West Wing
Southampton General Hospital
Southampton, SO16 6YD

Tel: 023 8079 5279
Fax: 023 8079 6711

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: **Healing in Osteoarthritis and Rheumatoid Arthritis; the Effect of Inflammatory Cytokines on Bioengineering Strategies to Replace Arthritic Joints**

Name of Researcher: **Dr Emma Williams**

Please initial box

1. I confirm that I have read and understand the information sheet dated June 2008 (version 2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Rheumatology research team, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to surplus tissue (synovial tissue) removed at the time of my joint replacement surgery being used for the purposes of this study. ☐
5. I understand that my additional consent will have to be sought before the synovial tissue samples could be used in any further studies. ☐
6. I agree to my GP being informed of my participation in the study ☐
7. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

A4 Study protocol

Healing in Osteoarthritis and Rheumatoid Arthritis; the Effect of Inflammatory Cytokines on Bioengineering Strategies to Replace Arthritic Joints

PROTOCOL

This study is aimed at determining the effects of inflammatory cytokines on bioengineering strategies currently under development for replacement of arthritic joints and potential ways of overcoming the perceived detrimental effects of these cytokines on the integrity of such cartilage and bone constructs.

We wish to look and see how the inflammatory cytokines present in patients with rheumatoid arthritis (RA) and osteoarthritis (OA) affect the normal growth of precursor cells into cartilage and bone and the effect on these processes when such cytokines are inhibited. Secondly, the effects of these inflammatory cytokines produced from samples of synovial tissue removed from patients with RA and OA on mesenchymal cell constructs (artificial cartilages) will also be assessed.

With the advent of mesenchymal cell constructs aimed at creating viable articular bone and cartilage in the future, as yet few studies have examined the effects of these cytokines from RA and OA synovial tissues on cell progress within these constructs. Equally, the effects of blocking individual cytokines in this situation have yet to be examined. Our hope is that this body of research will fill these gaps and provide us with a better understanding of the likely effects of the local cytokine/chemokine profile on in vivo articular cartilage constructs derived from mesenchymal stem cells of patients with OA, RA and traumatic injuries.

Design and Methodology

This is a pilot study to look and see how the inflammatory cytokines present in patients with RA and OA affect the normal growth of precursor cells into cartilage and bone and the effect on these processes when such cytokines are inhibited. Secondly, the effects of these inflammatory cytokines produced from samples of synovial tissue removed from patients with RA and OA on mesenchymal cell constructs (artificial cartilages and bone) will also be assessed. The study involves only small numbers of patients with RA and OA (total 50 patients). Individuals will be selected from Orthopaedic lists of patients due to undergo elective hip or knee replacement surgery or synovectomy at Southampton General Hospital. Their diagnosis of RA or OA will be established according to the American College of Rheumatology (ACR) criteria. Individuals will be approached by Dr Williams in the Orthopaedic or Rheumatology clinics at Southampton General Hospital.

The study will be conducted in the Wellcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital. The diagnosis of RA or OA will be determined by history and clinical examination to ensure patients meet the ACR criteria (Appendices 1 and 2) and assessment of disease activity using standard protocols (Disease Activity Score 28 joint count - DAS-28, Appendix 3). Demographic data and records of current drug therapies will also be recorded. The whole visit should take about an hour. The only samples patients will be agreeing to provide are surplus synovial tissue that would be removed routinely as part of hip or

knee replacement surgery and is usually just discarded. No change to their usual clinical care will occur.

Aim 1. Establishing cell cultures and usual patterns of differentiation

a) Establishing baseline state

Skeletal stem cell enriched populations, marrow stromal cells and progenitors respectively will be incubated in basal, osteogenic and chondrogenic culture media, together with bone morphogenetic proteins (BMPs) and their differentiation towards bone or cartilage will be assessed. The same process will also be carried out using mesenchymal stem cells on alginate templates developed in our local laboratory.

b) Assessing the effects of the addition of cytokines

Commercially available preparations of individual cytokines [Interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF alpha)] will then be added to each group of samples and their effects recorded. In each of these two phases, the main outcome measures recorded will be cell differentiation and therefore change in cell function, changes in cytokine expression, in messenger RNA (mRNA) production and finally cell death.

