Effects of inflammatory mediators on bone marrow mesenchymal stem cells

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Abstract

Despite osteoarthritis (OA) being the most prevalent form of joint disease, there is a striking unmet need for treatment strategies, with current treatments focused on the relief of pain symptoms. The inherent properties of mesenchymal stem cells (MSCs) make them an attractive candidate for a novel transplantation strategy, as they have both the potential to differentiate into chondrocytes for replacement of degraded cartilage, and the potential to exert immunosuppressive effects for modulation of the inflammatory environment. However, the inflammatory environment of the joint may affect the ability of these cells to functionally integrate into the host tissue and exert beneficial effects, as indicated by a lack of success seen in clinical trials. Identification of factors and cell signalling pathways that influence MSC function is therefore critical for ensuring their success in the clinic, and so this *in vitro* project aimed to identify the effects of inflammatory mediators on bone marrow MSCs (BMSCs). To investigate this, BMSCs were cultured in the presence of inflammatory mediators associated with OA pathology (IL-1β, IL-8, IL-10, MCP-1, NGF), either individually or in combination. Results showed that conditions containing IL-1β significantly affected the differentiation response of these cells under chondrogenic and osteogenic conditions. Gene expression analysis revealed changes in MAPK, Wnt and TLR signalling pathways, and highlighted the increased expression of pro-inflammatory cytokines and cartilage degrading enzymes. This work provides a basis for further investigation into factors present in the OA joint that may limit the therapeutic potential of MSCs, supporting further research into ways to circumvent this for novel OA treatments.

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Abbreviations

ALP	Alkaline phosphatase
AMSC	Adult muscle stem cell
ASC	Adult stem cell
BML	Bone marrow lesion
BMP	Bone morphogenetic protein
BMSC	Bone marrow derived mesenchymal stem cell
BSP	Bone sialoprotein
CM	Chondrogenic medium
COX	Cyclooxygenase
Ct	Threshold cycle
DAMP	Damage/danger-associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
DMMB	1,9-dimethylmethylene blue
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
(s)GAG	Sulphated glycosaminoglycan
GRADE	Grading of Recommendations, Assessment, Development and Evaluations
HA	Hyaluronic acid
HGF	Hepatocyte growth factor
HSC	Hematopoietic stem cell
IDO	Indoleamine 2,3-dioxygenase
IGF	Insulin growth factor
ihBMSC	Immortalised human bone marrow mesenchymal stem cell
IHH	Indian hedgehog
IL	Interleukin
ITS	Insulin-transferrin-sodium selenite
JNK	c-Jun N-terminal kinase
LRP	Low density lipoprotein receptor-related protein
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MIA	Monoiodoacetate
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
OA	Osteoarthritis
OM	Osteogenic medium
OPG	Osteoprotegerin
Osx	Osterix
PBS	Phosphate buffered saline
(RT-)PCR	(Reverse transcription-) polymerase chain reaction
PDGF	Platelet-derived growth factor receptor
PGE_2	Prostaglandin E2
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RUNX2	Runt related transcription factor 2
SDW	Sterile distilled water
SEM	Standard error of the mean
SF	Synovial fluid
SM	Standard medium
TAE	Tris-acetate-EDTA
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinases
TLDA	TaqMan Low Density Array
TLR	Toll like receptor
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor

Chapter 1 General Introduction

For review of the literature a short general introduction is provided here, followed by more targeted introductory sections for each results chapter.

1.1 Regenerative medicine

Regenerative medicine has gained significant attention, from both the scientific community and the public, since the identification of embryonic and adult stem cells. Although sporadically described in the mid 20th century, it was not until the latter half of the century that interest was gathered in the discovery and utilisation of these cells (Martin, 1981; Thomson *et al.*, 1998; Bajada *et al.*, 2008). Despite continuing efforts to expand knowledge, a persistent lack of insight into the basic biology and functioning of these cells is hindering their translation into the clinic, and as of yet their clinical applications have had limited impact (Forbes and Rosenthal, 2014). Nevertheless, their potential to target effects of localised disease, injury and aging on a systemic level is substantial, and provides hope for many alternative therapies in a host of scientific fields (Daley, 2012).

1.1.1 Stem cells: embryonic and adult

Laying the foundations for all tissues and organs, stem cells possess two defining characteristics – self-renewal and multipotency. The former means that these cells are able to divide and maintain their undifferentiated status, whilst the latter describes their capacity to differentiate into different cell lineages (Bajada *et al.*, 2008). There are two primary categories of stem cells: embryonic stem cells (ESCs), derived from the inner cell mass of blastocysts, and adult stem cells (ASCs), accessible from somatic tissue sources. ESCs are

described as pluripotent, with their ability to differentiate into any mature cell type. In contrast, ASCs are described as multipotent or unipotent, as they generally give rise to the specialised cells from the tissue in which they reside. Other stem cell types that fall outside of these two broad categories exist, and include engineered or induced pluripotent stem cells and cancer cells possessing stem-like qualities (Alvarez *et al.*, 2012). Although ESCs hold important potential for research in stem cell therapy, their current clinical uses are hindered by ethical and safety concerns, and so for many research strategies ASCs are the cells of choice (Bellayr and Li, 2009).

1.1.2 ASCs

ASCs contain functional properties that allow them to differentiate and replenish endogenous mature cell types within the tissue of which they reside. Co-localised with supporting cells, it is understood that their function is heavily influenced by bidirectional interactions with the surrounding specialised microenvironment (Schofield, 1978; Jones and Wagers, 2008). This means that, as well as being utilised to replenish lost endogenous cells, they could also be beneficial in exerting localised paracrine effects (Casarosa, Bozzi and Conti, 2014). Some of the best-characterised ASCs are derived from the bone marrow, and include haematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and endothelial progenitor cells. Although the majority of adult mammalian tissue displays a low cell turnover, with exceptions such as the skin, blood and gut, it was not until relatively recently that organs like the brain and heart were considered to harbour cells with self-renewal and regenerative potential. Now cardiac stem cells and neural stem cells are being demonstrated to have huge potential clinical implications (Mimeault, Hauke and Batra, 2007).

1.1.2.1 ASC potential

With less ethical considerations and easier accessibility, ASCs are a useful tool and candidate for stem cell research, and subsequent translation into the clinic (Bellayr and Li, 2009). A further important benefit of ASC therapy derives from the ability to harvest the cells directly from the patient requiring treatment, thereby minimising the risk of rejection through autologous transplantation. However, it should be noted that many of the diseases proposed as potential beneficiaries of these therapies are progressive and found in the aging population (Ahmed *et al.*, 2017). It is established that age negatively impacts on both ASCs and their niche (Carlson and Conboy, 2007), meaning that the cells derived from these patients may be less accessible and of limited therapeutic benefit if screening procedures to isolate cells expressing functional phenotypes are not developed and implemented (Dimmeler and Leri, 2008).

1.1.2.2 ASC microenvironment

The environment in which the specific ASC resides, coined the stem cell 'niche', is a key regulatory factor in the maintenance, differentiation and function of the cells, and influences their ability to be functionally integrated upon transplantation (Schofield, 1978). Within this niche, various factors are found, namely the extracellular matrix (ECM), cellular components and the vasculature; as well as constituents and effects of the immune response upon disease or injury (Rezza, Sennett and Rendl, 2014). The ECM highlights the bidirectional nature of this cell-environment relationship as the ASCs produce components of the ECM, which, in turn, is important in regulating their proliferation and divisional capabilities (Watt and Huck, 2013). Moreover, the close contact with capillary endothelium allows for delivery of oxygen,

metabolites and immunological signals, which can exert their effects on ASCs to regulate their function (Gómez-Gaviro *et al.*, 2012).

1.1.3 Challenges for stem cell research

Currently, there are two proposed methods of stem cell exploitation for therapy – mobilisation of endogenous stem cells and transplantation of exogenous stem cells. Challenges facing the latter shall be explored. Transplantation of cells functionally identical to the endogenous cells of the target tissue provides potential for high specificity and efficacy, but also brings with it a wide set of challenges (Dimmeler *et al.*, 2014). The challenges faced by the field from an exogenous transplantation perspective include, but are not limited to, sourcing of the most appropriate stem cells (embryonic vs. adult, and donor source), transportation and delivery of the cells into a patient, targeting of cells to the site of interest and retention there, and subsequent functional integration of the cells into the host tissue.

1.1.3.1 Challenge: stem cell delivery, targeting and retention strategies

The delivery of stem cell populations at the site of transplantation is a critical parameter for success, whereby efficient cell survival and targeting directly relates to success of a positive clinical outcome and minimisation of unspecific and harmful effects (Dimmeler *et al.*, 2014). ASCs have been shown to be migratory in nature, which has not only been described as advantageous for cell targeting at sites of injury, but can also enable their use as a therapeutic delivery agent to release substances at specific sites of damage (Müller, Snyder and Loring, 2006). However, there are hurdles in the targeting and retention of cells. This is highlighted by the fact that the routinely used HSC transplantation therapy for treatment of multiple myeloma and leukaemia still provides limited efficacy due to suboptimal cell survival, targeting and

retention (Bryder, Rossi and Weissman, 2006). One method to overcome such targeting and retention issues could lie in the reprogramming of cell gene expression, and despite safety hurdles, this approach is yielding some promising results (Dimmeler *et al.*, 2014). For example, treatment of HSCs with prostaglandin E2 induces expression of genes involved in targeting and retention of cells (such as *CXCR4*), thereby improving their potential efficacy (Cutler *et al.*, 2013).

A different method recently described for the targeting and retention of cells, is the use of magnetic micro- and nano-particles; extending the well-established foundations set by techniques such as magnetic resonance imaging (Pankhurst *et al.*, 2009). Stem cell studies, based on the theoretical idea that internalised magnetic particles, and therefore cells, can be mechanically controlled through magnetic fields, were reported *in vitro* by Sura, *et al.* (2008), whereby stem cells were magnetically trapped during fluid flow. Since then these results have been supported by *in vivo* studies, the first of which showed that magnetically labelled endothelial progenitor cells were successfully localised at a site of vascular injury (rat carotid artery) (Kyrtatos *et al.*, 2009). Advancement and optimisation of this technique could provide a great tool for circumventing the challenges associated with targeting and retention of cells at a site of injury or disease. It may also be extended to challenges against the differentiation ability of cells at a site of injury, as further studies utilising these magnetic particles have also shown the ability to direct differentiation by mechanical signals alone. An example of this is the activation of surface receptors by magnetic actuation, in order to promote gene expression that drives differentiation (Pankhurst *et al.*, 2009).

1.1.3.2 Challenge: stem cell functional integration

One of the most challenging aspects of stem cell therapy research may lie with the efficient integration of functional cells into the recipient tissue, to promote homeostatic function and subsequent repair. Investigation into both the intrinsic molecular pathways that underpin homeostatic stem cell function and the properties of the stem cell environment is essential for understanding how to successfully integrate transplanted ASCs (Dimmeler *et al.*, 2014). A positive approach to study this is to consider the diseased, injured or aged state, whereby trophic signalling is altered, and site-specific repair mechanisms are in place. An example of success lies with adult muscle stem cells (AMSCs) essential for muscle regeneration (Conboy *et al.*, 2003). With age this regeneration slows, resulting in muscle wasting. In parallel, at the molecular level, a delayed response of the Notch signalling pathway within the aged AMSC has been detected. Upon pharmacological stimulation of this pathway, the delayed response is reversed and symptoms improved, giving insight into a cell-specific response involved in this tissue regeneration strategy (Conboy *et al.*, 2003).

A particularly challenging area to functionally integrate transplanted stem cells is in joints afflicted by osteoarthritis (OA), a debilitating disease which currently lacks efficacious treatment options (Arthritis Research UK, 2013). There is, therefore, a huge potential for the advancement of methodologies to produce functional stem cells for tissue repair in OA, but this requires a greater understanding of OA pathogenesis and elucidation of key mediators that may influence the functional integration of stem cells in this disease.

1.2 Osteoarthritis

OA is the most prevalent form of joint disease. With 8.75 million people in the UK seeking treatment, this disease has a huge impact both economically and societally, and this is estimated to rise significantly due to an aging population and increasing levels of obesity – two of the disease's biggest risk factors (Arthritis Research UK, 2013). In fact, reports of knee OA, which currently afflicts 4.71 million people in the UK, are expected to rise to 6.5 and 8.3 million by 2020 and 2035 respectively (Arthritis Research UK, 2013).

Currently there is no effective treatment to help prevent disease progression or reverse the damage experienced by affected joints (Tonge, Pearson and Jones, 2014). Instead, symptoms are managed primarily through the use of analgesics or anti-inflammatory agents (e.g. non-steroidal anti-inflammatory drugs, such as ibuprofen, aspirin and naproxen) to relieve the pain frequently experienced with this condition. However, these themselves create further side effects and contribute to 4% of total hospital admissions (Arthritis Research UK, 2013). When symptoms become too difficult to manage joint replacements are undertaken, and these account for 97% of initial knee and 91% of initial hip replacements, costing an estimated £5 billion to health care providers each year (National Joint Registry, 2014). Therefore, there is an urgent need for alternative OA therapies. However, in order to identify potential therapeutic targets, further information regarding pathophysiological induction and progress of the disease needs to be elucidated.

1.2.1 Pathophysiology

OA is described as a cluster of diseases, as the etiology of disease varies between patients. However, it can universally be defined as a progressive, chronic disease which affects the whole joint (Little and Smith, 2008) resulting in a number of changes including, but not limited to, loss of articular cartilage integrity, subchondral bone remodelling and osteophyte formation, and synovial inflammation (synovitis) (Buckwalter and Mankin, 1998; Hunter, 2011) (Figure **1.1**). It should be noted that there is debate over whether loss of articular cartilage or bone remodelling occurs first, however synovitis can be indicated before either of these structural changes are observed (Ayral *et al.*, 2005; Krasnokutsky *et al.*, 2011; Roemer *et al.*, 2011).

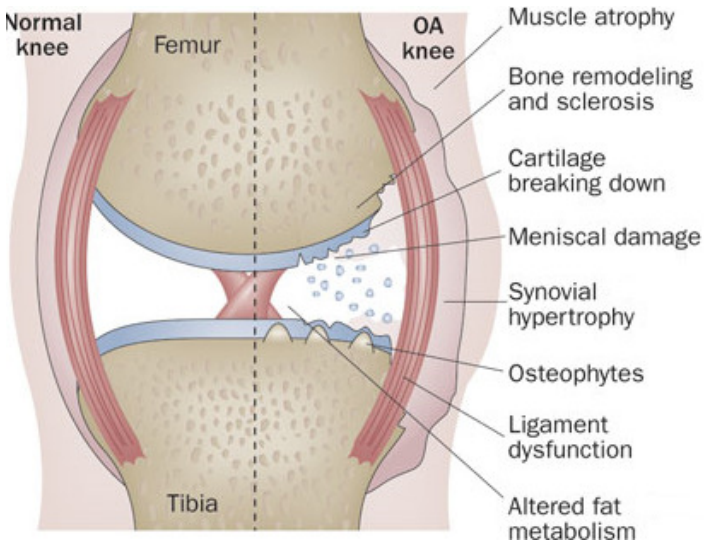


Figure 1.1 Osteoarthritis pathophysiology

Pathological changes observed in OA include cartilage breakdown, bone remodelling, osteophyte formation, synovitis and muscle damage (Adapted from Hunter, 2011).

1.2.1.1 Cartilage

In healthy joints, cartilage envelops the end of the bones, acting as a gliding structure that provides cushioning between the bones. Subsequently, this acts to prevent high load damage to the joint as a result of mechanical injury (Fox, Bedi and Rodeo, 2009). The cartilage itself comprises of chondrocytes (1-2%), a fluid phase (70-80%) and a solid phase (20-30%), the latter consisting of collagen (e.g. collagen type II) and proteoglycans (e.g. aggrecan) (Fox, Bedi and Rodeo, 2009).

In OA, disruption to cartilage homeostasis, and subsequent cartilage breakdown, is a hallmark of disease (Wieland *et al.*, 2005), an event to which matrix metalloproteinases (MMPs) have been directly and consistently linked (Iannone and Lapadula, 2003). In OA, normally inactive chondrocytes become activated and start to proliferate whilst also producing these MMPs (Goldring and Marcu, 2009). Some of the most widely studied MMPs implicated in this process include collagenase-1 (MMP-1) and collagenase-3 (MMP-13), which are involved in the degradation of native collagen (Freemont *et al.*, 1997), with the latter also able to degrade aggrecan (Burrage, Mix and Brinckerhoff, 2006). Moreover, the degradation of proteoglycans by stromelysins is primarily driven by stromelysin-1 (MMP-3) (Okada *et al.*, 1992), whilst the enzymatic destruction of denatured collagen is promoted by gelatinase 92kD (MMP-9) (Bigg *et al.*, 2007). The process of MMP activation starts with the release of proMMPs from chondrocytes, which are subsequently activated by cathepsin B and plasmin. Conversely, activity of MMPs is inhibited by tissue inhibitors of metalloproteinase (TIMPs), which bind to the active site of both MMPs and proMMPs (Dean and Woessner, 1984). In OA, the action of cathepsin B is crucial, as not only does it activate MMPs, but it also inhibits TIMPs. The subsequent reduction of these inhibitory and anti-angiogenic proteins

primarily ensures that MMP concentration accelerates and also stimulates angiogenesis (Kostoulas *et al.*, 1999), a process linked to bone changes in OA, such as osteophyte formation (Dodds *et al.*, 1994).

1.2.1.2 Bone

An imbalance between bone formation and resorption is observed during early stages of OA (Goldring and Goldring, 2010). Both the subchondral plate and trabecular bone are affected, with the former sustaining thickening, whilst the latter withstands irregular structural formation (Buckland-Wright, 2004). Emergence of vascularised, bony outgrowths called osteophytes also occurs, which is a hallmark of OA. Controversy surrounding their purpose is present; with some studies implicating their role in stabilisation of the joint to protect cartilage from further degradation (Brandt, 1999), whilst others suggest their role in OA pain (Lanyon *et al.*, 1998).

1.2.1.3 Synovium

Another essential component of the joint is the synovium. Also referred to as the synovial membrane, this specialised connective tissue lines diarthrodial joints, sealing the synovial cavity from surrounding tissues (Mathiessen and Conaghan, 2017). It is composed of two layers, the outer underlying tissue (subintima) containing a rich vascular and lymphatic supply as well as nerve fibres, and the inner layer of cells (intima) consisting of synoviocytes (Smith, 2011). In the healthy synovium, these synoviocytes are primarily resident fibroblasts, but macrophages are also present (Mathiessen and Conaghan, 2017). The synovium plays a crucial role in the maintenance of joint structure and function though a variety of mechanisms. One is by acting as a source of synovial fluid (SF) components, such as hyaluronic acid (HA) (Hui *et al.*, 2012), whose role includes protection of articular cartilage. Another is through

providing nutrients from the vascular and lymphatic systems, which are not present in articular cartilage (Smith, 2011). In this way, it is essential in both the provision and removal of products and metabolites.

During OA, the synovium becomes thickened (Wenham and Conaghan, 2010) and the intima layer, which is primarily comprised of fibroblasts in healthy tissue, has an influx of leukocytes from the vascular compartment (Scanzello and Goldring, 2012). These infiltrating cells are mainly macrophages and T-cell lymphocytes, though other cells can be observed to a lesser extent, including B cells, mast cells and plasma cells (de Lange-Brokaar *et al.*, 2012). Subsequently, pro-inflammatory mediators and enzymes are released into the synovium and SF, thereby contributing to joint destruction (Kennedy *et al.*, 2011). It is postulated that much of the innate immune activation observed in OA is a result of synovial macrophages (Sokolove and Lepus, 2013). Moreover, sensory innervation of the synovium is associated with nociception in OA, and the efferent function of these nerves can allow for release of pro-inflammatory mediators that drive the inflammatory response (McDougall, 2006; Takeshita *et al.*, 2012).

1.2.2 Role of the immune response

Chronic inflammation is of fundamental importance and detrimental in many age-related chronic diseases of the body, with OA being no exception. Originally characterised as a 'wear and tear' disease, this concept has been challenged and redefined in particular as a result of the presence of synovial inflammation (Sokolove and Lepus, 2013) and the bone marrow lesions (BMLs) evident on magnetic resonance imaging scans (Felson *et al.*, 2001). Importantly, both synovial inflammation (Neogi *et al.*, 2016) and BMLs (Felson *et al.*, 2001) are significantly associated with OA pain. The recent and continual

expansion of knowledge regarding the role of the immune response and synovitis in OA is vital in gaining a deeper understanding of OA pathophysiology.

Nettelbladt and Sundblad (1959) first hinted at the role of the immune response in OA after discovering elevated levels of inflammatory plasma proteins in the SF and blood of OA patients. However, follow up of this finding was largely ignored due to the comparison with the classical inflammatory disease rheumatoid arthritis (RA) (Farahat *et al.*, 1993; Smith *et al.*, 1997), in which inflammatory changes were much more marked. As well as leading to a dismissal of the role of the immune response, it even meant that OA samples acted as a control to RA experiments. Despite this, it has more recently been proven that OA tissue and fluids are rich in inflammatory mediators resulting in synovitis (Sokolove and Lepus, 2013).

The link between synovitis and OA initiation and progression has been indicated by a number of more recent studies (Ayral *et al.*, 2005; Krasnokutsky *et al.*, 2011; Roemer *et al.*, 2011) which highlight the initiation of the immune response before radiographical changes are observed for the classification and diagnosis of OA. Furthermore, those with minimal radiographic disease also show notable synovitis (Haywood *et al.*, 2003), contributing to the notion that inflammation is key during early stages of OA development and progression. Interestingly, the reported pain symptoms were worse in those patients who presented with synovial inflammation (Scanzello, 2012), and could also be used as a predictive tool for faster cartilage breakdown (Scanzello and Goldring, 2012).

Initiation of the innate immune response is observed in the OA joint. Pattern-recognition receptors (PRRs) primarily located on synovial macrophages

(Sokolove and Lepus, 2013), are activated by damage/danger-associated molecular patterns (DAMPs) as a result of joint damage. Subsequently, this activation initiates repair processes, which serve to perpetuate the cycle of activation and breakdown (Sokolove and Lepus, 2013). Many of these DAMPs, such as HA (Liu-Bryan and Terkeltaub, 2010), arise from the breakdown of the ECM in the OA joint. This directly has an adverse effect on cartilage integrity, and initiates the production of pro-inflammatory mediators, such as tumor necrosis factor-α (TNFα) and interleukin (IL)-1β (Homandberg, Hui and Wen, 1996). A whole host of soluble inflammatory mediators such as these have been implicated in the pathogenesis of OA (Sohn *et al.*, 2012; Sokolove and Lepus, 2013; Wojdasiewicz, Poniatowski and Szukiewicz, 2014), including other cytokines such as IL-6 and IL-15 (Kaneko *et al.*, 2000; Scanzello *et al.*, 2009), chemokines such as IL-8 and monocyte chemoattractant protein-1 (MCP-1) (Kaneko *et al.*, 2000; Stanokovic *et al.*, 2009), and adipokines such as leptin and adiponectin (Dumond *et al.*, 2003; Koskinen *et al.*, 2011). Moreover, plasma proteins such as α1-microglobulin and α2-macroglobulin are elevated as a result of this tissue damage and inflammation, resulting in further production of pro-inflammatory mediators from toll-like receptor (TLR)-4 mediated macrophages, including IL-6 and vascular endothelial growth factor (VEGF) (Sohn *et al.*, 2012). TLR-4 is the most widely activated PRR by DAMPs, which has also been implicated in the pain responses associated with OA (Miller *et al.*, 2015).

1.2.3 Pain

Despite current OA treatments only targeting pain symptoms most sufferers continue to experience pain to differing degrees (Sofat, Ejindu and Kiely, 2011). Anecdotal evidence suggests that pain experiences worsen from episodic to chronic over time (Schaible, 2012), though radiographic evidence

is unable to provide a positive correlation between pain and structural joint damage (Kidd, 2012). This could be in part due to the lack of nerve endings in articular cartilage, and interestingly it has been noted that pain frequency and severity is increased with higher levels of synovitis (Neogi *et al.*, 2016).

Joint nerves contain Aδ- and C-fibres in all structures of the joint, except normal cartilage, allowing for the induction of signals upon stimulation (Schaible *et al.*, 2009). However, evidence for the initiation and propagation of pain in OA is limited, in part due to a lack of information regarding neuronal organisation of joint pain. This is in stark contrast to the wealth of knowledge surrounding cutaneous pain (McDougall, 2006; Schaible, Schmelz and Tegeder, 2006).

Sensitisation of joint nociceptors is increased in OA models, in a damage dependent manner. This sensitisation occurs as a result of elevated inflammatory mediators, such as prostaglandins and cytokines (Schaible, Ebersberger and Natura, 2011). Subsequently, central sensitisation of the central neurophysiological pathway occurs, with hyperexcitability of ascending and non-ascending nociceptive spinal cord neurons and expansion of the receptive pain field, thereby contributing significantly to the mechanical hyperalgesia experienced (Schaible *et al.*, 2009). These signals are mediated by the brainstem, specifically in the periaqueductal gray region (Gwilym *et al.*, 2009). Furthermore, atrophy in the thalamus and gray matter of identified pain-related cortical areas occurs as a consequence of the chronic pain experienced by OA patients (Rodriguez-Raecke *et al.*, 2009).

With a focus on the peripheral neuronal mechanisms in the joint, animal models of OA pain have been undertaken primarily using the monoiodacetate (MIA) model, in which a MIA injection rapidly induces an OA state due to its

toxicity to chondrocytes (Schaible, 2012), however this induction is remarkably different to the slow, progressive nature of OA. Nevertheless, animals with MIA-induced OA begin to display mechanical hyperalgesia and it has been postulated that the initial inflammatory response contributes to this. TNFα and IL-6 are two pro-inflammatory cytokines that become elevated in this model (Orita *et al.*, 2011). These cytokines have been proven to act on nociceptive neurons as injection of them into non-OA joints results in the sensitisation of Aδ and C-fibres, which has even been shown to be partially reversed once an anti-TNFα mediator (e.g. etanercept) is administered (Richter *et al.*, 2010). At a later stage of disease, the MIA model highlights the elevation of nerve growth factor (NGF), a growth factor essential in the nociceptor development process and subsequent maintenance of the structural and functional properties (Orita *et al.*, 2011). Human studies have shown that anti-NGF treatments (e.g. tenazumab) relieve pain symptoms for an extended period of time, in a dose-dependent manner (Lane *et al.*, 2010). Therefore, it is possible that neutralisation of these inflammatory factors could be an effective way to reduce pain levels in OA patients.

1.2.4 Development of novel therapeutic strategies for OA

A lack of therapeutic options for OA highlights the urgent need for the development of new strategies that can target the described pain, inflammation and degeneration observed in the osteoarthritic joint. One potential treatment strategy is the use of stem cells, with MSCs being the leading candidate. First described by Friedenstein *et al.*, (1966), these multipotent stem cells demonstrate the ability to primarily differentiate into cells of the mesoderm lineage, including chondrocytes, osteoblasts and adipocytes. More recent findings have also highlighted the trophic and immunomodulatory abilities of these cells (Baraniak and McDevitt, 2010;

Kong *et al.*, 2017) that allow them to modulate their local environment and cells within it. Combined, these two biological functions could help to develop a treatment strategy that targets pain, inflammation and regeneration of the tissue.

Pre-clinical trials using animal models have successfully highlighted the potential of MSCs in OA repair (Gupta *et al.*, 2012). Interestingly, they have supported the role of paracrine effects for repair, as joint regeneration in these models have occurred primarily through mobilisation of native cells. However, current clinical trials assessing the effectiveness of MSCs for OA treatment do not provide unequivocal evidence for cartilage regeneration, and although significant improvements in pain and physical function of the treated joint have been reported, a systematic review of these trials suggests that poor study design, large heterogeneity of results and high-risk of bias impedes the quality of the results. In fact, utilisation of the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) rating system to analyse these clinical trials, revealed that the acquired evidence used to support the hypothesis that MSC transplantation leads to reduction in pain and improved physical function is considered very low to low (Iijima *et al.*, 2018). This indicates that these clinical trials are ineffective at providing evidence for MSC treatment and are not sufficient to support a clinical recommendation. However, as MSCs hold promise for future treatment strategies it is important to determine how these cells can be utilised effectively during transplantation.

1.2.5 Effect of the OA microenvironment on MSCs

One potential cause for the lack of observed success in clinical trials of MSC transplantation is the effect of the local inflammatory environment of the

osteoarthritic joint. As the local environment in which a stem cell resides conditions its maintenance, differentiation and function, the inflammatory osteoarthritic environment can affect the ability of transplanted MSCs to functionally integrate and exert their therapeutic effects, highlighting one of the biggest challenges facing clinical applications of stem cells.

In the OA joint, endogenous populations of MSCs have been shown to display an altered phenotype and differentiation potential (Coleman *et al.*, 2010; Wyles *et al.*, 2015), and properties of a recently described, novel population of MSCs suggest that they may even be contributing towards the OA phenotype (Jayasuriya *et al.*, 2018). Therefore, identification of factors and cell signalling pathways that influence MSC function is critical for ensuring MSCs promote reparative effects and do not contribute to disease pathology. These findings will subsequently help to develop targeted MSC-based treatments for OA that can regenerate joint tissue, modulate the immune response and relieve pain. Some key inflammatory mediators that are involved in OA pathology are described below.

1.2.5.1 Interleukin-1β

IL-1β is a pro-inflammatory, catabolic cytokine (Dinarello, 2011) that has been shown to have a positive correlation between IL-1β levels and OA changes (Kubota *et al.*, 1997), as the limited amount of IL-1β found in healthy tissue is markedly increased in chondrocytes, synovial cells and the SF of OA patients. This cytokine is recognised by chondrocytes through their expression of the type I receptor (IL-1RI), which is upregulated during OA, explaining the catabolic effects of IL-1β in this condition (Shlopov, Gumanovskaya and Hasty, 2000). Subsequently, an autocrine-positive feedback loop is initiated in which the chondrocytes themselves produce higher levels of IL-1β, as well as

another pro-inflammatory marker IL-6 (Fan *et al.*, 2007). Moreover, genetic studies show that IL-1β antagonists are downregulated during this process (Fernandes, Martel-Pelletier and Pelletier, 2002).

Recent studies have implicated the use of MSCs as a way to reverse this IL-1β mediated cartilage degradation (Tang *et al.*, 2015). In contrast to its inflammatory function, IL-1β evokes the immunosuppressive activity of MSCs, and in a colitis study pre-treatment of transplanted MSCs with IL-1β led to more efficacious results (Fan *et al.*, 2012). Additionally, IL-1β has been shown to induce osteogenic differentiation of MSCs primarily through the Wnt-5a/receptor tyrosine kinase-like orphan receptor 2 pathway (Sonomoto *et al.*, 2012).

1.2.5.2 Interleukin-8

The chemokine family are crucial in the inflammatory process through their induction of chemotaxis in a variety of immune cells, including neutrophils, lymphocytes and monocytes (Moser and Willimann, 2004). Specifically, with regards to OA, their ability to contribute to the release of cartilage degrading enzymes and pro-inflammatory cytokines, such as IL-1β, TNFα and IL-6 (Pierzchala, Kusz and Hajduk, 2011), plays a critical role in joint pathology. One such chemokine is IL-8 (or CXCL8), which is increased in the SF of OA patients (Kaneko *et al.*, 2000). Interestingly, IL-8 directly affects MSCs by increasing their ability to produce VEGF, thereby making the chemokine pro-angiogenic, and is also endogenously secreted by MSCs (Hou *et al.*, 2014).

1.2.5.3 Interleukin-10

An anti-inflammatory cytokine increased in an osteoarthritic joint is IL-10 (Iannone *et al.*, 2001). Its importance in OA has been identified by the fact that

genetically engineered MSCs, which overexpress IL-10, have the ability to inhibit the development and progression of arthritis in a mouse model (Choi *et al.*, 2008). MSCs express IL-10 endogenously, however upon differentiation cytokine expression profiles are altered and IL-10 expression is lost. In a rat cardiac model, restoration of this expression restored the immunological function of MSCs, indicating that IL-10 is a key mediator in the immunoprivileged status of MSCs (Dhingra *et al.*, 2015).

1.2.5.4 Monocyte chemoattractant protein-1

Another chemokine increased in the SF of OA patients is MCP-1 (or CCL2) (Stanokovic *et al.*, 2009). Secreted by a number of cell types involved in the OA inflammatory process (including fibroblasts, monocytes and T cells), MCP-1 is a key mediator in the initiation of inflammation due to its chemoattractant properties (Deshmane *et al.*, 2009) as it stimulates monocyte, macrophage and lymphocyte chemotaxis and transendothelial migration to inflammatory lesions (Melgarejo *et al.*, 2009). A member of the C-C chemokine family, this signalling protein directly contributes to chondrocyte catabolism in OA through promotion of apoptosis and inhibition of proliferation (Xu *et al.*, 2015). These observations may be due to increased proteinase expression and inhibition of proteoglycan synthesis, respectively. Nevertheless, although migration of MSCs is in part promoted by MCP-1 (Xu *et al.*, 2010), the ability of MSCs to undergo chondrogenic differentiation to replace damaged or lost chondrocytes is shown to be inhibited by this chemokine (Harris *et al.*, 2013). Another function of MCP-1 in OA is its role in pain regulation via central sensitisation, whereby increased protein expression levels mediate excitatory effects in both the dorsal root ganglia and the spinal cord (Miller *et al.*, 2012).

1.2.5.5 Nerve growth factor

NGF is elevated at later stages of OA, and as previously described, it is essential for nociceptor development and maintenance (Orita *et al.*, 2011). Therapies against this growth factor (e.g. tenazumab) reversed reported OA pain symptoms and attenuated pain behaviour and aspects of joint pathology in models of OA (Lane *et al.*, 2010). Although addition of NGF to MSCs improved functional recovery of rat spinal cord injury (Huang, Wang and Chen, 2006), in a more relevant study NGF slowed the rate of chondrogenic differentiation in murine cells (Huang *et al.*, 2013).

1.3 Aims and hypotheses

1.3.1 Scientific need

Despite MSCs holding promise as a novel treatment for OA, current clinical trials assessing their effectiveness are unable to provide sufficient evidence that would support their clinical recommendation (Iijima *et al.*, 2018). The inability of MSCs to regenerate cartilage in these studies could be due to the inflammatory osteoarthritic environment that they have been transplanted into. This is particularly important as it is known that the local environment in which a stem cell resides (it's 'niche') regulates many aspects of its biology, including its ability to differentiate (Schofield, 1978). Furthermore, it is known that MSCs found within the osteoarthritic joint display an altered phenotype and differentiation potential, providing further evidence that the osteoarthritic inflammatory environment affects the function of MSCs (Coleman *et al.*, 2010; Wyles *et al.*, 2015; Jayasuriya *et al.*, 2018).

If MSCs are to be used for regeneration of cartilage, their ability to differentiate is a key parameter for success. Therefore, if their reparative capacity is to be

harnessed for future OA treatment strategies, it is necessary to identify specific mediators that are found in the osteoarthritic joint and assess their effects on MSCs.

1.3.2 Aims and hypotheses

The work within this thesis aimed to explore the effect of the inflammatory osteoarthritic environment on BMSCs by studying the *in vitro* effects of a selection of inflammatory mediators, that are known to be upregulated in the osteoarthritic joint, on these cells. In this way, changes to proliferation, viability, chondrogenic and osteogenic differentiation potential, and gene expression were measured.

The inflammatory mediators chosen in this study are all known to contribute to the pathogenesis of OA and included both pro-inflammatory mediators (IL-1β, IL-8, MCP-1), anti-inflammatory mediators (IL-10) and growth factors involved in OA pain (NGF). These mediators were specifically chosen in order to identify if changes to MSC function occur due to a mediator's inflammatory properties, and to assess whether MSC functional changes could be enhanced or recovered during combined exposure, which is more representative of the osteoarthritic joint. This specific set of mediators were also chosen to help widen the current understanding of these mediators' roles in MSC (dys)function.

It was hypothesised that pro-inflammatory mediators (e.g. IL-1β, IL-8, MCP-1) would stimulate expression of genes involved in catabolism in human BMSCs, as these mediators are proposed to form a cytokine network that contributes to the pathogenesis of inflammatory joint disease (Harigai *et al.*, 1993). Within this, it was hypothesised that these mediators would increase

the expression of cartilage degrading enzymes, such as MMPs, thereby increasing the breakdown of matrix proteins produced during chondrogenic differentiation and decreasing the rate of chondrogenesis observed *in vitro* (Merz *et al.*, 2003; Xu *et al.*, 2015; Liu *et al.*, 2017). Although largely unstudied, it was hypothesised that NGF could also have a catabolic effect on human BMSCs, similar to pro-inflammatory mediators. This is due to this growth factors pivotal role in the propagation of OA pain (Orita *et al.*, 2011) and previous findings that have shown NGF to slow the rate of chondrogenic differentiation in murine cells (Huang *et al.*, 2013).

On the other hand, it was hypothesised that anti-inflammatory mediators (e.g. IL-10) could, to some extent, counteract the catabolic effects of pro-inflammatory mediators, particularly as IL-10 is a key mediator of immunosuppression and is known to inhibit OA progression in mice (Choi *et al.*, 2008; Dhingra *et al.*, 2015). This cytokine has also been shown to stimulate production of proteoglycans located in articular cartilage (van Roon *et al.*, 1996).

As bone remodelling is also an important component of OA pathogenesis, the effects of inflammatory mediators on BMSCs was also studied. In contrast, it was hypothesised that pro-inflammatory mediators and growth factors (e.g. IL-1β, IL-8, MCP-1, NGF) would increase the rate of osteogenic differentiation of human BMSCs, as IL-1β has previously been shown to have this effect *in vitro* (Sonomoto *et al.*, 2012). On the other hand, it was hypothesised that anti-inflammatory mediators (e.g. IL-10) would slow the rate of osteogenic differentiation, as it has previously been seen to inhibit early osteogenesis in murine BMSCs (Van Vlasselaer *et al.*, 1994).

However, due to a wealth of conflicting data over the effects of inflammatory mediators (such as IL-1β) on the rate of osteogenesis (Lacey *et al.*, 2009; Sonomoto *et al.*, 2012) it was hypothesised that cell type and species may influence any observed effects. Therefore, osteogenic differentiation was undertaken using an immortalised murine BMSC cell line, an immortalised human BMSC cell line (ihBMSCs) and human primary BMSCs in order to investigate this hypothesis.

1.3.3 Objectives

The specific objectives of this study were:

1. Identification of key inflammatory mediators that alter the biological functions of BMSCs
2. Identification of key pathways that inflammatory mediators may use to alter the function of BMSCs
3. Assessment of individual and combined inflammatory mediators on the proliferation and viability of ihBMSCs during *in vitro* culture
4. Assessment of individual and combined inflammatory mediators on the gene expression profile of ihBMSCs during *in vitro* culture
5. Assessment of individual and combined inflammatory mediators on the chondrogenic potential of ihBMSCs, including changes to gene expression
6. Assessment of individual and combined inflammatory mediators on the osteogenic potential of a murine BMSC cell line, ihBMSCs and human primary BMSCs, including changes to gene expression in ihBMSCs

Chapter 2 General Methods

General methods regarding MSC cell culture and molecular biology are described here, followed by more targeted methods sections for each results chapter. Materials were purchased from Sigma-Aldrich unless otherwise stated.

2.1 MSC cell culture

All cell culture was performed in a class II microbiological safety cabinet (ThermoFisher), providing unidirectional flow of sterile air to prevent external contamination. All cells were maintained at 37°C with 5% CO_2 in a Hera Cell 150 incubator (Thermo Electron Corporation).

2.1.1 Bone marrow derived MSCs

Three types of bone marrow derived MSCs (BMSCs) were utilised in this project. The most commonly used cell type was an immortalised human BMSC cell line (ihBMSCs) (Okamoto *et al.*, 2002), but both an immortalised murine BMSC cell line (D1, ATCC CRL-12424) and human primary BMSCs (Lonza, UK) were also utilised. In this early stage of investigation, readily available and well documented immortalised human (passage 55-75) and murine (passage 35-39) BMSC cell lines were utilised for comparison to, and extension of, the existing literature. The use of two species also allowed for the identification of species differences, which is particularly important when considering future *in vivo* work. Human primary BMSCs (passage 4) were further utilised as they constitute a clinically relevant cell type and provide an enhanced reflection of potential human *in vivo* conditions.

All of these cell types were cultured in low glucose (1g/L) Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies), supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine and 1% (v/v) penicillin streptomycin, which is referred to as standard medium (SM) throughout this project. DMEM is a defined media containing components such as essential amino acids, salts, vitamins and iron that help to provide a suitable environment that promotes cellular growth and maintenance. The media is further supplemented by foetal bovine serum, which contains a variety of growth factors to promote cell proliferation and prevent apoptosis. The unstable essential amino acid, L-glutamine, is also added along with non-essential amino acids to reduce the metabolic burden and allow increased cellular proliferation. The addition of the antibiotic penicillin streptomycin prevents contamination from both gram-positive and gram-negative bacteria.

2.1.2 Passaging cells

Once cells had achieved 70-80% confluency, BMSCs were passaged using a standard trypsinisation protocol. To achieve this, cells were first washed with phosphate buffered solution (PBS) to remove any trypsin-neutralising serum, before addition of 0.25% (v/v) trypsin/0.02% (v/v) ethylene diaminetetraacetic acid (EDTA) at 37°C for 1 minute. Trypsin is a proteolytic enzyme that breaks down proteins anchoring cells to the culture vessel surface, whilst EDTA removes calcium required for the function of adhesion molecules at cell-to-cell junctions. The flask then underwent mechanical stimulation to help detach any remaining cells from the tissue culture plastic, and fresh SM was added to halt the actions of trypsin and EDTA. The resulting cell suspension was transferred to a universal tube and centrifuged for 5 minutes at 200 x *g* in a Sigma 3-16 centrifuge. The subsequent pellet formed from centrifugation was

resuspended in fresh SM and seeded into tissue culture flasks for continuation of culture, or counted using the Countess II FL (ThermoFisher) automated system and seeded into plates for experimental purposes. Typically, the cells were seeded at a ratio between 1:3 and 1:6 for passaging after 2-3 days.

2.1.3 Inflammatory mediator exposure

Recombinant proteins IL-1β (Abnova), IL-8 (R&D systems), IL-10 (Source Bioscience), MCP-1 (ThermoFisher) and NGF (ThermoFisher) were reconstituted in PBS containing 0.1% (v/v) bovine serum albumin (ThermoFisher) and were stored and aliquoted at suitable stock concentrations at -20°C. These proteins were thawed and added to cell culture medium at the concentrations displayed in Table **2.1** each time medium was changed during initial cell proliferation and viability assays, whilst the concentrations displayed in Table **2.2**. were used for gene expression analysis studies and during chondrogenic and osteogenic differentiation protocols.

Mediator	Low dose	Medium dose	High dose
IL-1β	1pg/ml	10pg/ml	100pg/ml
IL-8	0.5ng/ml	1ng/ml	2ng/ml
IL-10	10pg/ml	50pg/ml	100pg/ml
MCP-1	0.5ng/ml	2.5ng/ml	12.5ng/ml
NGF	1ng/ml	10ng/ml	100ng/ml

Table 2.1 Doses of inflammatory mediators cultured with BMSCs for cell proliferation and viability assays

Mediator	Dose
IL-1β	100pg/ml
IL-8	2ng/ml
IL-10	100pg/ml
MCP-1	10ng/ml
NGF	10ng/ml

Table 2.2 Doses of inflammatory mediators cultured with BMSCs for gene expression expression analysis, chondrogenic and osteogenic differentiation

2.2 Molecular biology analysis

2.2.1 RNA extraction

BMSCs in culture were washed twice with PBS and resuspended in Tri-reagent for subsequent RNA extraction. First, cells were centrifuged in an Eppendorf 5415R microcentrifuge at 3,000 x *g* for 5 minutes to pellet any insoluble material. The supernatant was transferred to a fresh eppendorf tube and chloroform (Fisher Scientific) was added at a 1:5 ratio. Samples were vortexed and incubated at room temperature for 10 minutes to allow phase separation. The samples were then centrifuged at 12,000 x *g* for 15 minutes and following this the upper aqueous phase, containing nucleic acids, was transferred to a new eppendorf tube. A 1:1 volume of 70% ethanol (EtOH) was added and thoroughly vortexed before being transferred to a RNeasy MinElute spin column from a RNeasy Micro Kit (Qiagen). RNA was then extracted using this kit, according to manufacturer's instructions. This extraction included a DNase I treatment to remove DNA contamination from the samples. On the final step, RNA samples were eluted in 14µl of sterile distilled water (SDW) and the RNA concentration and purity was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA).

2.2.2 cDNA synthesis

cDNA was synthesised from the extracted RNA via reverse transcription using a High Capacity RNA-to-cDNA Kit (ThermoFisher), according to manufacturer's instructions. Briefly, 500ng-2µg of RNA was utilised to synthesise the equivalent amount of cDNA by preparing a reaction mix composed of reverse transcriptase (RT) buffer (containing dNTPs, random octamers and oligo dT-16), RT enzyme (containing MuLV and RNase inhibitor protein) and the RNA sample. The samples were subsequently mixed thoroughly and incubated at 37°C for 1 hour in a heat block. After this time the samples were transferred to a heat block at 95°C for 5 minutes. cDNA samples were stored at -20°C until used in reverse transcription-PCR (RT-PCR) for validation of successful cDNA synthesis, or used to analyse gene expression by TaqMan Low Density Arrays.

2.2.3 Reverse transcription PCR

Successful cDNA synthesis was validated using RT-PCR with primers for the housekeeping gene GAPDH. Upon receipt lyophilised primers were reconstituted in SDW to 5µM and stored at -20°C. RT-PCR was conducted using Platinum Taq DNA polymerase (Invitrogen) in a reaction mixture consisting of 100ng cDNA, 2µl 10x PCR buffer (Invitrogen), 0.6µl $MgCl_2$ (Invitrogen), 0.6µl 2.5mM dNTPs (Invitrogen), 0.75µl 5mM forward primer, 0.75µl 5mM reverse primer and 0.15µl Taq polymerase made up to a total of 20µl with SDW. The PCR reaction was carried out in a Labnet Multigene Thermal Cycler (Labnet International Inc., USA) with the following conditions: 5 minute initial denature step at 90°C, 40x cycles of 94°C for 30 seconds, 60°C for 45 seconds, 72° for 60 seconds, and a final 10 minute 72°C extension step. Reactions were held at 4°C until removed for analysis using agarose gel electrophoresis.