Aim 2. Producing synovial tissue cultures

a) Application of supernatants to all cell lines under assessment

Synovial tissue cultures will be produced from each of the OA and RA patient samples. To do this, whole synovial membranes obtained at the time of surgery will be placed immediately in RPMI 1640 Foetal Calf Serum (FCS). The tissue will then be dissected into small pieces with forceps and scissors in order to remove the synovial membrane from the underlying fibrous joint capsule. It will then be digested in a medium consisting of 0.15mg/ml DNase type I and 5mg/ml collagenase for 1-2 hours at 37°C. Cells will be washed and re-suspended in RPMI 1640 10% FCS to a density of $1x10^6$ cells/ml. The suspension will then be centrifuged and the supernatants from each explant culture retained. These supernatants will then be added to mesenchymal stem cells cultured in basal, osteogenic and chondrogenic media. The same outcome measures of cell death, changes in cellular function, cytokine expression and mRNA expression will be used.

b) Assessment of the effects of cytokine inhibition

Inhibitors of individual cytokines will then be added to these co-cultures and their effects on the aforementioned outcome measures noted. The effects of the biologic therapies for RA, including anti-TNF, anti-IL-1, anti-IL6 and anti-B cell therapies on these co-cultures in terms of these same four outcome measures will also be described.

Appendix 1

The 1987 revised American College of Rheumatology (ACR) criteria for the classification of rheumatoid arthritis

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (Bilateral involvement of PIPs, MCPs or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony rominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded. Designation as classic, definite, or probable rheumatoid arthritis is *not* to be made.

Appendix 2

ACR Classification Criteria for Osteoarthritis of the Hip

Traditional format

Hip pain plus at least two of the following:

- ESR of less than 20 mm per hour
- Femoral or acetabular osteophytes on radiographs
- Joint space narrowing on radiographs

Classification-tree format

Hip pain plus femoral or acetabular osteophytes on radiographs

or

Hip pain plus joint space narrowing on radiographs and an ESR of less than 20 mm per hour

ESR = erythrocyte sedimentation rate.

ACR Classification Criteria for Idiopathic Osteoarthritis of the Knee

Traditional format

Knee pain plus osteophytes on radiographs and at least one of the following:

- Patient age older than 50 years
- Morning stiffness lasting 30 minutes or less
- Crepitus on motion

Classification-tree format

Knee pain and osteophytes on radiographs

or

Knee pain plus patient age of 40 years or older, morning stiffness lasting 30 minutes or less and crepitus on motion

ACR Classification Criteria for Osteoarthritis of the Hand

Hand pain, aching or stiffness

plus

Hard tissue enlargement of two or more of 10 selected joints*

plus

Fewer than three swollen metacarpophalangeal joints

plus

Hard tissue enlargement of two or more distal interphalangeal joints

or

Deformity of two or more of 10 selected joints*

* - 10 selected joints are the second and third distal interphalangeal joints, the second and third proximal interphalangeal joints and the first carpometacarpal joints (of both hands).

Appendix 3

DAS 28 proforma

Patient name…… Date of Birth ……-……-…….

Observer name……..… Date ……-……-……

		Left		**Right**	
		Swollen	Tender	Swollen	Tender
Shoulder					
Elbow					
Wrist					
MCP	1				
	2				
	3				
	4				
	5				
PIP	1				
	2				
	3				
	4				
	5				
Knee					
Subtotal					
Total		Swollen		Tender	

No disease activity High disease activity

Swollen (0-28)	
Tender (0-28)	
ESR	
VAS observers disease activity (0-100mm)	

DAS28 = 0.56*√(t28) + 0.28*√(sw28) + 0.70*Ln(ESR) + 0.014*GH

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