2.2.4 Agarose gel electrophoresis

RT-PCR products for cDNA validation were visualised using agarose gel electrophoresis. Here a 2% agarose gel containing 0.0001% ethidium bromide was made using Tris-acetate-EDTA (TAE) buffer and allowed to set for 20 minutes. The PCR samples were mixed with 6x loading dye (New England BioLabs) and loaded onto the gel along with a 50bp hyperladder (Bioline) for determination of product size. The gel was run at 85V, 400mA for 1 hour in TAE, after which time PCR products were visualised using a FujiFilm LAS 4000 imager (FujiFilm UK Ltd, UK).

2.2.5 TaqMan Low Density Arrays

Custom TaqMan Low Density Array (TLDA) 384-well microfluidic cards (ThermoFisher) were designed and used for analysis of gene expression. Cards with a 96 gene x 4 sample layout were designed using probes from ThermoFisher, which were then preloaded onto the card. Where possible probes spanning an exon boundary and that were not in the untranslated region were chosen. A list of the genes studied can be found in Table **2.3**.

To load the microfluidic cards, 200ng of each cDNA sample was diluted in a total of 50µl RNase free water in a LightSafe microcentrifuge tube, and an additional 50µl of TaqMan Universal Master Mix II with UNG was added. The samples were vortexed thoroughly and this 100µl mix was added to each reservoir of the TLDA plate. To disperse the cDNA across the wells, the plate was centrifuged twice for 1 minute at 200 x *g*, after which the plate was sealed. TLDA plates were run in a 7900HT Fast Real Time PCR System using SDS RQ Manager Software (Applied Biosystems) using the thermal cycler settings in Table **2.4**.

Relevance	Gene	Name
Inflammatory mediators and receptors	*IL1B*	Interleukin 1 beta
	IL1R1	Interleukin 1 receptor type 1
	CXCL8	C-X-C motif chemokine ligand 8
	CXCR1	C-X-C motif chemokine receptor 1
	IL10	Interleukin 10
	IL10RB	Interleukin 10 receptor subunit beta
	CCL2	C-C motif chemokine ligand 2
	CCR2	C-C motif chemokine receptor 2
	NGF	Nerve growth factor
	NGFR	Nerve growth factor receptor
	NTRK1	Neurotrophic receptor tyrosine kinase 1
Osteogenic markers	*RUNX2*	Runt related transcription factor 2
	SP7	Sp7 transcription factor
	ALPL	Alkaline phosphatase
	COL1A1	Collagen type 1 alpha 1 chain
	BGLAP	Bone gamma-carboxyglutamate protein
	SPP1	Secreted phosphoprotein 1
	IBSP	Integrin binding sialoprotein
Chondrogenic markers	*SOX4*	SRY-box 4
	SOX9	SRY-box 9
	P4HA2	Prolyl 4-hydroxylase subunit alpha 2
	SDC2	Syndecan 2
	SDC3	Syndecan 3
	ACAN	Aggrecan
	COL2A1	Collagen type 2 alpha 1 chain
	COL10A1	Collagen type X alpha 1 chain
Immuno-modulatory mediator	*PTGS2*	Prostaglandin-endoperoxide synthase 2
	PTGES2	Prostaglandin E synthase 2
	HMOX1	Heme oxygenase 1
	HLAG	Major histocompatibility complex, class I, G
	CSF3	Colony stimulating factor 3
	IDO1	Indoleamine 2,3-dioxygenase 1
	IFNG	Interferon gamma
	NOS1	Nitric oxide synthase 1
	NOS2	Nitric oxide synthase 2
	IL1A	Interleukin 1 alpha
	IL6	Interleukin 6
	TNF	Tumor necrosis factor
	TNFRSF1A	TNF receptor superfamily member 1A
	NFKB1	Nuclear factor kappa B subunit 1
	PPARA	Peroxisome proliferator activated receptor alpha
	PPARG	Peroxisome proliferator activated receptor gamma
	MMP1	Matrix metalloproteinase 1
	MMP3	Matrix metalloproteinase 3
	MMP13	Matrix metalloproteinase 13
Growth factors and receptors	*EGFR*	Epidermal growth factor receptor
	FGF2	Fibroblast growth factor 2
	FGFR1	Fibroblast growth factor receptor 1

	HGF	Hepatocyte growth factor
	IGF1	Insulin like growth factor 1
	PDGFRB	Platelet derived growth factor receptor beta
	VEGFA	Vascular endothelial growth factor A
TGF-β/BMP signalling	*TGFB1*	Transforming growth factor beta 1
	TGFB3	Transforming growth factor beta 3
	BMPR1B	Bone morphogenetic protein receptor type 1B
	BMP2	Bone morphogenetic protein 2
	BMP4	Bone morphogenetic protein 4
	BMP6	Bone morphogenetic protein 6
	GDF2	Growth differentiation factor 2
	SMAD1	SMAD family member 1
	SMAD3	SMAD family member 3
	SMAD5	SMAD family member 5
	SMAD9	SMAD family member 9
	NOG	Noggin
MAPK signalling	*MAPK1*	Mitogen-activated protein kinase 1
	MAPK3	Mitogen-activated protein kinase 3
	MAPK9	Mitogen-activated protein kinase 9
	MAPK14	Mitogen-activated protein kinase 14
Wnt signalling	*WNT5A*	Wnt family member 5A
	ROR2	Receptor tyrosine kinase like orphan receptor 2
	ITPR3	Inositol 1,4,5-trisphosphate receptor type 3
	WNT3A	Wnt family member 3A
	WNT7A	Wnt family member 7A
	LRP5	LDL receptor related protein 5
	CTNNB1	Catenin beta 1
Toll like receptor signalling	*TLR1*	Toll like receptor 1
	TLR2	Toll like receptor 2
	TLR3	Toll like receptor 3
	TLR4	Toll like receptor 4
	TLR5	Toll like receptor 5
	TLR6	Toll like receptor 6
	TLR9	Toll like receptor 9
Other signalling pathways	*IHH*	Indian hedgehog
	SHH	Sonic hedgehog
	NOTCH1	Notch 1
	HES1	Hes family bHLH transcription factor 1
	NELL1	Neural EGFL like 1
	JAK2	Janus kinase 2
	STAT3	Signal transducer and activator of transcription 3
	TAZ	Tafazzin
	YAP1	Yes associated protein 1
Housekeeping genes	*RNA18S*	18S ribosomal RNA
	ACTB	Actin beta
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
	GUSB	Glucuronidase beta
	TBP	TATA-box binding protein

Table 2.3 List of genes analysed using TLDAs

Stage	Temp (°C)	Time (mm:ss)
Hold	50	02:00
Hold	94.5	10:00
Cycle (40 cycles)	92	00:15
	60	01:00

Table 2.4 Thermal cycling conditions for TaqMan Low Density Arrays

2.3 Statistical analysis

Statistical analyses were carried out using GraphPad Prism 7 (GraphPad Software, San Diego, CA).

All results were plotted as mean ± standard error of the mean (SEM). A Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's test for post hoc multiple comparisons. Results were considered statistically significant with a p-value of less than 0.05.

Chapter 3 Analysis of the MSC response after exposure to inflammatory mediators

3.1 Introduction

3.1.1 Paracrine activity of MSCs

The emergence of knowledge on the critical role that MSC paracrine activity plays in the establishment of a regenerative microenvironment has broadened the therapeutic potential of MSCs (Kusuma *et al.*, 2017). This has been shown by the many pre-clinical and clinical models that utilise MSCs in clinical conditions that do not require differentiation into phenotypic lineages of these cells (Caplan and Correa, 2011b). Instead, the trophic activities and immunomodulatory actions of MSCs have been suggested to mediate up to 80% of the therapeutic effects seen during MSC treatment (Caplan and Dennis, 2006). They achieve this through secretion of a diverse range of bioactive factors, including cytokines (e.g. IL-1β, IL-6), chemokines (e.g. IL-8, MCP-1), growth factors (e.g. NGF, transforming growth factor-β (TGF-β)) and angiogenic factors (e.g. VEGF), which can act in both autocrine and paracrine manners (Hofer and Tuan, 2016). This action allows MSCs to guide the fate of other cell types in the local environment, including cells of the immune response and local tissue cells of the cartilage and bone (Kusuma *et al.*, 2017). Therefore, harnessing MSC paracrine activity is being widely investigated for consideration of OA repair strategies (Liang *et al.*, 2014).

3.1.2 Effect of the inflammatory environment on MSCs

As the fate of MSCs is influenced by its local microenvironment, the presence of an inflammatory environment, like that seen in OA, has the capacity to alter the intrinsic biological functions of these cells (Park *et al.*, 2009). These

alterations could be beneficial through the promotion of an immunosuppressive MSC response. Here, identification of specific factors that interact with MSCs, and the understanding of their subsequent effects on cellular signalling pathways, would allow for exploitation of MSCs for improved therapeutic outcomes (Kusuma *et al.*, 2017). Alternatively, these alterations could be detrimental to the functions of MSCs, and in fact endogenous MSCs in the OA joint have been shown to present with an altered phenotype and differentiation capacity, which may allow them to augment OA progression (Coleman *et al.*, 2010; Wyles *et al.*, 2015). Therefore, identification of cellular interactions that alter the functions of MSCs, including their immunomodulatory capacity, may help to prevent these cells from contributing to the pathogenesis of the disease.

3.1.3 Aims and hypotheses

The general aims of the study presented in this chapter was to identify the effect of inflammatory mediators, known to contribute to OA pathology, on the proliferation, viability and gene expression profile of ihBMSCs during *in vitro* culture. An immortalised human cell line was chosen as this cell type has been widely used in the literature, and would therefore allow for both direct comparison to and expansion of the current knowledge and understanding in this cell type.

To achieve these aims, ihBMSCs were cultured in standard medium (SM) supplemented with inflammatory mediators, and the proliferative capacity, viability and gene expression profile of these cells was assessed, the latter by the use of TaqMan Low Density Arrays. Based on the existing literature, it was hypothesised that pro-inflammatory mediators and growth factors (IL-1β, IL-8, MCP-1, NGF) would stimulate the expression of immunomodulatory genes

(Redondo-Castro *et al.*, 2017) as well as genes involved in catabolic processes (Harigai *et al.*, 1993). On the other hand, it was hypothesised that anti-inflammatory mediators (IL-10) could counteract the altered expression profile caused by pro-inflammatory mediators (Choi *et al.*, 2008; Dhingra *et al.*, 2015).

3.2 Methods

For more detailed methods describing MSC culture and mediator exposure please see Chapter 2 Materials and Methods. Figure **3.1** provides a schematic of the experimental design.

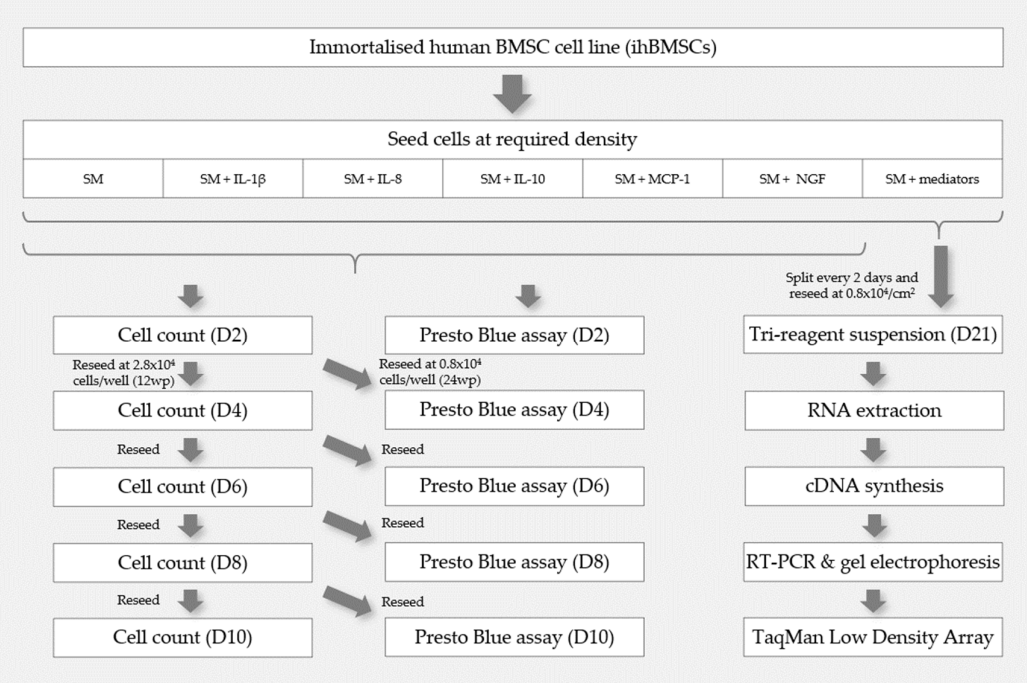


Figure 3.1 Schematic of the exposure experimental design

3.2.1 Cell proliferation

ihBMSCs were seeded onto 12-well plates (Grenier Bio-One) at a density of $2.8x10^4$ cells/well and incubated at 37°C with 5% CO_2. After 48 hours, the cells were viewed using an Eclipse TS100 inverted microscope (Nikon) in order to assess morphology, and the cells were passaged and counted using the Countess II FL (ThermoFisher) automated system in order to assess proliferation. Cells were re-seeded at $2.8x10^4$ cells/well and incubated again for 48 hours, whereby the process was repeated until day 10.

3.2.2 Cell viability

To determine the viability of ihBMSCs, the resazurin based reagent PrestoBlue, was utilised. In this assay, the reducing potential of metabolically active cells causes the PrestoBlue reagent to be modified into a fluorescent form, thereby allowing for the quantification of viable cells (Xu, McCanna and Sivak, 2015). ihBMSCs were seeded into 24-well plates (ThermoFisher) at a density of $0.8x10^4$ cells/well and incubated at 37°C with 5% CO_2. After 48 hours, the cells were washed 3x with PBS and incubated with 500µl 10% (v/v) PrestoBlue (Invitrogen) diluted in SM for 30 minutes at 37°C, 5% CO_2. After this time 100µl media was transferred to a 96-well plate (ThermoFisher) in triplicate and read using an Infinite 200 Pro plate reader and i-control software (Tecan), with an excitation wavelength of 560nm and emission wavelength of 590nm. Cells were re-seeded from the concurrent cell proliferation assay at $0.8x10^4$ cells/well and incubated again for 48 hours, whereby the process was repeated until day 10.

3.3.3 TaqMan Low Density Arrays

TLDAs (ThermoFisher) were used to measure changes in gene expression of ihBMSCs during *in vitro* culture. For more detailed methods on cDNA preparation and validation, and TLDAs please see Chapter 2 Materials and Methods.

Briefly, ihBMSCs were cultured in SM, SM + IL-1β, SM + IL-8, SM + IL-10, SM + MCP-1, SM + NGF, SM + all mediators for 21 days, after which time cells were washed twice with PBS and resuspended in Tri-reagent (Sigma Aldrich). RNA was subsequently extracted from the cells using a RNeasy Micro Kit (Qiagen), according to manufacturer's instructions. The RNA concentration and purity were then assessed using a Nanodrop ND-1000 spectrophotometer

(Nanodrop Technologies, USA) and, following this, cDNA was synthesised from 2μg total RNA using a High-Capacity RNA-to-cDNA Kit (ThermoFisher), according to manufacturer's instructions. Successful cDNA synthesis was confirmed by RT-PCR and gel electrophoresis, upon which 384-well microfluid TaqMan array cards were loaded with cDNA and run on the 7900HT Fast-Real Time PCR System using SDS RQ Manager Software (Applied Biosystems). The generated cycle threshold (Ct) values were normalised to the geometric mean of beta-actin (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which were identified as the most stable reference genes. The data was then corrected for baseline and the $\log_2$ fold change from SM plotted using GraphPad Prism 7 (GraphPad Software, San Diego, CA). A Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's test for post hoc multiple comparisons.

3.3 Results

3.3.1 Effects of inflammatory mediators on ihBMSC proliferation

Initial studies investigated the effects of inflammatory mediators (IL-1β, IL-8, IL-10, MCP-1, NGF) on the proliferative capacity of ihBMSCs during *in vitro* culture. Each of these mediators were added to SM at 3 different concentrations (See Table **2.1**) to allow for any dose response effects. Effects of these treatments were assessed through cell counts every 2 days, for a total of 10 days (Figure **3.2**).

None of the tested inflammatory mediators affected the proliferative capacity of the cells compared to SM. This lack of effect was apparent in all of the doses used over a 10 day culture protocol.

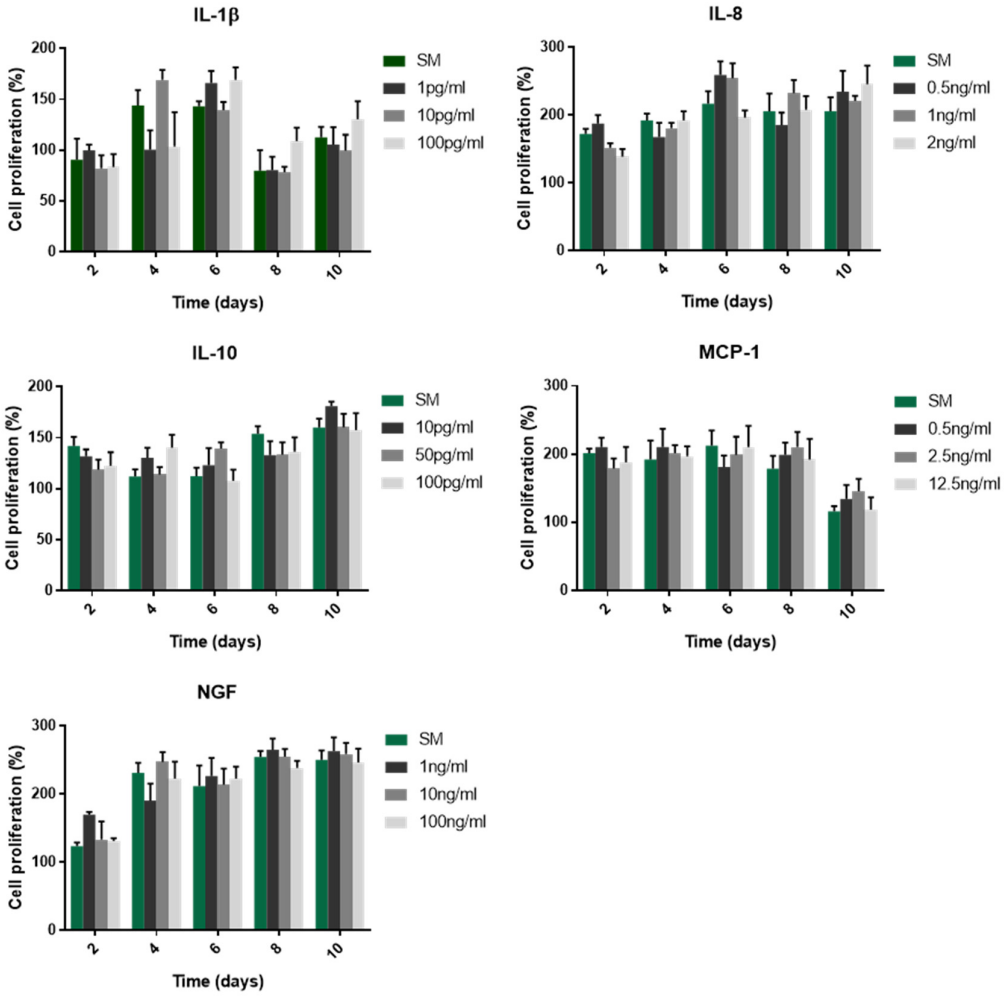


Figure 3.2 Cell proliferation of ihBMSCs in response to inflammatory mediators

Cell proliferation of ihBMSCs was measured through cell counts every 2 days, for a total of 10 days. A Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The presence of inflammatory mediators caused no significant effect on the proliferative capacity of the cells over a 10 day period, at any dose used. n=4 technical replicates, * $p<0.05$. Error bars represent SEM.

3.3.2 Effects of inflammatory mediators on ihBMSC viability

To investigate the effects of inflammatory mediators on the viability of ihBMSCs during *in vitro* culture, cells were exposed to 3 doses of each of the mediators (IL-1β, IL-8, IL-10, MCP-1, NGF) (See Table **2.1**). Effects of these treatments were assessed through a Presto Blue viability assay every 2 days, for a total of 10 days (Figure **3.3**).

None of the tested inflammatory mediators affected the proliferative capacity of the cells compared to SM. This lack of effect was apparent in all of the doses used over a 10 day culture protocol.

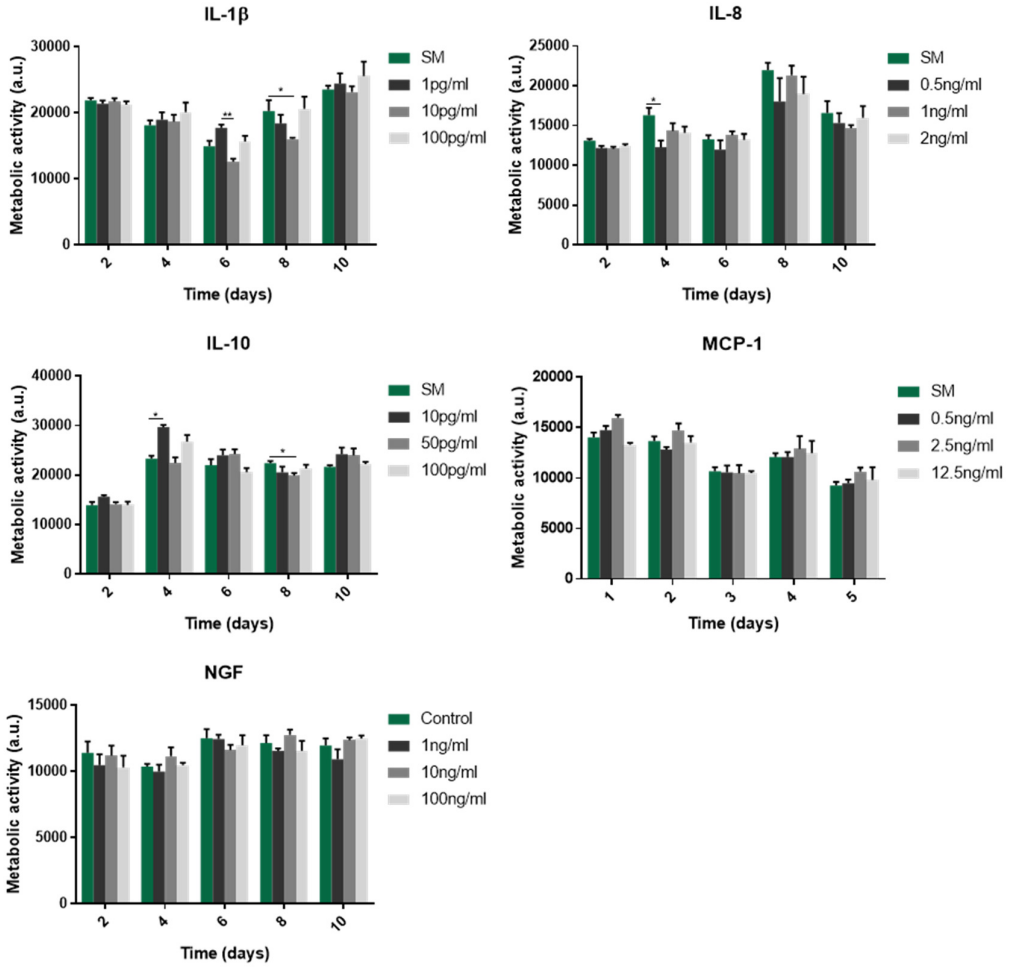


Figure 3.3 Viability of ihBMSCs in response to inflammatory mediators

ihBMSC viability was measured using a Presto Blue Assay every 2 days, for a total of 10 days. A Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The presence of inflammatory mediators caused no significant effect on the viability of the cells over a 10 day period, at any dose used. n=4 technical replicates, * $p<0.05$. Error bars represent SEM.

3.3.3 Effects of inflammatory mediators on ihBMSC gene expression

As no effect of inflammatory mediators could be observed on the proliferative capacity of ihBMSCs, nor their viability, the highest dose mediator was taken forward for further experiments (See Table **2.2**). To identify the effects of inflammatory mediators on ihBMSCs, the gene expression profile of these cells was analysed after 21 days exposure. The genes analysed in this study are described in Table **2.3**.

3.3.3.1 Inflammatory mediators and their receptors

Expression of genes encoding for inflammatory mediators used in this study were analysed, along with their receptors, to assess the autocrine effects of these mediators on ihBMSCs (Figure **3.4** and Figure **3.5**).

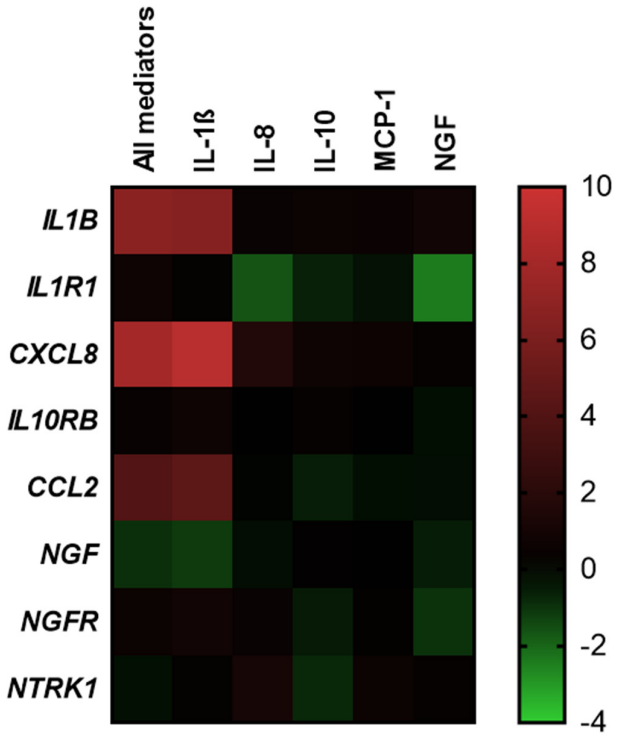


Figure 3.4 Heatmap of inflammatory mediator and receptor gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of inflammatory mediators and their receptors following ihBMSC inflammatory mediator exposure were calculated and displayed as $\log_2$ fold change relative to SM. Expression of genes encoding for pro-inflammatory mediators (*IL1B*, *CXCL8*, *CCL2*) were significantly increased by the presence of combined inflammatory mediators and IL-1β alone.

Expression of genes encoding for inflammatory mediators used in this study were primarily affected by the presence of combined inflammatory mediators and IL-1β alone. Here, these two treatment groups increased expression of each of the pro-inflammatory mediators measured: *IL1B*, *CXCL8* and *CCL2*. *CXCL8* saw the biggest change, as combined inflammatory mediators caused a $\log_2$ fold change of 7.96 in ihBMSCs, whilst IL-1β alone caused a larger $\log_2$ fold change of 9.02 compared to SM. Next, *IL1B* expression was increased

following exposure to combined inflammatory mediators by a 6.72 log_2 fold change, and treatment with IL-1β alone caused a 6.50 log_2 fold change from SM. Expression of *CCL2* was also significantly increased by these treatment conditions, as seen by a log_2 fold change of 3.97 in medium containing combined mediators and a log_2 fold change of 4.46 in medium containing IL-1β alone. In contrast expression of *NGF* was significantly decreased by the presence of IL-1β alone, though this was measured as a 0.56 log_2 fold change.

Changes in the gene expression levels of receptors of the studied inflammatory mediators went largely unchanged, as seen in *IL10RB* and *NTRK1*. However, the presence of IL-1β prevented a significant decrease in both *IL1R1* and *NGFR* expression when compared to NGF treatment. None of the other individually added mediators appeared to significantly alter levels of gene expression compared to SM, and expression of *CXCR1* and *CCR2* could not be detected in this array.

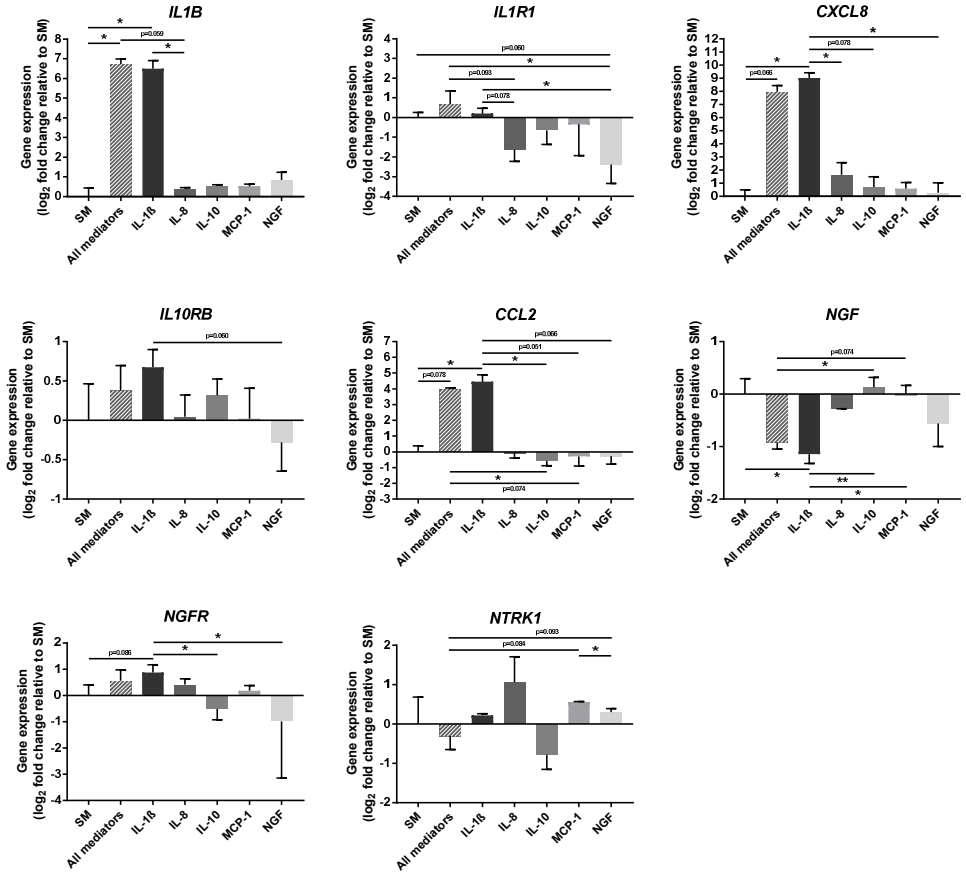


Figure 3.5 Gene expression of inflammatory mediators and their receptors following ihBMSC inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for inflammatory mediators and their receptors were analysed by TLDAs following 21 days exposure. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *IL1B* was significantly increased in ihBMSCs by the presence of combined inflammatory mediators ($p=0.026$) and IL-1β alone ($p=0.013$). Furthermore, expression of *CXCL8* ($p=0.013$) and *CCL2* ($p=0.049$) were also significantly increased in response to IL-1β treatment, and this increased trend was also observed in ihBMSCs treated with combined inflammatory mediators. n=3 (all mediators, IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates, * $p<0.05$. Error bars represent SEM.

3.3.3.2 Osteogenic differentiation markers

Expression of genes encoding for osteogenic differentiation markers were analysed to assess the effects of inflammatory mediators on ihBMSCs during *in vitro* culture (Figure **3.6** and Figure **3.7**).

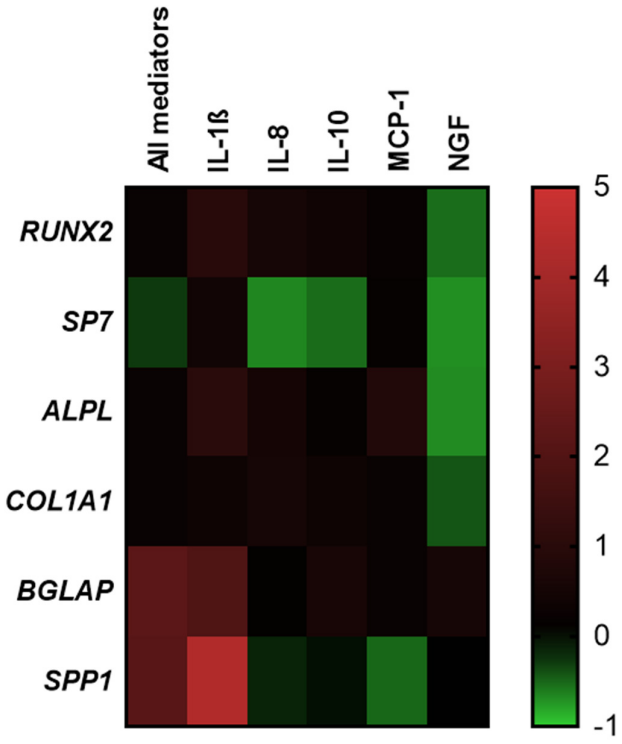


Figure 3.6 Heatmap of osteogenic marker gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of osteogenic markers following ihBMSC inflammatory mediator exposure were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *RUNX2* and *ALPL* was increased following IL-1β treatment, whilst *BGLAP* and *SPP1* expression was increased by combined inflammatory mediators as well as IL-1β alone.

The treatment of ihBMSCs with IL-1β increased gene expression of a number of osteogenic markers. This included *RUNX2*, which showed a $\log_2$ fold change of 0.96 compared to SM, and *ALPL*, which showed a $\log_2$ fold change of 1.02. A larger increase was observed in both *BGLAP* and *SPP1* expression following IL-1β treatment, with a 1.90 and 4.30 $\log_2$ fold change respectively. The presence of combined inflammatory mediators also increased expression of *BGLAP* (2.22 $\log_2$ fold change) and *SPP1* (2.14 $\log_2$ fold change) compared to SM, though the latter did not display such a marked effect compared to IL-1β alone. ihBMSC expression of *SP7* and *COL1A1* was unchanged by the presence of inflammatory mediators.

None of the other individually added mediators appeared to significantly alter levels of gene expression compared to SM, though it was noted that NGF treatment caused a small decreased trend in many of the analysed genes (*RUNX2, SP7, ALPL* and *COL1A1)*. Expression of *IBSP* could not be detected in this array.

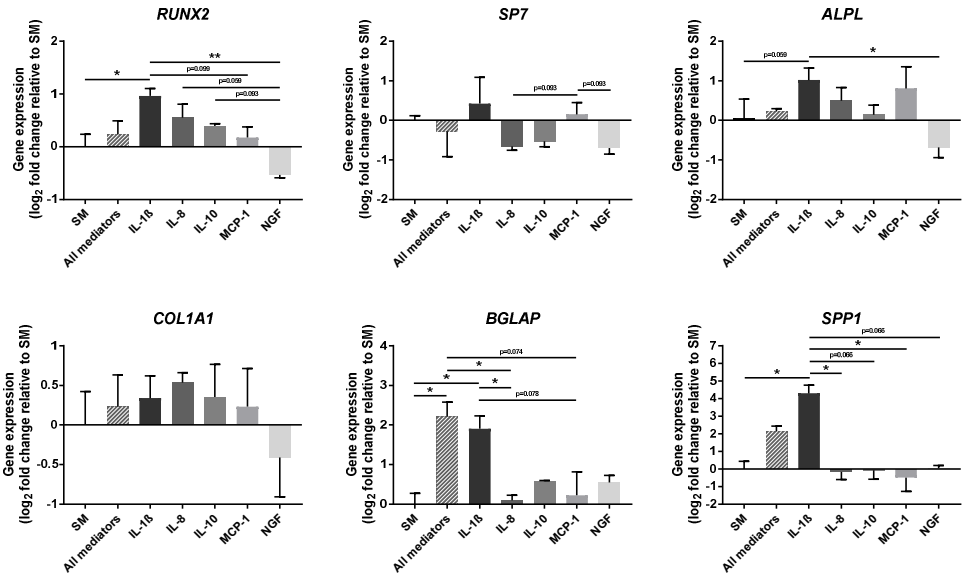


Figure 3.7 Gene expression of osteogenic markers following ihBMSC inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for osteogenic markers were analysed by TLDAs following 21 days exposure. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *RUNX2* was significantly increased by ihBMSC treatment with IL-1β ($p=0.021$) n=3 (all mediators, IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

3.3.3.3 Chondrogenic differentiation markers

Expression of genes encoding for chondrogenic differentiation markers were analysed to assess the effects of inflammatory mediators on ihBMSCs during *in vitro* culture (Figure **3.8** and Figure **3.9**).

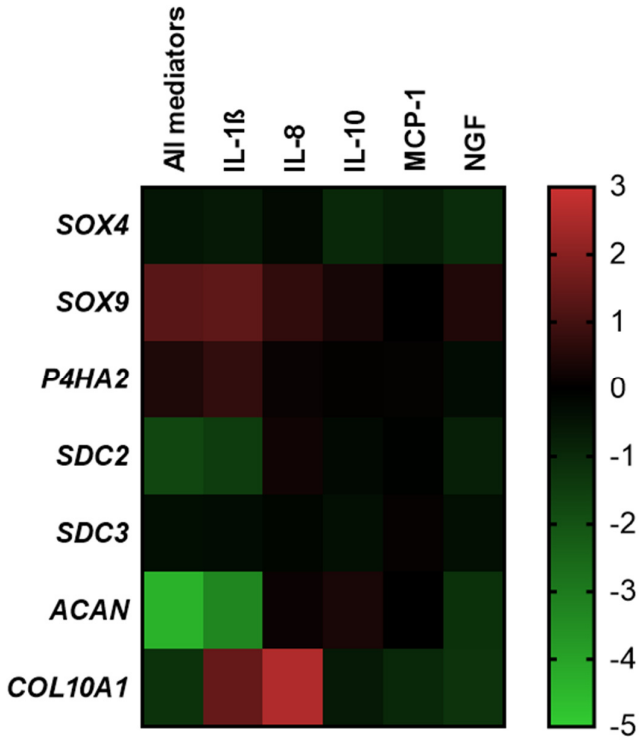


Figure 3.8 Heatmap of chondrogenic marker gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of chondrogenic markers following ihBMSC inflammatory mediator exposure were calculated and displayed as $\log_2$ fold change relative to SM. Treatment of IL-1β resulted in an increased expression of *SOX9, P4HA2* and *COL10A1* by ihBMSCs. Combined inflammatory mediator treatment also increased *SOX9* expression, whilst treatment with IL-8 increased levels of *COL10A1*.

Expression of *SOX9* was significantly increased in ihBMSCs following treatment with combined inflammatory mediators (1.28 log_2 fold change) and IL-1β alone (1.39 log_2 fold change). IL-1β also caused a significant increase in expression of *P4HA2* compared to SM alone (0.76 log_2 fold change), and increased expression of *COL10A1* by 1.47 log_2 fold change. *COL10A1* was increased further by the presence of IL-8 alone (2.57 log_2 fold change).

In contrast, expression of *SDC2* and *ACAN* was decreased in the presence of combined inflammatory mediators and IL-1β alone. *SDC2* saw a log_2 fold change of -1.73 compared to SM following exposure to combined inflammatory mediators, and IL-1β caused a log_2 fold change of -1.48. Similarly, *ACAN* expression was decreased by -4.36 log_2 fold change in the presence of combined inflammatory mediators and -3.29 log_2 fold change in the presence of IL-1β alone. Although not significant, NGF showed a decreased trend in expression of these genes.

The expression of *SDC3* and *SOX4* was not significantly affected by the presence of inflammatory mediators. Moreover, none of the other individually added mediators appeared to significantly alter levels of gene expression compared to SM, and *COL2A1* could not be detected by this array.

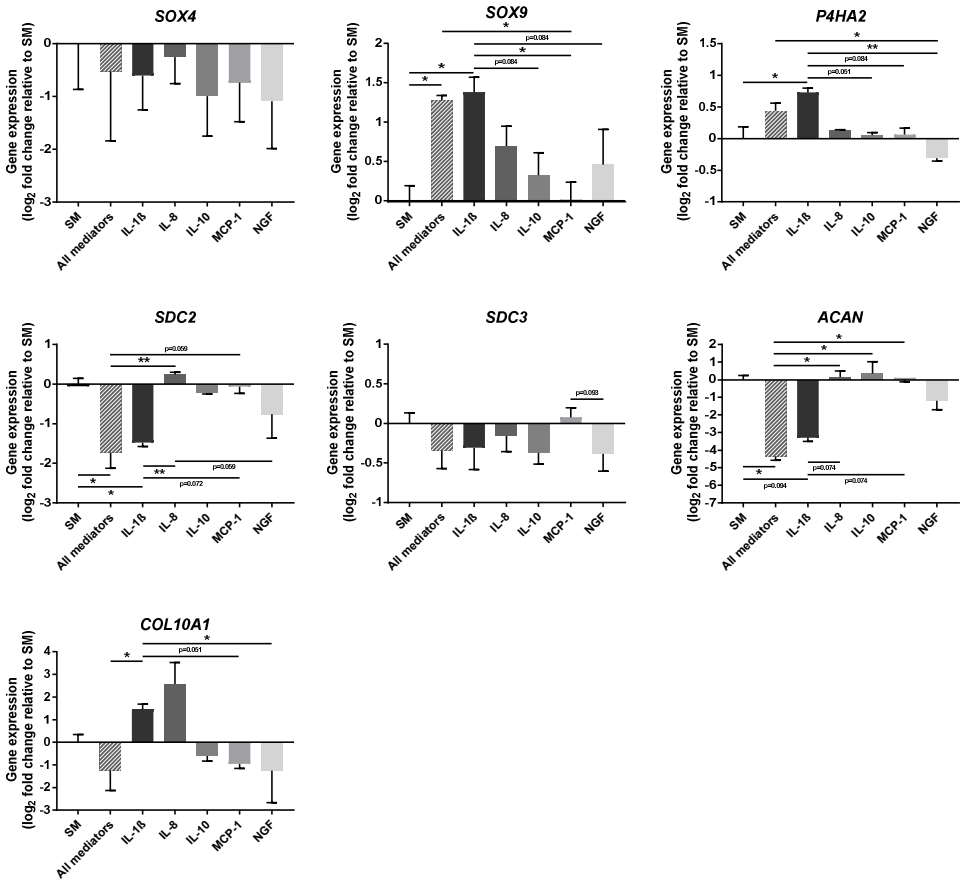


Figure 3.9 Gene expression of chondrogenic markers following ihBMSCs inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for chondrogenic markers were analysed by TLDAs following 21 days exposure. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *SOX9* was significantly increased upon exposure to combined inflammatory mediators (p=0.035) and IL-1β alone (p=0.013). IL-1β also significantly increased the expression of *P4HA2,* and along with IL-8 treatment increased expression of COL10A1 though this was not significant. Expression of *SDC2* was decreased following exposure to combined inflammatory mediators (p=0.035) and IL-1β (p=0.040), in a similar way to expression of *ACAN* (all mediators p=0.031; IL-1β p=0.094). n=3 (all mediators, IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates, * p<0.05, ** p<0.005. Error bars represent SEM.

3.3.3.4 MSC immunomodulatory mediators

Expression of genes encoding for MSC immunomodulatory mediators were analysed to assess the effects of inflammatory mediators on ihBMSCs during *in vitro* culture (Figure **3.10** and Figure **3.11**).

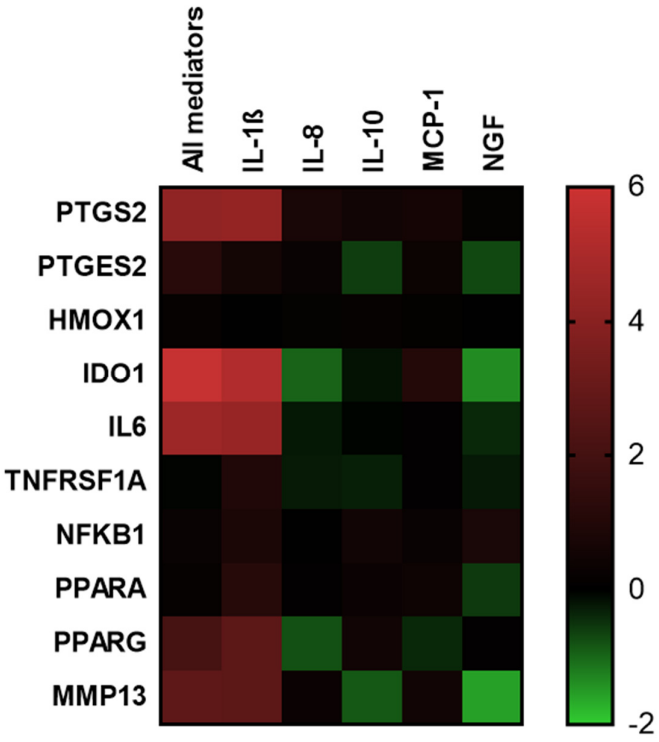


Figure 3.10 Heatmap of immunomodulatory mediator gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of immunomodulatory mediators following ihBMSC inflammatory mediator exposure were calculated and displayed as $\log_2$ fold change relative to SM. Treatment of ihBMSCs with combined inflammatory mediators and IL-1β increased expression of many immunomodulatory genes, including *PTGS2, PTGES2, IDO1, IL6, PPARG* and *MMP13*

Treatment of ihBMSCs with IL-1β increased the expression of all of the analysed immunomodulatory genes, except *HMOX1* which showed unchanged expression following treatment with any inflammatory mediators.

This was observed by a 4.30 log_2 fold increase in *PTGS2*, 5.18 log_2 fold increase in *IDO1*, 4.42 log_2 fold increase in *IL6*, 0.96 log_2 fold increase in *TNFRSF1A*, 0.82 log_2 fold increase in *NFKB1*, 1.10 log_2 fold increase in *PPARA*, and 2.69 log_2 fold increase in both *PPARG* and *MMP13*. In a similar way, combined inflammatory mediators increased many of the genes studied including *PTGS2* (4.2 log_2 fold increase), *IDO1* (5.79 log_2 fold increase), *IL6* (4.58 log_2 fold increase), *PPARG* (2.05 log_2 fold increase) and *MMP13* (2.76 log_2 fold increase), though unlike IL-1β alone expression of *TNFRSF1A*, *NFKB1* and *PPARA* was not observed. None of the other inflammatory mediators appeared to significantly affect expression of these genes compared to SM.

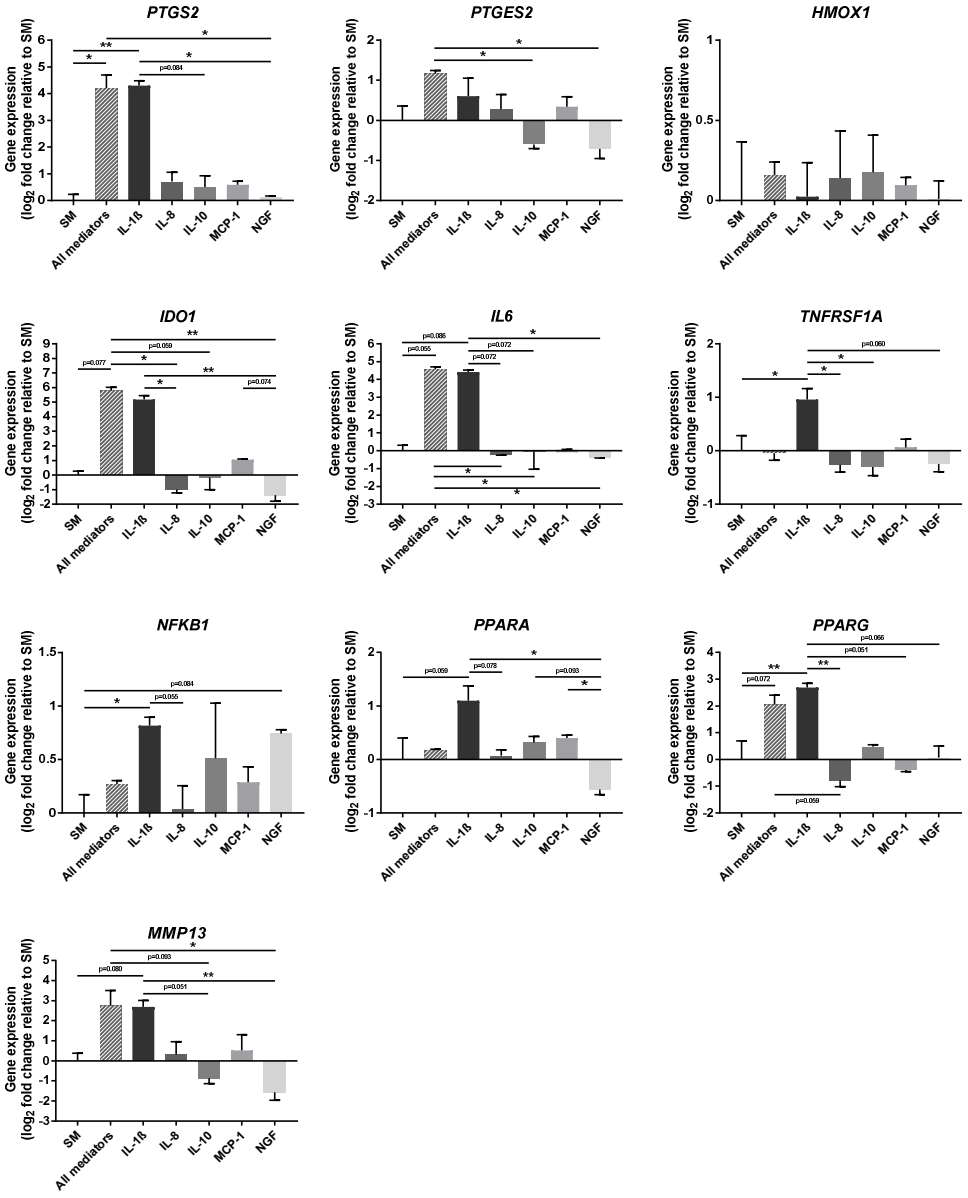


Figure 3.11 Gene expression of immunomodulatory mediators following ihBMSCs inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for immunomodulatory mediators were analysed by TLDAs following 21 days exposure. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of all immunomodulatory markers were increased during exposure to IL-1β alone, except *HMOX1.* The same trend was observed during exposure to combined inflammatory mediators, though they did not increase expression of

TNFRSF1A, NFKB1 or *PPARA*. n=3 (all mediators, IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

3.3.3.5 Growth factors and receptors

Expression of genes encoding for growth factors and their receptors were analysed to assess the effects of inflammatory mediators on ihBMSCs during *in vitro* culture (Figure **3.12** and Figure **3.13**).

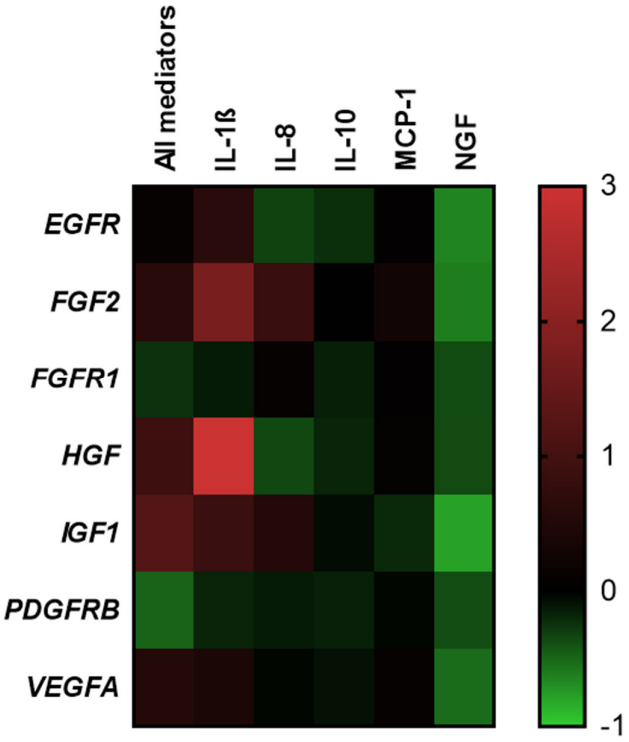


Figure 3.12 Heatmap of growth factor and receptor gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of growth factors and their receptors following ihBMSC inflammatory mediator exposure were calculated and displayed as $\log_2$ fold change relative to SM. Treatment of ihBMSCs with IL-1β primarily increased expression of *FGF2* and *HGF* after 21 days, but slight increases in *EGFR* and *IGF1* were seen. Combined inflammatory mediators also resulted in an increased trend of growth factor expression.

Expression of *FGF2* (1.76 log_2 fold change) and *HGF* (2.96 log_2 fold change) was significantly increased in ihBMSCs following 21 days exposure to IL-1β. This cytokine also caused an observed increased trend in *EGFR* and *IGF1*, though this was not significant. An increased trend could also be observed in combined inflammatory mediator treatments when measuring *HGF* and *IGF1*, but was not significant compared to SM.

The expression of *FGFR1*, *PDGFRB* and *VEGFA* was not significantly affected by the presence of inflammatory mediators. Moreover, none of the other individually added mediators appeared to significantly alter levels of gene expression compared to SM. However, interestingly, treatment with NGF caused decreased expression in each of the genes analysed compared to SM, although significance could not be detected.

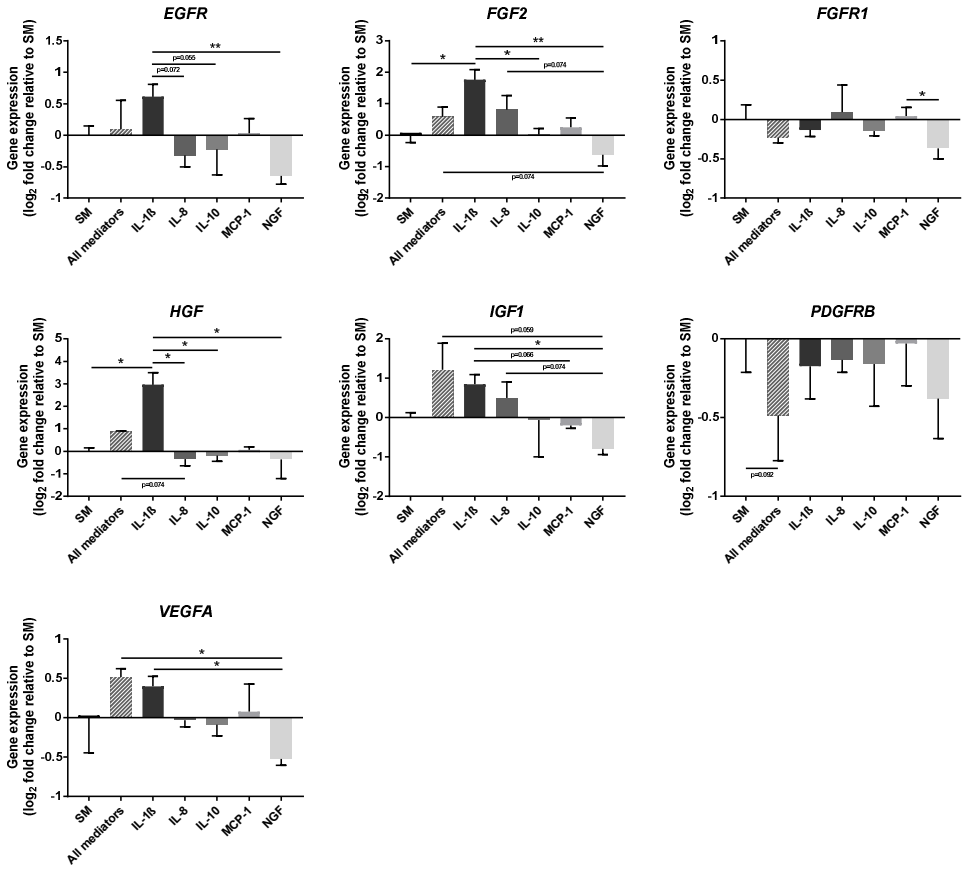


Figure 3.13 Gene expression of growth factors and their receptors following ihBMSCs inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for growth factors and their receptors were analysed by TLDAs following 21 days exposure. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. ihBMSC expression of *FGF2* (p=0.026) and *HGF* (p=0.032) was significantly increased following treatment with IL-1β. n=3 (all mediators, IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

3.3.3.6 TGF-β/BMP signalling

Expression of genes encoding for members of the TGF-β/bone morphogenetic protein (BMP) signalling pathways were analysed to assess the effects of inflammatory mediators on ihBMSCs during *in vitro* culture (Figure **3.14** and Figure **3.15**).

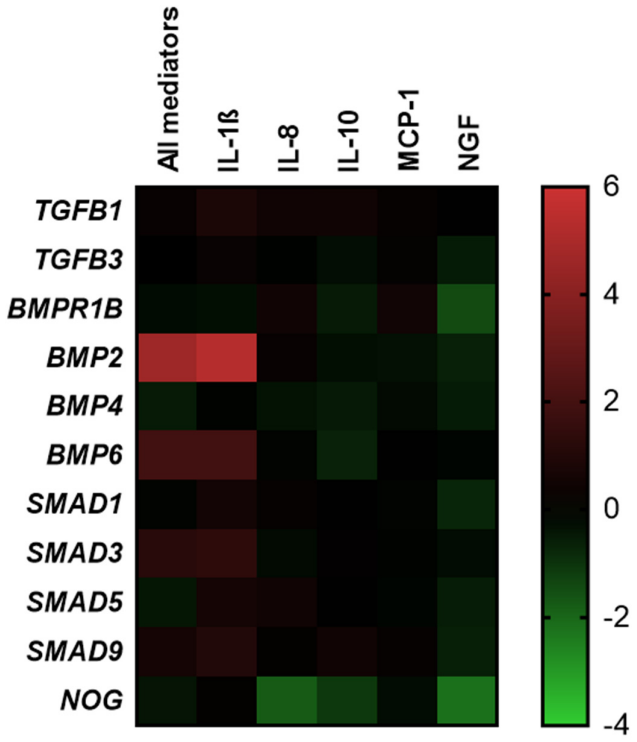


Figure 3.14 Heatmap of TGF-β/BMP signalling gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of TGF-β/BMP signalling molecules following ihBMSC inflammatory mediator exposure were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *BMP2, BMP6, SMAD3* was increased in the presence of IL-1β and combined inflammatory mediators, whilst *TGFB1* and *SMAD9* were increased in the presence of IL-1β alone.

Expression of *BMP2* was increased in the presence of IL-1β and combined inflammatory mediators following 21 days exposure with a 5.30 log_2 fold change and 4.62 log_2 fold change respectively. In a similar way expression of *BMP6* was increased by both IL-1β and combined inflammatory mediators by a log_2 fold change of 1.89, whilst expression of *SMAD3* was increased by both of these conditions (1.32 and 1.15 log_2 fold change respectively). However, the presence of IL-1β alone further significantly increased expression of *TGFB1* with a log_2 fold change of 0.81 from SM, as well as caused an increased trend in expression of *SMAD1, SMAD5* and *SMAD9.* NGF significantly decreased expression of *NOG,* but none of the other inflammatory mediators appeared to cause a significant change in expression of these genes.

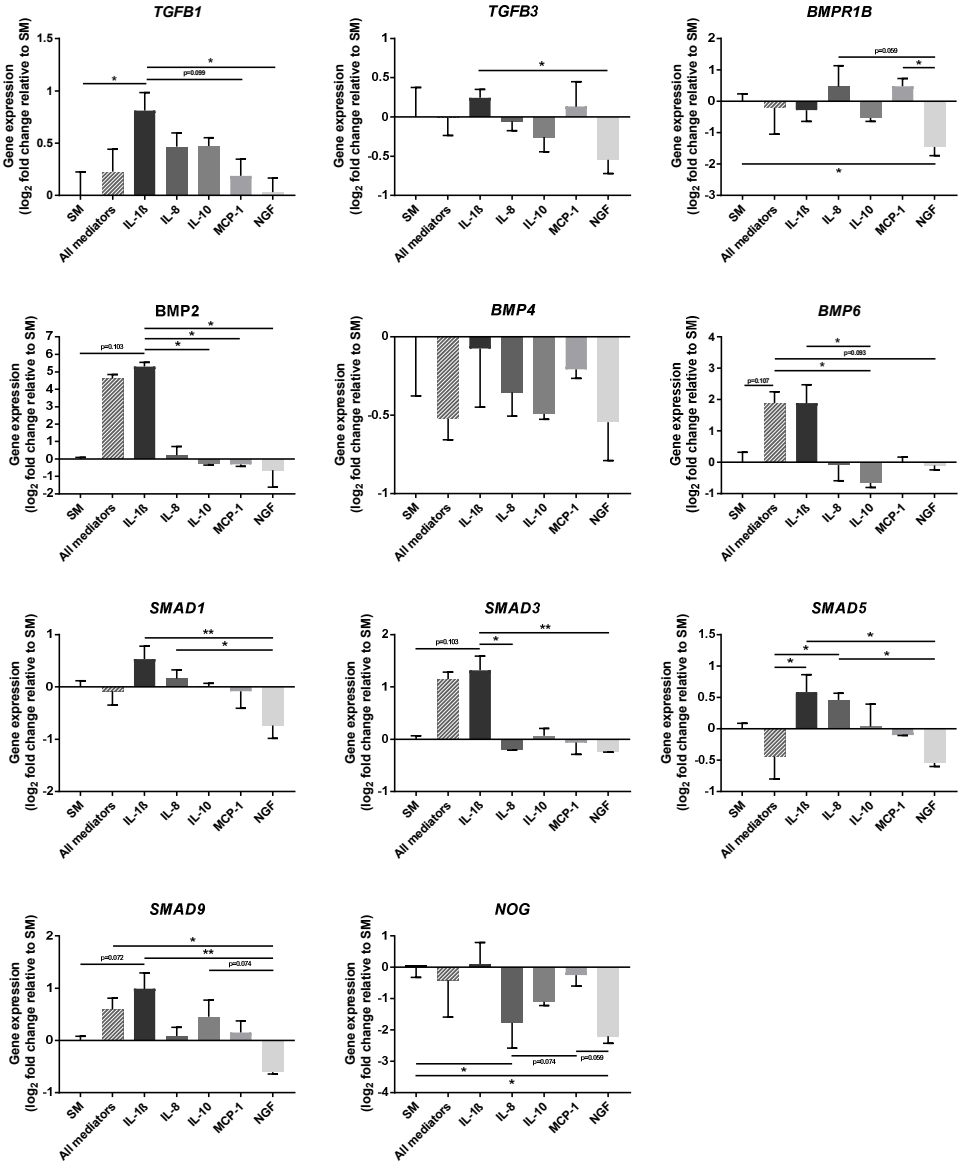


Figure 3.15 Gene expression of TGF-β/BMP signalling molecules following ihBMSCs inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for TGF-β/BMP signalling molecules were analysed by TLDAs following 21 days exposure. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of combined inflammatory mediators and IL-1β alone caused an increase in expression of *BMP2*, *BMP6* and *SMAD3*, however only IL-1β alone caused an increase in expression of *TGFB1*, *SMAD1*, *SMAD5* and *SMAD9*. n=3 (all mediators,

IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates, * p<0.05, ** p<0.005. Error bars represent SEM.

3.3.3.7 MAPK signalling

Expression of genes encoding for members of the mitogen-activated protein kinase (MAPK) signalling pathways were analysed to assess the effects of inflammatory mediators on ihBMSCs during *in vitro* culture (Figure **3.16** and Figure **3.17**).

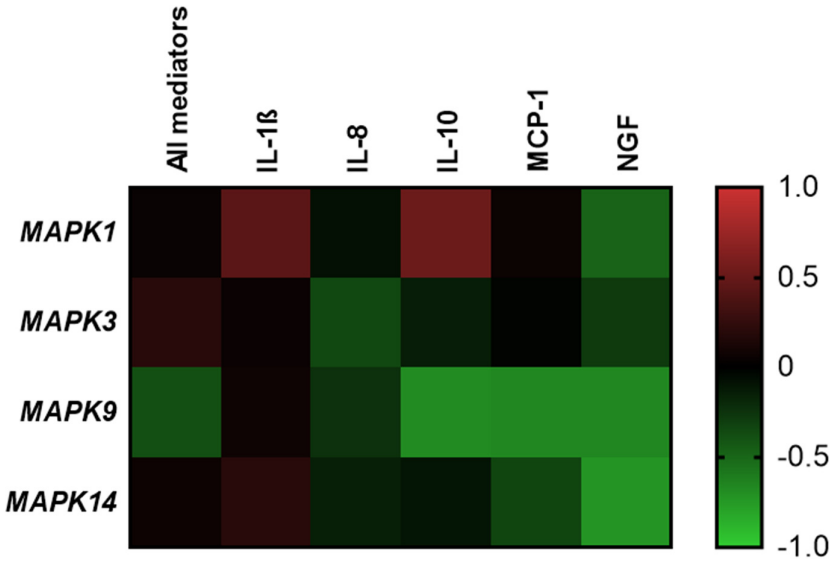


Figure 3.16 Heatmap of MAPK signalling gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of MAPK signalling molecules following ihBMSC inflammatory mediator exposure were calculated and displayed as $\log_2$ fold change relative to SM. Exposure of ihBMSCs to IL-1β and IL-10 caused a slight increase in *MAPK1* expression, whilst IL-10, MCP-1 and NGF caused a noticeable decrease in *MAPK9* expression.

Expression of genes encoding for MAPK molecules were largely unaffected by the presence of inflammatory mediators, although IL-1β and IL-10 caused a slight increase in *MAPK1* expression with a log_2 fold change of 0.44 and 0.52 from SM respectively. Expression of *MAPK9* was slightly decreased (-0.67 log_2 fold change) in the presence of IL-10, MCP-1 and NGF. Expression of *MAPK3* and *MAPK14* were not significantly affected by the presence of inflammatory mediators.

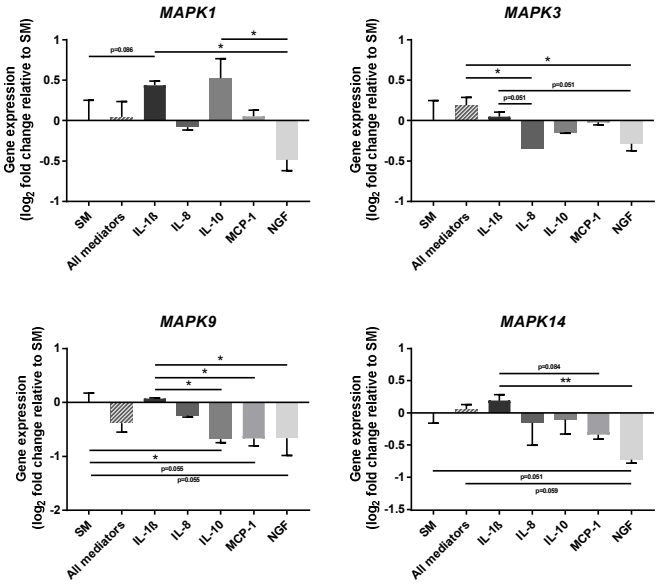


Figure 3.17 Gene expression of MAPK signalling molecules following ihBMSCs inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for MAPK signalling molecules were analysed by TLDAs following 21 days exposure. Results were reported as log_2 fold change relative to SM and a Kruskal Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The presence of IL-1β and IL-10 caused a slight increase in expression of *MAPK1* compared to SM, though this was not significant. Moreover, IL-10, MCP-1 and NGF caused a decrease in expression of *MAPK9,* with IL-10 being significant compared to SM (p=0.042). n=3 (all mediators, IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates,
* p<0.05, ** p<0.005. Error bars represent SEM.

3.3.3.8 Wnt signalling

Expression of genes encoding for members of the Wnt signalling pathways were analysed to assess the effects of inflammatory mediators on ihBMSCs during *in vitro* culture (Figure **3.18** and Figure **3.19**).

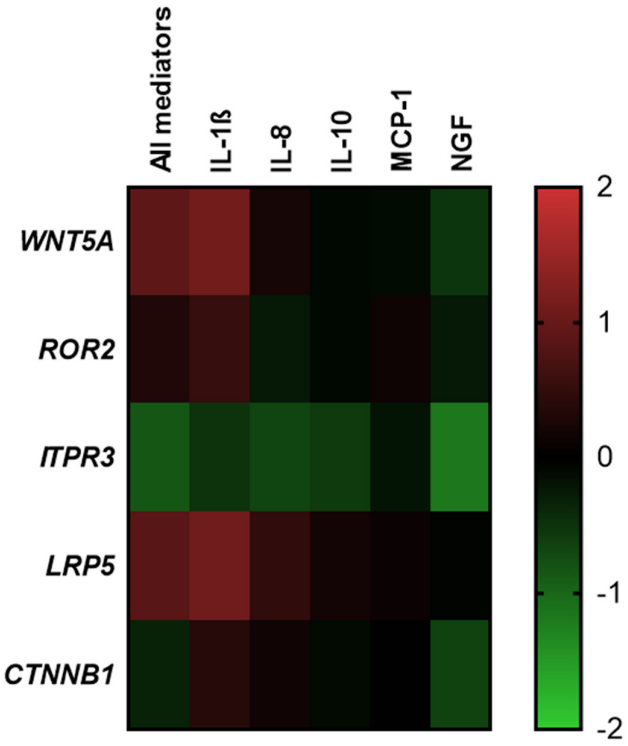


Figure 3.18 Heatmap of Wnt signalling gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of Wnt signalling molecules following ihBMSC inflammatory mediator exposure were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *WNT5A, ROR2, LRP5* and *CTNNB1* was increased by the presence of IL-1β, whilst combined inflammatory mediators increased expression of *WNT5A* and *LRP5.*

The presence of IL-1β alone increased expression of a number of Wnt signalling molecules compared to SM, including *WNT5A* (1.11 $\log_2$ fold change), *ROR2* (0.53 $\log_2$ fold change), *LRP5* (1.08 $\log_2$ fold change) and

CTNNB1 (0.37 log_2 fold change). However, its presence with other combined inflammatory mediators reduced this effect and increases could mainly be observed in *WNT5A* (0.91 log_2 fold change) and *LRP5* (0.86 log_2 fold change). Expression of *ITPR3* showed a decreased trend in expression that was significant in the presence of combined inflammatory mediators (-0.84 log_2 fold change) and NGF (-1.17 log_2 fold change). None of the other inflammatory mediators appeared to significantly affect expression of these genes compared to SM.

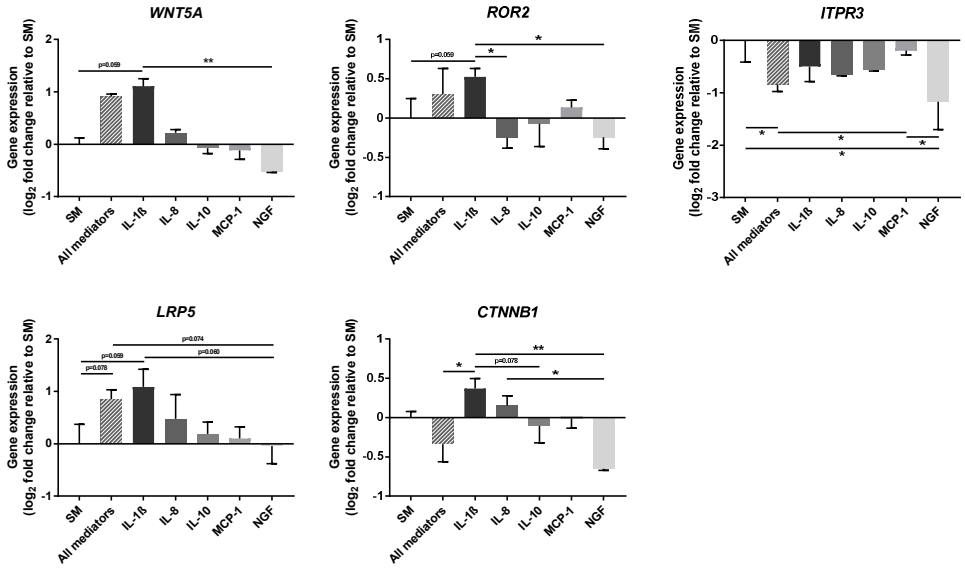


Figure 3.19 Gene expression of Wnt signalling molecules following ihBMSCs inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for Wnt signalling molecules were analysed by TLDAs following 21 days exposure. Results were reported as log_2 fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *WNT5A, ROR2, LRP5* and *CTNNB1* were all increased in the presence of IL-1β compared to SM, but these were not significant. n=3 (all mediators, IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

3.3.3.9 Toll like receptor signalling

Expression of genes encoding for TLRs were analysed to assess the effects of inflammatory mediators on ihBMSCs during *in vitro* culture (Figure **3.20** and Figure **3.21**).

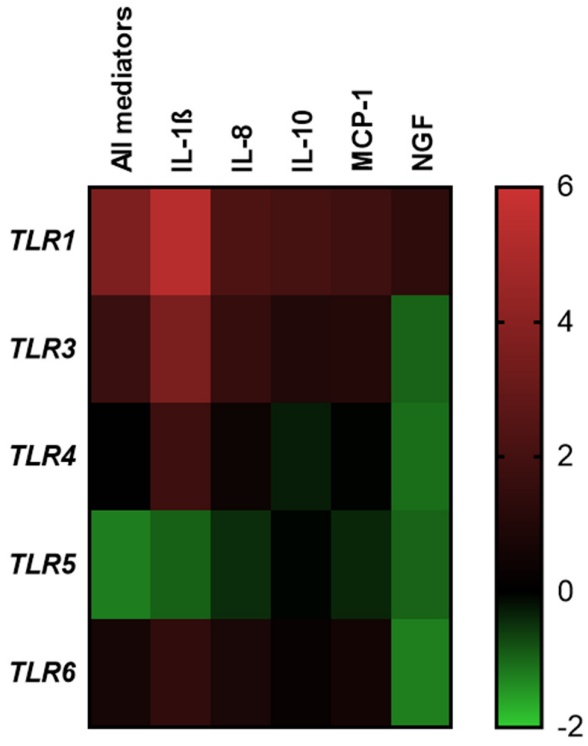


Figure 3.20 Heatmap of TLR signalling gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of TLR signalling molecules following ihBMSC inflammatory mediator exposure were calculated and displayed as log_2 fold change relative to SM. Expression of *TLR1, TLR4, TLR4* and *TLR6* were all increased following ihBMSC exposure to IL-1β. However, combined inflammatory mediators only saw an increase in *TLR1* expression.

Expression if TLRs was significantly increased in ihBMSCs following 21 days culture with IL-1β alone. This effect of IL-1β was most prominent in *TLR1* and *TLR3* in which gene expression levels were increased with a 5.35 log_2 fold change and 3.59 log_2 fold change from SM respectively. IL-1β also increased

expression of *TLR4* with a $\log_2$ fold change of 1.76 from SM, and *TLR6* with a 1.39 $\log_2$ fold change. In contrast, combined inflammatory mediators only significantly increased the expression of *TLR1*, as seen by a $\log_2$ fold change of 3.64 from SM. Expression of *TLR5* was significantly decreased in the presence of combined inflammatory mediators and individual exposure to IL-1β and NGF. None of the other inflammatory mediators appeared to significantly affect expression of these genes compared to SM.

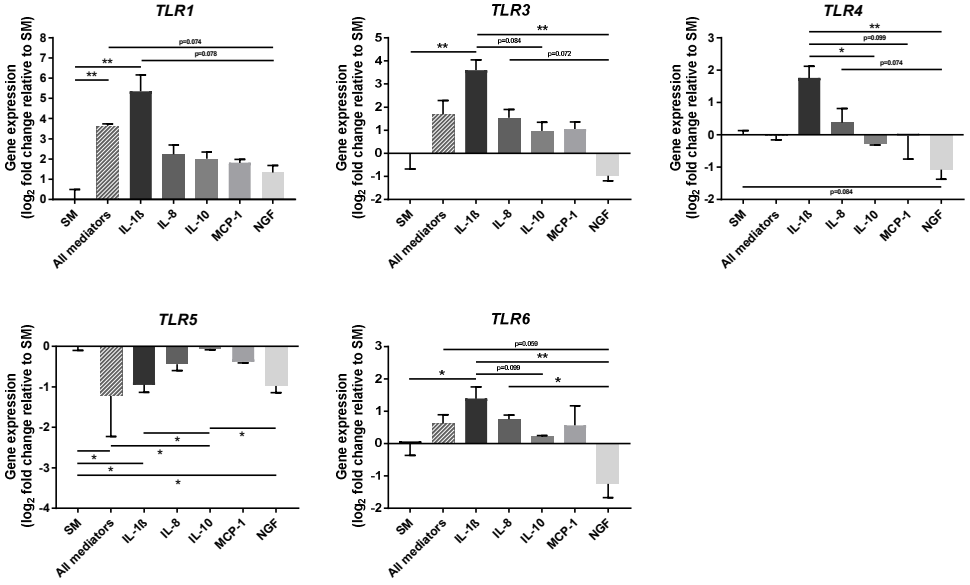


Figure 3.21 Gene expression of TLR signalling molecules following ihBMSCs inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for TLR signalling molecules were analysed by TLDAs following 21 days exposure. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The expression of *TLR1, TLR3, TLR4* and *TLR6* were all increased in the presence of IL-1β, however combined inflammatory mediators only saw the significant increase in *TLR1* expression. n=3 (all mediators, IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

3.3.3.10 Other signalling pathways

Expression of genes encoding for members of other signalling pathways of interest were analysed to assess the effects of inflammatory mediators on ihBMSCs during *in vitro* culture (Figure **3.22** and Figure **3.23**).

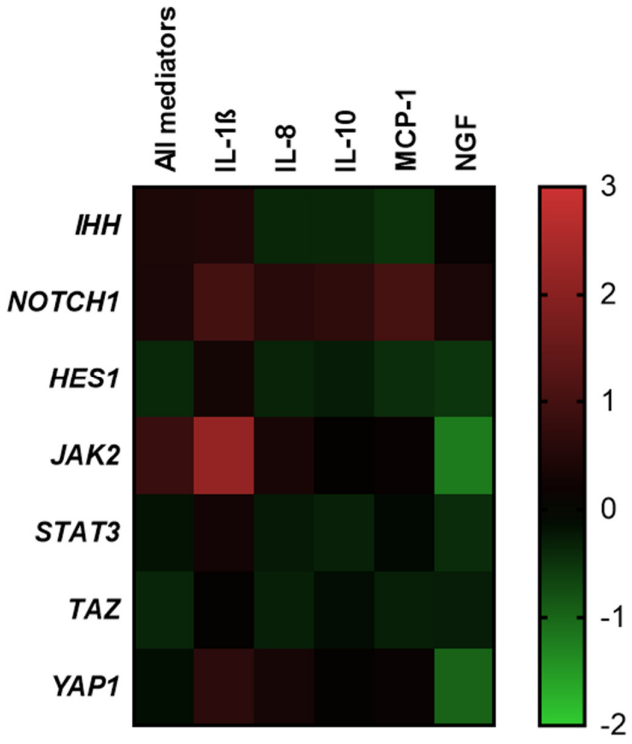


Figure 3.22 Heatmap of other signalling gene expression levels following ihBMSC inflammatory mediator exposure

Gene expression levels of other signalling molecules of interest following ihBMSC inflammatory mediator exposure were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *JAK2* and *YAP1* were increased in the presence of IL-1β alone.

Expression of *JAK2* was significantly increased (2.16 log_2 fold change) in ihBMSCs following 21 days exposure to IL-1β, whilst the presence of combined inflammatory mediators did not cause this effect. Moreover, expression of *YAP1* was slightly increased by the presence of IL-1β (log_2 fold change 0.62). Expression of *NOTCH1* was not significantly affected by any of the inflammatory mediators though it did show an increased trend in expression. Expression of *IHH*, *HES1*, *STAT3*, *TAZ* and *YAP1* was not significantly affected by the presence of inflammatory mediators. None of the other inflammatory mediators appeared to significantly affect expression of these genes compared to SM.

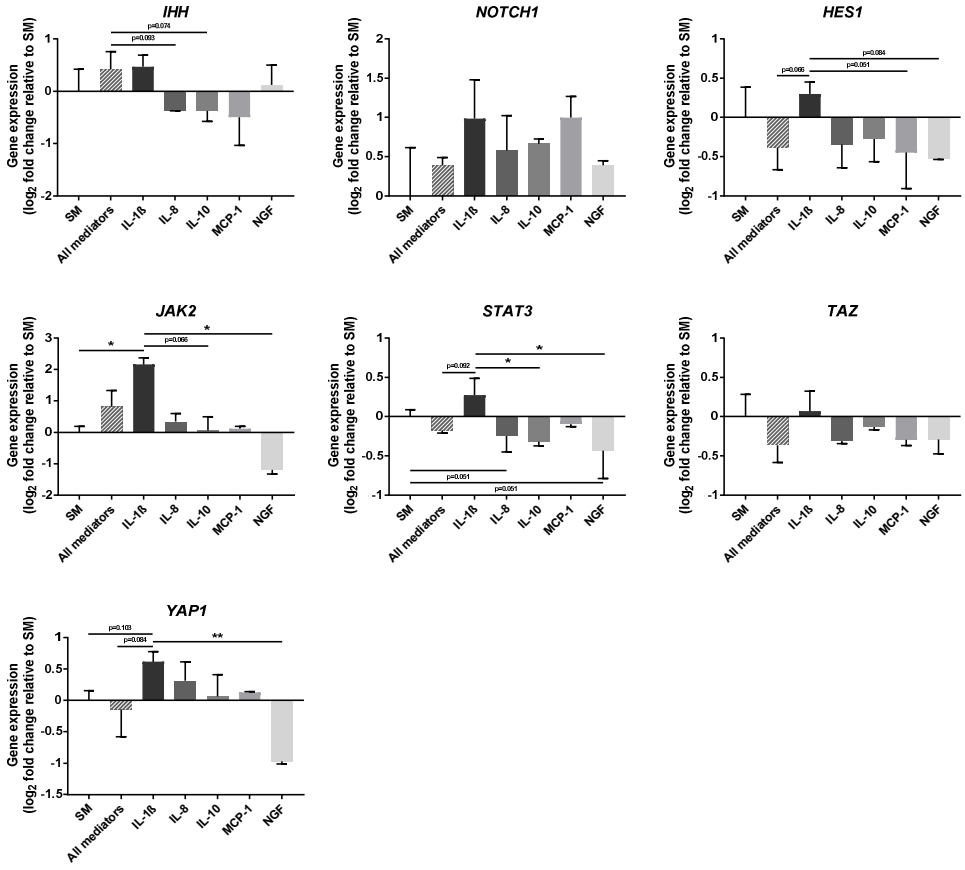


Figure 3.23 Gene expression of other signalling molecules following ihBMSCs inflammatory mediator exposure

To assess the effects of inflammatory mediators on the ihBMSC gene profile, the expression of genes encoding for other signalling molecules of interest were analysed by TLDAs following 21 days exposure. Results were reported as $\log_2$ fold change relative to SM and a Kruskal Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *JAK2* was significantly increased in the presence of IL-1β, whilst *YAP1* expression showed an increased trend during IL-1β exposure. n=3 (all mediators, IL-1β), n=2 (IL-8, IL-10, MCP-1, NGF) biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

3.4 Discussion

As the fate of MSCs is influenced by its local microenvironment, the presence of an inflammatory environment, like that seen in OA, has the capacity to alter the intrinsic biological functions of these cells (Park *et al.*, 2009). Identification of cellular interactions that alter the functions of MSCs, including their immunomodulatory capacity, may help to promote the use of MSCs for OA therapy and reduce the likelihood that they could contribute to disease.

To contribute to the expanse of knowledge in the field of MSC paracrine activity, this study aimed to identify the effects of specific inflammatory mediators on MSCs during standard *in vitro* culture. The mediators assessed in this study are each found to be upregulated in the osteoarthritic joint and contribute to OA pathology. Importantly, this research aimed to extend findings in the literature by analysing the effects of a combined range of pro- and anti-inflammatory mediators, to better represent conditions found in the osteoarthritic joint. To measure this, the effects on cell proliferation and viability were ascertained to identify if these mediators resulted in apoptosis that would prevent transplanted MSCs from exerting their effects. Moreover, a more specific look at the effects of these mediators on the transcriptional regulation of ihBMSCs was undertaken via gene expression analysis, to identify changes to cell signalling pathways and the potential shifts in the secretion profile of these cells.

3.4.1 Effects of inflammatory mediators on ihBMSC proliferation and viability

Initial studies assessed the effects of osteoarthritic inflammatory mediators on the proliferative capacity and viability of ihBMSCs *in vitro*. The data presented

here showed that neither ihBMSC proliferation nor viability was affected by the presence of IL-1β, IL-8, IL-10, MCP-1 or NGF, and therefore suggests that these particular mediators might not trigger apoptosis of the cells upon transplantation into an inflammatory environment. However, these results are limited by a short time course of 10 days, and would be strengthened by analysis over a longer time period.

3.4.2 Effects of inflammatory mediators on gene expression of ihBMSCs during *in vitro* culture

Results presented in this chapter primarily illustrated the role of IL-1β in modulation of the ihBMSC gene expression profile. Interestingly, this pro-inflammatory cytokine was the only tested mediator that not only affected gene expression across a range of inflammatory and immunomodulatory factors, but also stimulated its autocrine activity, as indicated by increased expression of *IL1B*. Importantly, results from this study highlighted a lack of observable effects of IL-8, IL-10, MCP-1 and NGF on the gene expression profile of ihBMSCs.

3.4.2.1 Inflammatory mediators

Inflammatory cytokines are considered central mediators of OA pathogenesis, as they can exert their effects on a wide range of cells in the joint, influencing cellular signalling pathways that result in autocrine production of the mediators themselves and secretion of destructive enzymes. MSCs naturally secrete inflammatory mediators such as IL-1β, IL-6, IL-8 and MCP-1 (Park *et al.*, 2009), and it has been hypothesised that these mediators form a cytokine network that contributes to the pathogenesis of inflammatory joint diseases (Harigai *et al.*, 1993). Results from this study substantiate this hypothesis for an OA model, as IL-1β induction of ihBMSCs resulted in the significant

increase in expression of each of these mediators. This was observed in treatment conditions that included IL-1β alone, or in combination with other inflammatory mediators.

Along with TNF-α, IFN-γ and IL-1α, IL-1β is a key mediator of OA pathogenesis, stimulating catabolic events and contributing towards the observed inflammatory response of OA (Wojdasiewicz, Poniatowski and Szukiewicz, 2014). The primary effects of this cytokine have been reported in cartilage catabolism, a process in which it contributes two-fold. Firstly, it enhances production of MMPs and aggrecanases that stimulate the breakdown of cartilage (Koshy *et al.*, 2002), and secondly it prevents recovery of this degradation through interference of both type II collagen and aggrecan synthesis (Stöve *et al.*, 2000; Shakibaei *et al.*, 2005). Both of these effects could be observed in the treated ihBMSCs of this experiment, as *MMP13* expression was increased, whilst *ACAN* expression was decreased. Moreover, this cytokine is reported to act in an autocrine manner to stimulate its own synthesis, whilst also promoting production of other pro-inflammatory factors such as IL-6 and IL-8 (Wojdasiewicz, Poniatowski and Szukiewicz, 2014). Again, results presented here corroborated these findings as expression of *IL1B*, *IL6* and *CXCL8* were all significantly increased in the treatment conditions containing IL-1β, thereby suggesting that these MSCs may be able to participate in the destructive processes of OA following exposure to IL-1β. This is particularly important considering this cytokines widespread presence in the osteoarthritic joint, as it has been detected in the cartilage, subchondral bone, SF and synovial membrane (Farahat *et al.*, 1993; Melchiorri *et al.*, 1998; Massicotte *et al.*, 2002).

In a similar way to the increased expression of *IL1B*, the increased expression of *IL6* by IL-1β treatment further signified that these MSCs have the potential

to exert destructive OA functions. IL-6 is considered a predictive marker for disease development as its concentration in SF and serum has been correlated with intensity of OA lesions (Livshits *et al.*, 2009; Stannus *et al.*, 2010). Along with its ability to stimulate production of cartilage degrading enzymes and reduce synthesis of ECM proteins (Rowan *et al.*, 2001; Porée *et al.*, 2008; Sui *et al.*, 2009), it is important in stem cell proliferation and protection against apoptosis (Pricola *et al.*, 2009), and thus may help to promote survival of these cells in the inflammatory environment. It has also been observed to have detrimental effects on the subchondral bone through formation of bone resorbing osteoclasts, suggesting that these MSCs may have an enhanced potential to alter bone remodelling in the osteoarthritic joint (Huh and Lee, 2013).

Moreover, IL-8 functions as a mitogenic and angiogenic factor (Xie, 2001), and its pro-angiogenic nature has been investigated by Hou *et al.,* (2014) who showed that this chemokine enhances the angiogenic potential of human BMSCs through the increased production of VEGF; in part via the MAPK/extracellular signal-regulated kinases (ERK) pathway. Although data presented here showed an increased trend in expression of *MAPK1* and *VEGFA* following IL-1β treatment, it was not significant, indicating that increased production of IL-8 did not enhance the pro-angiogenic nature of ihBMSCs directly. However, this may be a limitation of the assay as culture medium was continually replaced throughout the 21-day treatment, thereby causing removal of any secreted mediators and limiting their actions on the cells. Moreover, the increased change in *VEGFA* expression observed by Hou *et al.,* (2014) was seen with 100ng/ml IL-8 treatment, which is considerably higher than both the concentration used in this study and of that found in the OA joint, indicating that physiological levels of IL-8 may not promote such a response *in vivo*. Although IL-8 did not appear to directly enhance the

angiogenic potential of these ihBMSCs, it could be contributing to the increased expression of *MMP13* detected in these cells (Borzi *et al.*, 2014).

Finally, the other inflammatory mediator involved in this hypothesised MSC cytokine network is MCP-1, and gene expression of this chemoattractant protein was also increased in response to IL-1β and combined inflammatory mediator exposure. Like the other described mediators this chemokine may be contributing to the increased expression of *MMP13*, as it has been linked to both increased *MMP3* and *MMP13* expression (Xu *et al.*, 2015).

3.4.2.2 MSC immunomodulatory mediators

Although the increased expression of pro-inflammatory cytokines is linked to initiation of catabolic effects, these mediators can also be involved in supporting the immunomodulatory function of MSCs. For example, IL-6 is involved in the generation of anti-inflammatory M2 macrophages (Najar *et al.*, 2016). This result is highlighted by the increased gene expression of immunomodulatory genes in response to treatment with IL-1β and combined inflammatory mediators, corroborating findings by Redondo-Castrol *et al.*, (2017) who showed that pre-treatment of MSCs with IL-1β increased expression of trophic factors, thereby allowing for the induction of an immunosuppressive environment. It is hypothesised that MSCs have to be primed by specific inflammatory mediators in order to exert their immunomodulatory effects (DelaRosa, Dalemans and Lombardo, 2012), therefore it is interesting to note that the only mediator that was able to stimulate gene expression in ihBMSCs was IL-1β. This suggests that other studied inflammatory mediators might not be involved in the induction of MSC immunosuppressive effects, and individual exploitation of these mediators may not improve these cells' immunomodulatory functions.

Prostaglandin E2 (PGE_2) is one of the primary factors in MSC-mediated immunomodulation, as its effects have been linked to many cells involved in the immune response. This includes its action on NK cell suppression, DC maturation inhibition, B cell proliferation and development of T regulatory cells, which all culminate into the establishment of an immunosuppressive environment (Zhao, Ren and Han, 2016). Although expression of *PGE2* was not assessed in this study, this prostaglandin is generated by cyclooxygenase (COX) enzymes, and in the stress environment the COX-2 isoform is linked to high levels of PGE_2 generation (Zhang *et al.*, 2002). Data presented here showed that the gene encoding for COX-2, *PGTS2*, was increased in the presence of IL-1β and combined inflammatory mediators, substantiating findings that COX-2 can be induced by inflammatory cytokines (Arikawa, Omura and Morita, 2004). The upregulation of a number of genes in this study are postulated to engage in a synergistic relationship in order to promote their continued expression. For example, PGE_2 is known to upregulate production of cytokines such as IL-6, which positively regulates COX-2 production and thereby PGE_2 in a positive feedback loop (Arikawa, Omura and Morita, 2004; Najar *et al.*, 2016). In contrast to the catabolic effects of IL-6 previously described, this cytokine has also been linked to regulation of the immune response as it is involved in the generation of anti-inflammatory M2 macrophages (Najar *et al.*, 2016), which subsequently contribute to the inhibition of T cells. However, both COX-2 and IL-6 are linked to pain in OA (Laine *et al.*, 2008; Lin *et al.*, 2017). Importantly, this indicates that ihBMSCs exposed to IL-1β may enhance pain through release of these factors, thereby contributing to the prevailing symptom of OA.

Another key mediator of MSC driven immunomodulation is indoleamine 2,3-dioxygenase (IDO). Expression of this mediator by MSCs can be induced by inflammatory mediators such as IFN-γ, TNF-α and IL-1β. Results presented

here validated findings that IL-1β stimulates expression of this gene. In a similar way to PGE_2 and COX-2 this enzyme exerts its immunosuppressive effects in a number of ways, including T-cell suppression through direct actions or indirectly through generation of anti-inflammatory M2 macrophages (François *et al.*, 2012; Kyurkchiev, 2014). Another mediator of T cell suppression is hepatocyte growth factor (HGF), which can exert its effects with TGF-β (Nauta and Fibbe, 2007). Results presented here show that genes encoding for both of these growth factors are upregulated in response to IL-1β treatment, highlighting the generation of a number of immunomodulatory factors that form complex interactions to modulate the immune response.

3.4.2.3 Toll like receptor signalling

Expression of TLRs by ihBMSCs following culture with inflammatory mediators showed that individual exposure of IL-1β increased expression of these genes. Part of the innate immune system, TLRs have paradoxically been identified to contribute to both immune suppression (Opitz *et al.*, 2009) and inflammation-induced catabolism (Gómez *et al.*, 2014). This is evidenced by studies that show that TLRs are important for the induction of *IDO1* (Opitz *et al.*, 2009), but in contrast, the generation of signalling cascades that result in the induction of inflammatory mediators, such as pro-inflammatory cytokines and chemokines (DelaRosa, Dalemans and Lombardo, 2012). In particular, IL-6 and IL-8 have been identified as two mediators increased in response to TLR activation. Interestingly, both *IL6* and *IL8* expression was significantly increased in response to IL-1β treatments, suggesting that TLR activation may in part account for the generation of these inflammatory mediators.

These seemingly counterintuitive mechanisms of immune regulation have been explained by MSC priming studies that identify that the type of TLR

depends on the response (Waterman *et al.*, 2010). TLR-4 results in induction of pro-inflammatory cytokines that can activate T cells, whilst TLR-3 activation results in the well-known immunosuppression properties of MSCs as genes for anti-inflammatory mediators such as IDO, PGE_2, TGF-β and HGF are expressed, and these can inhibit T cell proliferation (Waterman *et al.*, 2010). Part of this anti-inflammatory effect of TLR-3 stimulation is reported to be mediated through the JAK2/STAT1 pathway as Tomchuck *et al.*, (2012) reported this to be involved in expression of suppressor of cytokine signalling proteins. Interestingly, the array presented in this chapter revealed that *JAK2* expression was increased in ihBMSCs in response to IL-1β only, in a similar way to *TLR3*. This suggests that the IL-1β mediated induction of *TLR3* could result in subsequent signalling through the JAK/STAT pathway.

Interestingly, ihBMSCs cultured with combined inflammatory mediators showed altered gene expression of TLRs as they either dampened the response (*TLR1*) or the effect appeared to be lost (*TLR3, TLR4, TLR6*). This suggests that other mediators are able to counteract the effects of IL-1β and so further exploration would be necessary to identify these mechanisms of action.

3.4.3 Conclusions

In conclusion, this work identifies opposing effects of IL-1β on a population of ihBMSCs. On the one hand it stimulates production of inflammatory mediators known to contribute to the pathogenesis of OA. However, on the other it increases expression of key immunomodulatory mediators of ihBMSCs that contribute towards an immunosuppressive environment. Interestingly, IL-1β also preferentially increased expression of genes involved in bone remodelling and thus it could be hypothesised that IL-1β might be priming these ihBMSCs towards differentiation into the osteoblast lineage.

Work by Sonomoto *et al.,* (2012) has shown that IL-1β can induce osteogenesis, and this hypothesis would fit with the knowledge that MSCs from the osteoarthritic joint have an altered capacity to differentiate that favours osteogenesis (Murphy *et al.*, 2002). If MSCs are transplanted into the osteoarthritic joint with a view to regenerate cartilage, this effect may hinder effective outcomes.

Furthermore, the expression profile of ihBMSCs treated individually with IL-1β differs from the profile of ihBMSCs treated with a combination of inflammatory mediators. In particular, this was seen for expression of genes encoding for TLRs. This provides new evidence that although individual exposure of IL-1β *in vitro* might produce significant effects on the gene expression of ihBMSCs, an environment in which combined inflammatory mediators can work synergistically and antagonistically allows for differential gene expression. Further investigation into the mediators counteracting these effects should be undertaken as it may begin to identify therapeutic targets for the prevention of IL-1β mediated catabolic events. A summary of the key changes in ihBMSC gene expression during exposure to combined inflammatory mediators and IL-1β alone can be observed in Table **3.1**.

	SM + mediators	SM + IL-1β
Gene	Fold change (log_2)	Fold change (log_2)
Pro-inflammatory mediators		
IL1B	6.72	6.50
CXCL8	7.96	9.02
CCL2	3.97	4.46
IL6	4.58	4.42
MMP13	2.76	2.69
Immunomodulatory mediators		
PTGS2	4.20	4.30
IDO1	5.79	5.18
HGF	0.88	2.96
TGFB1	0.23	0.81
MSC differentiation markers		
ACAN	-4.36	-3.29
RUNX2	0.24	0.96
ALPL	0.23	1.02
BGLAP	2.22	1.90
SPP1	2.14	4.30
Toll like receptor signalling		
TLR1	3.64	5.35
TLR3	1.69	3.59
TLR4	-0.01	1.76
TLR6	0.62	1.39

Table 3.1 Key changes in ihBMSC gene expression during exposure to inflammatory mediators

Exposure of ihBMSCs to combined inflammatory mediators and IL-1β alone resulted in significant changes to ihBMSC gene expression. Shown as the log_2 fold change from SM, genes encoding for pro-inflammatory mediators, immunomodulatory mediators, TLRs and MSC differentiation markers associated with bone remodelling were significantly increased following exposure to these conditions. In contrast, the expression of *ACAN* was significantly decreased compared to SM. Furthermore, the gene expression profile of ihBMSCs treated individually with IL-1β was seen to differ to the expression profile of ihBMSCs treated with combined inflammatory mediators. This phenomenon was observed through changes in gene expression for specific TLR signalling molecules, such as *TLR4* and *TLR6*.

Chapter 4 Analysis of the MSC chondrogenic response after exposure to inflammatory mediators

4.1 Introduction

4.1.1 Cartilage in health and disease

4.1.1.1 Articular cartilage homeostasis

Hyaline cartilage, found covering the articular surface of bones (articular cartilage), is a highly specialised tissue that functions to provide a low-friction gliding surface with high compressive strength, thereby preventing high load damage to the joint (Buckwalter and Mankin, 1998; Bhosale and Richardson, 2008). Aneural and avascular in nature, maintenance of articular cartilage is controlled by one resident cell type, chondrocytes (Palfrey and Davies, 1966; Chen, Frenkel and Di Cesare, 1999; Goldring and Marcu, 2009). Derived from mesenchymal progenitor cells and found in low abundance (1-2% total volume) (Fox, Bedi and Rodeo, 2009), these cells exist in a stable, quiescent phase in order to preserve the structural integrity of cartilage (Mariani, Pulsatelli and Facchini, 2014). They achieve this through the synthesis of matrix proteins that form the extracellular matrix (ECM), giving the cartilage its tensile and compressive strength (Bhosale and Richardson, 2008).

The two major components of the ECM produced and secreted by chondrocytes, are collagens and proteoglycans (Fox, Bedi and Rodeo, 2009). Type II collagen is principally found in articular cartilage, comprising 90-95% of the total collagen, however presence of types VI, IX, X and XI can also be detected. The collagen itself provides tensile strength and forms a fibrillar network that entraps proteoglycans. In articular cartilage, the most prevalent

proteoglycan is aggrecan, which is composed of glycosaminoglycan (GAG) subunits bound to a protein core (Bhosale and Richardson, 2008). The turnover of these ECM components by chondrocytes is regulated by a complex network of signalling molecules, and identifying these pathways is particularly important when understanding disruption of cartilage homeostasis in the aged or diseased joint (Mariani, Pulsatelli and Facchini, 2014).

4.1.1.2 Cartilage in the osteoarthritic joint

Once seen as inert, chondrocytes within the cartilage are now known to respond to external factors, such as mechanical injury and presence of inflammatory mediators, thereby altering the cellular mechanisms that regulate their function. In joint disease, such as OA, this chondrocyte response results in an imbalance between the catabolic and anabolic mechanisms that govern the turnover of ECM proteins (Goldring and Marcu, 2009). The net result of this, is breakdown of cartilage, which is fundamentally mediated by the actions of MMPs and aggrecanases (Iannone and Lapadula, 2003).

During OA pathogenesis, the once stable chondrocytes begin to exhibit a transient phenotype, in which cells mature and progress towards terminal differentiation (Singh *et al.*, 2018). This shift is characterised by chondrocyte hypertrophy and subsequent ossification, whereby chondrocytes undergo cell death, allowing for angiogenic invasion and infiltration of cells required for the deposition of bone on the cartilage matrix (Mackie *et al.*, 2008) (Figure **4.1**). During skeletal development and growth, endochondral ossification is essential for the formation of bone, however in the adult joint this process leads to abnormalities such as osteophyte development (van der Kraan and van den Berg, 2007; Van der Kraan and Van den Berg, 2012).

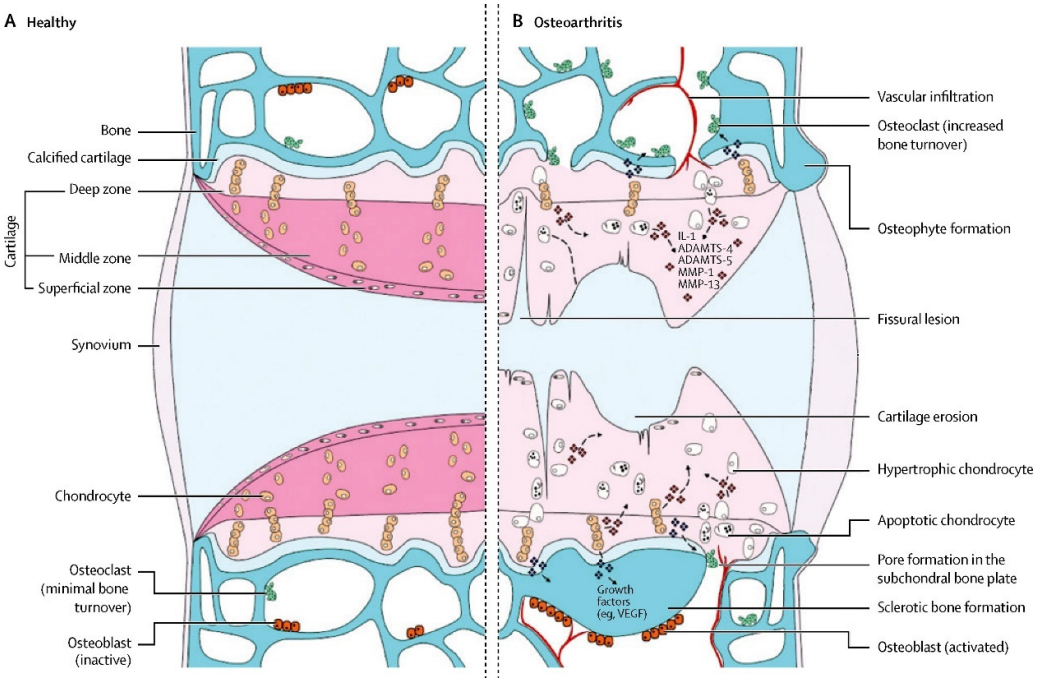


Figure 4.1 Cartilage changes observed in the osteoarthritic joint

In the healthy joint hyaline cartilage covers the articular surface of bones (**A**). Chondrocytes within this specialised tissue help to maintain its structure and function by existing in a stable phase that allows for the synthesis of matrix proteins. However, in response to external factors (such as inflammatory mediators) in the osteoarthritic joint, chondrocytes begin to undergo terminal differentiation, as observed by cell hypertrophy and apoptosis, and begin to release cartilage degrading enzymes such as aggrecanases and MMPs (**B**). This subsequently leads to dysregulation of matrix protein turnover and results in the breakdown of cartilage, characterised by erosion and fissural lesions. These changes also allow for the invasion of blood vessels and the infiltration of cells required for deposition of bone on the cartilage matrix, a process considered to contribute to osteophyte formation (Adapted from Kuyinu *et al.*, 2016).

4.1.1.3 Signalling pathways in cartilage homeostasis and disease

Cartilage homeostasis in healthy adult bone relies on precise regulatory mechanisms that maintains chondrocyte ECM turnover, and prevents chondrocyte hypertrophy and endochondral ossification (Bhosale and Richardson, 2008). Although a lot of research into the individual signalling pathways governing this process has been undertaken, the exact mechanisms

and relationships between them remain unclear, thereby limiting the progress of targeted therapies. To gain a better understanding of these results some of the most widely studied pathways will be explored.

Growth factors in the joint provide one important means of signalling in homeostatic cartilage. For example, the growth factor TGF-β1 is crucial for the development of chondrocytes, and prevention of chondrocyte hypertrophy (Mariani, Pulsatelli and Facchini, 2014). It achieves this via Smad 2-3 signalling, by which TGF-β1 engagement with the ALK5 receptor results in phosphorylation of these Smad proteins for subsequent transcriptional regulation. Animal models have shown that deletion of *Smad3* results in an OA phenotype, characterised by progressive articular cartilage degradation. This occurs in conjunction with increased levels of hypertrophic chondrocytes, highlighting the role of this TGF-β signalling pathway in chondrocyte maintenance (Yang *et al.*, 2001). Moreover, patients with *SMAD3* single nucleotide polymorphisms show an increased frequency of hip and knee OA development (Valdes *et al.*, 2010). In the osteoarthritic joint, however, a shift in TGF-β signalling is observed. Instead, TGF-β1 engagement of the ALK1 receptor initiates activation of the Smad 1-5-8 pathway (Blaney Davidson *et al.*, 2009). This pathway coordinates its effects with the help of the transcription factor runt-related transcription factor 2 (RUNX2), stimulating the hypertrophic differentiation of chondrocytes and production of factors such as Collagen type X, MMP-13, alkaline phosphatase (ALP), osteopontin, osteocalcin and VEGF (van der Kraan, Blaney Davidson and van den Berg, 2010; Hellingman *et al.*, 2011; Mariani, Pulsatelli and Facchini, 2014). One potential cause of this change in signalling is seen to be a switch in TGF-β1 interaction with ALK5 to ALK1, as the former is significantly reduced in both human and mouse OA conditions (Blaney Davidson *et al.*, 2009).

Insulin-like growth factor 1 (IGF-1) has also been linked to maintenance of cartilage integrity. This growth factors presence stimulates chondrocyte proteoglycan synthesis and retains chondrocytes in their quiescent state (Bohme *et al.*, 1992), whilst its absence results in the development of articular cartilage lesions (Ekenstedt *et al.*, 2006). Although it can exert these effects alone, it has been shown to have a functional relationship with TGF-β1, whereby their combined action enhances cartilage production (Fukumoto *et al.*, 2003). Nevertheless, its effects are limited in the osteoarthritic joint, as chondrocytes derived from this environment display a dampened response to IGF-1 (Loeser *et al.*, 2002). In contrast, the roles of fibroblast growth factors (FGFs) remain controversial. Some studies have suggested that FGF2 has a positive regenerative effect through stimulation of ECM protein synthesis (Hiraide *et al.*, 2005; Kaul *et al.*, 2006; Somoza *et al.*, 2014), whilst others have linked it to the acceleration of chondrocyte hypertrophy and subsequent upregulation of cartilage degrading enzymes, such as MMP-13 and aggrecanases (Im *et al.*, 2007; Muddasani *et al.*, 2007). Work by Li *et al.,* has identified that these opposing effects are dependent on species, with FGF2 exerting catabolic effects in human articular cartilage. This highlights the importance of considering species differences when extrapolating findings.

Another important signalling pathway in chondrocyte function is the MAPK signalling cascade, whereby existence of an inverse relationship between p38 and ERK1/2 allows this signalling family to exert its control over chondrocyte homeostasis (Oh *et al.*, 2000). For matrix synthesis, activation of p38 is essential, but a switch to ERK1/2 signalling results in development of a hypertrophic phenotype (Prasadam *et al.*, 2010). This has been succinctly proven by studies that show delayed development of hypertrophy and endochondral ossification upon inhibition of ERK1/2. Importantly, this

inhibition also decreases expression of MMPs that are key mediators of cartilage destruction (Wang *et al.*, 2011).

Roles of other signalling pathways in chondrocyte regulation are continually being uncovered. This includes, but is not limited to, Hedgehog signalling, whereby ligands such as Indian hedgehog (IHH) are expressed by chondrocytes in response to stress, subsequently inducing activation of downstream transcription factors that drive terminal differentiation (Lin *et al.*, 2009). Moreover, TLR expression is increased in OA cartilage and has a positive correlation with severity of the disease. Specifically, TLR-2 and TLR-4 have been identified in areas closest to cartilage lesions (Houard, Goldring and Berenbaum, 2013), and their expression activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent MMP release (Liu-Bryan and Terkeltaub, 2010). As well as regulating expression of MMPs, NF-κB has also been shown to exert its effects on downstream regulators of terminal chondrocyte differentiation, including RUNX2 and β-catenin, an important member of the Wnt signalling family (Mariani, Pulsatelli and Facchini, 2014). This brief overview begins to reveal the multitude of signalling pathways, and their intricate interactions, that govern the complex system of cartilage homeostasis and dysregulation. With this in mind, it is unsurprising to learn that significant shifts in the microenvironment, as seen in diseased states, alters the process and role of chondrocytes.

4.1.2 Cartilage repair therapies

The intrinsic regenerative capacity of cartilage is limited by the low density of local chondrocytes, whilst a lack of vascularisation and innervation in cartilaginous tissue restricts recruitment of progenitor cells to a site of injury (Heng, Cao and Lee, 2004). Therefore, to promote cartilage repair in

degenerative diseases such as OA, it is imperative to explore and utilise cell-based strategies that can circumvent these limitations and provide a population of reparative cells. Tissue engineering has primarily investigated two key approaches: the use of chondrocytes and the use of MSCs.

Autologous chondrocyte implantation is an approved therapeutic option for the treatment of cartilage defects, and is proven to be clinically effective in the short term through reduction of clinical symptoms (NICE, 2017). However, therapies exploiting the use of expanded chondrocytes are problematic two-fold. Firstly, these therapies produce disappointing results due to an inability of these cells to maintain a stable chondrocyte phenotype (Clar *et al.*, 2005). Secondly, isolating chondrocytes requires a tissue biopsy. This results in damage to the healthy cartilage, and is therefore counterintuitive to the aim of treatment (Solchaga, Penick and Welter, 2011). Consequently, an alternative cell-based therapy utilising MSCs has become an attractive candidate for repair.

4.1.2.1 MSCs for cartilage repair

MSCs are an exciting prospect for cartilage repair therapy, as not only can they exert immunomodulatory effects, but they can differentiate into chondrocytes (chondrogenesis) given the right local environment (Kolf, Cho and Tuan, 2007). Current therapeutic options exploiting MSCs for repair includes the stimulation of endogenous MSCs through microfracture techniques (Murawski, Foo and Kennedy, 2010). As articular cartilage does not classically have direct access to a significant source of reparative progenitor cells, this bone marrow stimulation technique creates a passage between the bone marrow and articular space to allow and encourage migration of MSCs to the site of injury. Despite providing direct access to endogenous cells, this

technique results in fibrocartilage formation instead of a hyaline cartilage phenotype (Kreuz *et al.*, 2006). This finding is supported by animal models that have successfully demonstrated differentiation of implanted MSCs *in vivo*, but noted that these cells display calcification, fibrogenesis and hypertrophy, rather than features of hyaline cartilage (Chen *et al.*, 2009).

Together, these findings indicate that the signalling pathways governing chondrogenesis are altered to produce an alternative phenotype. The process of MSC chondrogenic differentiation itself is defined by a series of consecutive stages: MSC proliferation and condensation, chondroprogenitor differentiation, chondrogenesis, hypertrophy and terminal differentiation, and ossification (Chen *et al.*, 2009). In the healthy joint, MSC derived chondrocytes do not progress to the hypertrophy and terminal differentiation stage unless undergoing endochondral ossification; a process only observed in pathogenic conditions following skeletal maturity (Van der Kraan and Van den Berg, 2012). Therefore, the development of a hypertrophic phenotype in reparative therapies would be detrimental to the regeneration of cartilage. For this reason, it is crucial to determine why these cells are terminally differentiating, and how external factors might influence this.

When considering the osteoarthritic joint, there is inappropriate mechanical stimulation and presence of inflammatory mediators, which can both influence the differentiation capacity of MSCs. As the induction and mediation of MSC differentiation is tightly controlled by growth factors and cytokines found in the local environment (Yoo *et al.*, 2011), the investigation into the effect of inflammatory mediators is paramount.

Ultimately, a clearer understanding on the action of ligands and respective cellular signalling will help to overcome the detrimental effects of external

stimuli that result in chondrocyte hypertrophy, as it will allow for development of targeted treatment strategies. Identification of suitable candidates could allow for treatments that increase the reparative potential of MSCs via pre-conditioning, for example through the addition or removal of growth factors. Priming the cells in this way would alter the cell receptor profile and allow for exploitation of intracellular signalling pathways involved in the desired route of chondrogenesis (Somoza *et al.*, 2014).

4.1.3 Chondrogenic differentiation of MSCs *in vitro*

To initiate and assess the differentiation capacity of MSCs *in vitro*, inclusion of lineage specific growth factors such as TGF-β1, dexamethasone, ascorbic acid, sodium pyruvate and insulin-transferrin-selenium (ITS) in serum free culture medium can be adopted (Johnstone *et al.*, 1998; Yoo *et al.*, 1998). 3D culture systems allow for successful chondrogenic differentiation of MSCs, in part due to the provision of cell-to-cell interactions. The use of pellet culture was introduced by Johnstone *et al.,* (1998) and has since become a standard method to study chondrogenic differentiation of MSCs and the signalling pathways that govern this. However, it does not come without its limitations. In particular, the chondrocytes that develop display a fibrocartilage phenotype and hypertrophy is observed (Chen *et al.*, 2015). This indicates that MSCs might have an intrinsic, functional drive towards endochondral ossification, and it is in fact specific environmental conditions that retain the chondrocyte phenotype in the healthy joint. Although efforts have been made to avoid hypertrophy of chondrocytes *in vitro* progress is still ongoing (Zhang *et al.*, 2010; Chen *et al.*, 2015).

Identification of successful MSC chondrogenic differentiation is primarily obtained through the measurement of proteoglycans. This can either be

through histological evaluation techniques, such as Safranin O or Toluidine Blue staining (Schmitz *et al.*, 2010), or through spectrophotometric assays such as the 1,9-dimethylmethylene blue (DMMB) assay (Stone *et al.*, 1994). Allowing for quantification, DMMB is a cationic dye that specifically binds to sulphated GAGs (sGAGs) and is frequently used during assessment of chondrogenic differentiation. Furthermore, gene and protein expression analysis techniques can also be utilised to assess differentiation, as specific markers such as SOX9, aggrecan and collagen type II are increased during this process. This method is also useful for identifying hypertrophy as a different expression profile can be observed, in which collagen type X, MMP-13 and RUNX2 constitute key markers (Van der Kraan and Van den Berg, 2012).

4.1.4 Aims and hypotheses

The general aim of the *in vitro* study presented in this chapter was to identify the effect of inflammatory mediators, found to be upregulated in osteoarthritic tissue, on the chondrogenic differentiation capacity of ihBMSCs. An immortalised human cell line was chosen as this cell type has been widely used in the literature to study chondrogenic differentiation *in vitro*. Furthermore, this cell line was also chosen to reduce variation of results due to species differences.

To achieve this aim, pellets of ihBMSCs were cultured in chondrogenic medium supplemented with inflammatory mediators, and the level of sGAGs were measured to establish any effects on the chondrogenic process. Following this, expression of candidate genes in ihBMSCs was evaluated by TaqMan Low Density Arrays (TLDAs) in order to identify any changes to signalling pathways governing differentiation.

Based on the existing literature, it was hypothesised that the inclusion of pro-inflammatory mediators and growth factors (IL-1β, IL-8, MCP-1, NGF) in chondrogenic medium would decrease the rate of chondrogenesis in ihBMSCs, with decreased levels of aggrecan and increased levels of hypertrophic markers and proteases that contribute to cartilage breakdown (Merz *et al.*, 2003; Huang *et al.*, 2013; Xu *et al.*, 2015; Liu *et al.*, 2017). On the other hand, it was hypothesised that the anti-inflammatory mediator IL-10 would increase the rate of chondrogenesis (van Roon *et al.*, 1996).

4.2 Methods

For more detailed methods describing MSC culture and mediator exposure please see Chapter 2 Materials and Methods.

4.2.1 Chondrogenic Differentiation

Chondrogenic differentiation was induced in ihBMSC pellets cultured in chondrogenic medium (CM) over a period of 21 days. CM consisted of high glucose (4.5g/L) DMEM supplemented with 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine, 1% (v/v) penicillin streptomycin, 110mg/L (w/v) sodium pyruvate, 0.1uM dexamethasone, 50uM L-ascorbic acid-2-phosphate, 40ug/ml (w/v) L-proline, 1% (v/v) ITS supplement and 10ng/ml TGF-β1. ihBMSCs were seeded in standard medium (SM) at $1x10^6$ cells per 1.5ml eppendorf and centrifuged at 500 x *g* for 5 min in a Sigma 1-14 microfuge. Pellets were incubated and allowed to attach for 24 hours at 37°C before replacement of medium with CM with or without inflammatory mediators. The medium was replaced three times a week for 21 days, and the differentiation status assessed by a glycosaminoglycan assay, and gene expression levels gained through TLDAs. Figure **4.2** provides a schematic of the experimental design.

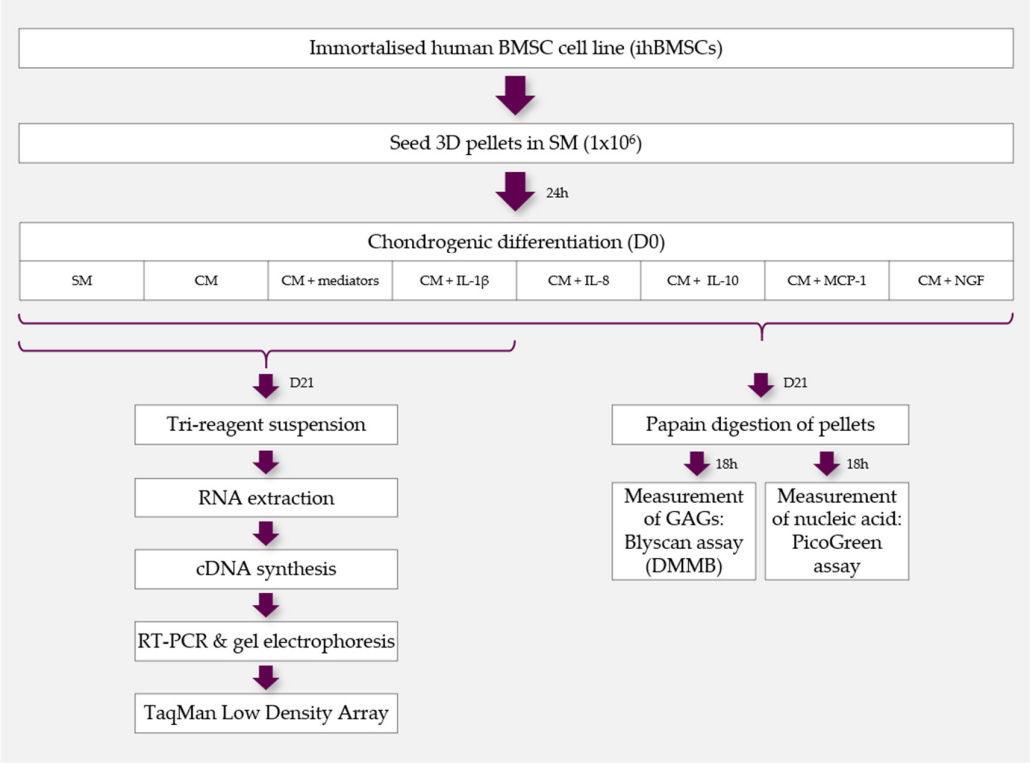


Figure 4.2 Schematic of the chondrogenic experimental design

4.2.2 Quantification of glycosaminoglycans

To measure the GAG content of chondrogenic pellets, sGAGs were extracted using a papain extraction reagent consisting of 0.2M sodium phosphate buffer with 8mg/ml (w/v) sodium acetate (Sigma-Aldrich), 4mg/ml (w/v) EDTA (Acros Organics), 0.8mg/ml (w/v) cysteine hydrochloride (Sigma-Aldrich) and 100ug/ml papain (Sigma-Aldrich), at pH 6.4. Pellets were incubated in 1ml papain extraction reagent at 65°C for 18 hours to aid digestion of protein. Following this, the samples were centrifuged at 10,000 x *g* for 10 minutes (Eppendorf centrifuge 5415 R) and the supernatant decanted for use with the Blyscan Sulfated Glycosaminoglycan Assay kit (Biocolor Life Science Assays, UK) according to manufacturer's instructions. This assay utilises the DMMB cationic dye that specifically binds to sGAGs.

Briefly, 100µl of papain digested sample was transferred to a fresh eppendorf and 1ml of Blyscan dye reagent, containing DMMB in an inorganic buffer, was added. Samples were mixed by inversion every 5 minutes, for a total of 30 minutes, to allow for the formation of an sGAG-dye complex. Samples were then centrifuged at 13,000 x *g* for 10 minutes to pellet the bound sGAGs. The supernatant was carefully drained and 500µl of dissociation reagent, containing sodium salt of an anionic surfactant, was added to dissociate the sGAG-dye complex and enhance the spectrophotometric absorption profile of the unbound dye. Samples were vortexed thoroughly until the bound dye had been released and dissolved, after which samples were centrifuged at 13,000 x *g* for 5 minutes to remove foam. To quantify sGAG content, 200µl of each sample was immediately transferred to a 96 well plate and absorbance was read using an Infinite 200 Pro plate reader and i-control software (Tecan) at 656nm. Samples were measured alongside a reagent blank and GAG standards, containing 1-5µg of bovine tracheal chondroitin 4-sulfate, that provided a standard curve to calculate the sGAG concentration of the samples.

4.2.3 PicoGreen Assay

To normalise the sGAG concentration to nucleic acid concentration the Quant-iT™ PicoGreen® dsDNA kit (Invitrogen) was used according to manufacturer's instructions. Briefly, a five-point standard curve using bacteriophage lambda DNA was ascertained with concentrations between 1ng/ml to 1µg/ml. For this, the DNA stock solution was diluted in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) to a known concentration and 100µl was incubated in a 96 well plate with 100µl of Quant-iT™ PicoGreen® Reagent for 5 minutes at room temperature, protected from light. The sample fluorescence was measured using an Infinite 200 Pro plate reader and i-control software (Tecan), with an excitation wavelength of 480nm and an emission

wavelength of 520nm. This data was used to generate a standard curve of fluorescence vs DNA concentration to determine the DNA concentration of unknown samples.

The nucleic acid concentration of papain digested samples was then calculated using the standard curve, whereby 100µl of sample was incubated with 100µl of Quant-iT™ PicoGreen® Reagent for 5 minutes at RT, protected from light. The sample fluorescence was measured and plotted onto the standard curve to ascertain DNA concentration.

The measurement of dsDNA was used to normalise GAG concentration levels and the data plotted using GraphPad Prism 7 (GraphPad Software, San Diego, CA). A Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's test for post hoc multiple comparisons.

4.2.4 TaqMan Low Density Arrays

TLDAs (ThermoFisher) were used to measure changes in gene expression of ihBMSCs during chondrogenic differentiation. For more detailed methods on cDNA preparation and validation, and TLDAs please see Chapter 2 Materials and Methods.

Briefly, ihBMSCs were cultured in SM, CM, CM + mediators or CM + IL-1β for 21 days, after which time cells were washed twice with PBS and resuspended in Tri-reagent (Sigma Aldrich). RNA was subsequently extracted from the cells using a RNeasy Micro Kit (Qiagen), according to manufacturer's instructions. The RNA concentration and purity were then assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA) and, following this, cDNA was synthesised from 500ng total RNA using a High-Capacity RNA-to-cDNA Kit (ThermoFisher), according to manufacturer's

instructions. Successful cDNA synthesis was confirmed by RT-PCR and gel electrophoresis, upon which 384-well microfluid TaqMan array cards were loaded with cDNA and run on the 7900HT Fast-Real Time PCR System using SDS RQ Manager Software (Applied Biosystems). The generated Ct values were normalised to the geometric mean of beta-actin (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which were identified as the most stable reference genes. The data was then corrected for baseline and the $\log_2$ fold change from SM plotted using GraphPad Prism 7 (GraphPad Software, San Diego, CA). A Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's test for post hoc multiple comparisons.

4.3 Results

4.3.1 Response of ihBMSCs to inflammatory mediators during chondrogenic differentiation

Initial studies investigated the effects of inflammatory mediators (IL-1β, IL-8, IL-10, MCP-1, NGF) on ihBMSCs during chondrogenic differentiation. Each of these mediators were added to CM either individually or in combination, the latter of which was chosen to better replicate the osteoarthritic microenvironment, where a range of both pro- and anti-inflammatory mediators are present. Effects of these treatments were assessed through the measurement of sGAGs after 21 days (Figure **4.3**).

The concentration of sGAGs produced by CM-treated chondrogenic pellets was significantly increased compared to pellets cultured in SM ($p=0.002$), confirming successful differentiation of the cells. Interestingly, exposure to CM + IL-1β significantly decreased the concentration of sGAGs ($p=0.016$), whilst exposure to CM + mediators caused a decreased trend ($p=0.097$). This same effect could be observed when measuring the concentration of dsDNA, as CM + IL-1β significantly decreased nucleic acid levels ($p=0.022$). Normalisation of sGAG concentration to nucleic acid content confirmed that the CM + IL-1β ($p=0.011$) and CM + mediators ($p=0.077$) treatment groups decreased detection of sGAGs during the chondrogenic protocol, though the latter was not significant. None of the other inflammatory mediators individually altered sGAG concentration compared to CM alone.

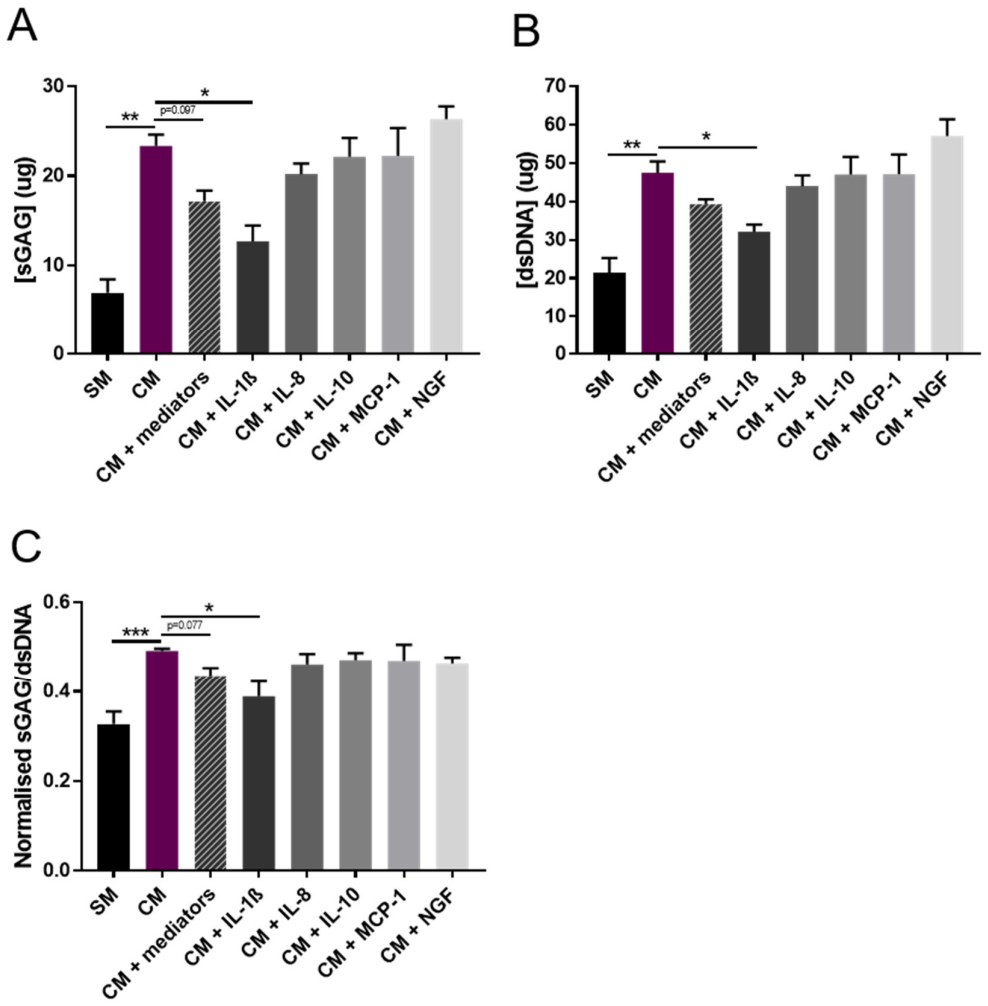


Figure 4.3 sGAG concentration of chondrogenic pellets after exposure to inflammatory mediators

sGAG concentration (μg) was quantitatively measured in chondrogenic pellets after exposure to inflammatory mediators for 21 days. A Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The presence of IL-1β during chondrogenic differentiation, significantly decreased the concentration of sGAGs (p=0.016) (**A**) and dsDNA (p=0.022) (**B**) compared to CM alone. The presence of combined inflammatory mediators also decreased the concentration of sGAGs (p=0.077) (**A**) and dsDNA (**B**) compared to CM alone, though it was not significant. The same results could be observed when sGAG concentration was normalised to dsDNA concentration (C). n=3 biological replicates, * $p<0.05$, ** $p<0.005$, *** $p<0.001$. Error bars represent SEM.

4.3.2 Effects of inflammatory mediators on ihBMSC gene expression during chondrogenic differentiation

Results from ihBMSC chondrogenic differentiation experiments (in section 4.3.1) showed that the addition of IL-1β to CM significantly reduced the chondrogenic potential of the cells, as evidenced by decreased levels of sGAGs after 21 days. This reduction was observed in CM treatment groups containing IL-1β alone, or in combination with other inflammatory mediators (IL-1β, IL-8, IL-10, MCP-1, NGF). To gain insight into the mechanisms governing this phenomenon, the gene expression profile of these cells was analysed at day 21 of the differentiation protocol. The genes analysed in this study are described in Table **2.3**.

4.3.2.1 Inflammatory mediators and their receptors

Expression of genes encoding for the inflammatory mediators used in this study were analysed, along with their receptors, to assess the autocrine effects of these mediators on ihBMSCs, as well as the effect of chondrogenesis itself (Figure **4.4** and Figure **4.5**).

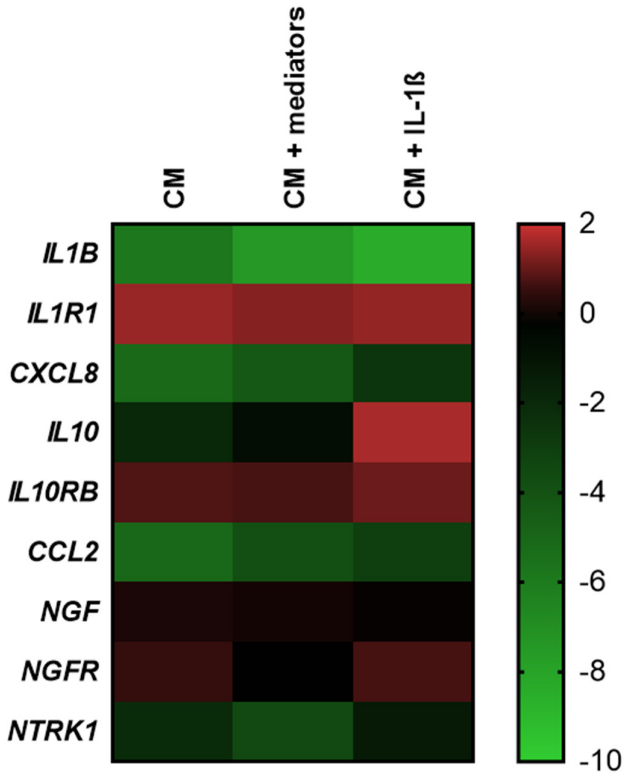


Figure 4.4 Heatmap of inflammatory mediator and receptor gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of inflammatory mediators and their receptors during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *IL10* was increased in CM + IL-1β compared to CM.

Expression of genes encoding for the inflammatory mediators used in this study were largely unaffected by the process of chondrogenesis, including during exposure to inflammatory mediators. *IL1B, CXCL8* and *CCL2* all displayed decreased expression in ihBMSCs undergoing chondrogenesis, though the presence of IL-1β did prevent the significant decrease in *IL10* expression, instead causing a 1.64 $\log_2$ fold increase from SM. Although the presence of combined inflammatory mediators did prevent a significant decrease in *IL10* expression it did not show the same effect as IL-1β alone, as expression was still decreased with a -0.68 $\log_2$ fold decrease from SM. Moreover, expression of *NGF* was unaffected by the chondrogenic treatment.

When analysing the expression of receptors, *IL1R1* was seen to be increased in all ihBMSCs cultured in CM, in a similar way to *IL10RB*. On the other hand, *NTRK1* expression was decreased, whilst *NGFR* expression was unaffected by chondrogenic differentiation. Expression of *CXCR1* and *CCR2* could not be detected by this array.

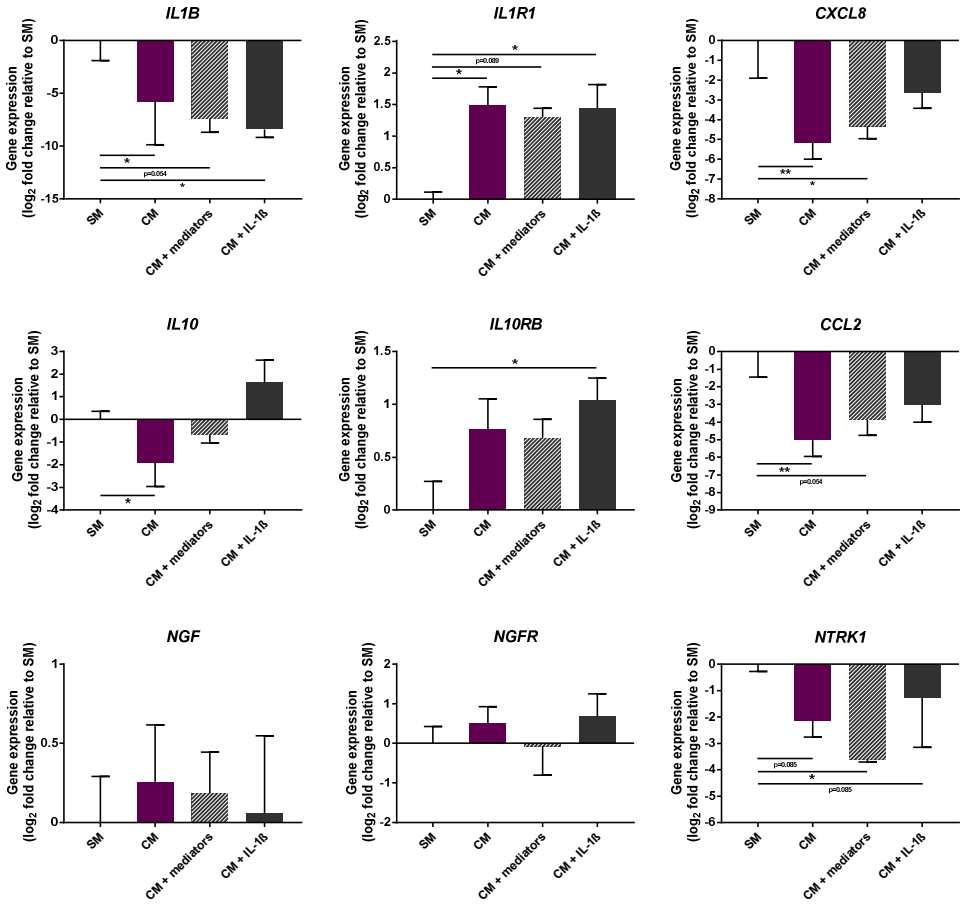


Figure 4.5 Gene expression of inflammatory mediators and their receptors during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for inflammatory mediators and their receptors were analysed at day 21 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The presence of IL-1β in CM prevented a significant decrease in expression of *IL10* during chondrogenic differentiation of ihBMSCs. n=3 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

4.3.2.2 Chondrogenic differentiation markers

Expression of genes encoding for classical chondrogenic differentiation markers were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **4.6** and Figure **4.7**).

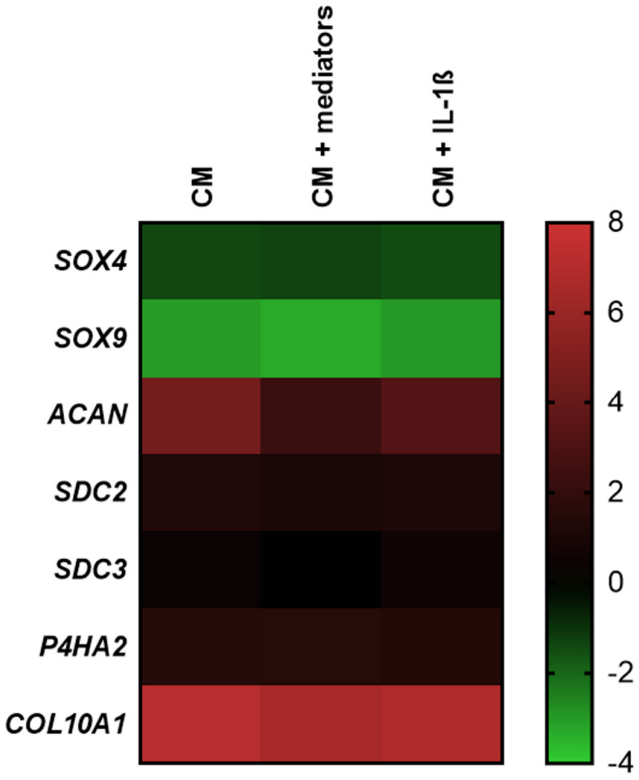


Figure 4.6 Heatmap of chondrogenic marker gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of chondrogenic markers during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *ACAN* was reduced in CM treatment groups containing IL-1β or combined inflammatory mediators.

Expression of *ACAN* was significantly increased in ihBMSCs cultured in CM (4.50 $\log_2$ fold change) compared to SM. However, the presence of IL-1β alone, or in combination with other inflammatory mediators, resulted in a decreased level of expression compared to CM, with a 3.19 and 2.26 $\log_2$ fold change from SM respectively. None of the other chondrogenic markers were seen to be

affected by the presence of inflammatory mediators as *SOX4* and *SOX9* were decreased in all CM conditions, whilst *SDC2*, *P4HA2*, *COL10A1* were all increased in CM conditions. The expression of *SDC3* in ihBMSCs was not seen to be affected by chondrogenic differentiation, and expression of *COL2A1* could not be detected by this array.

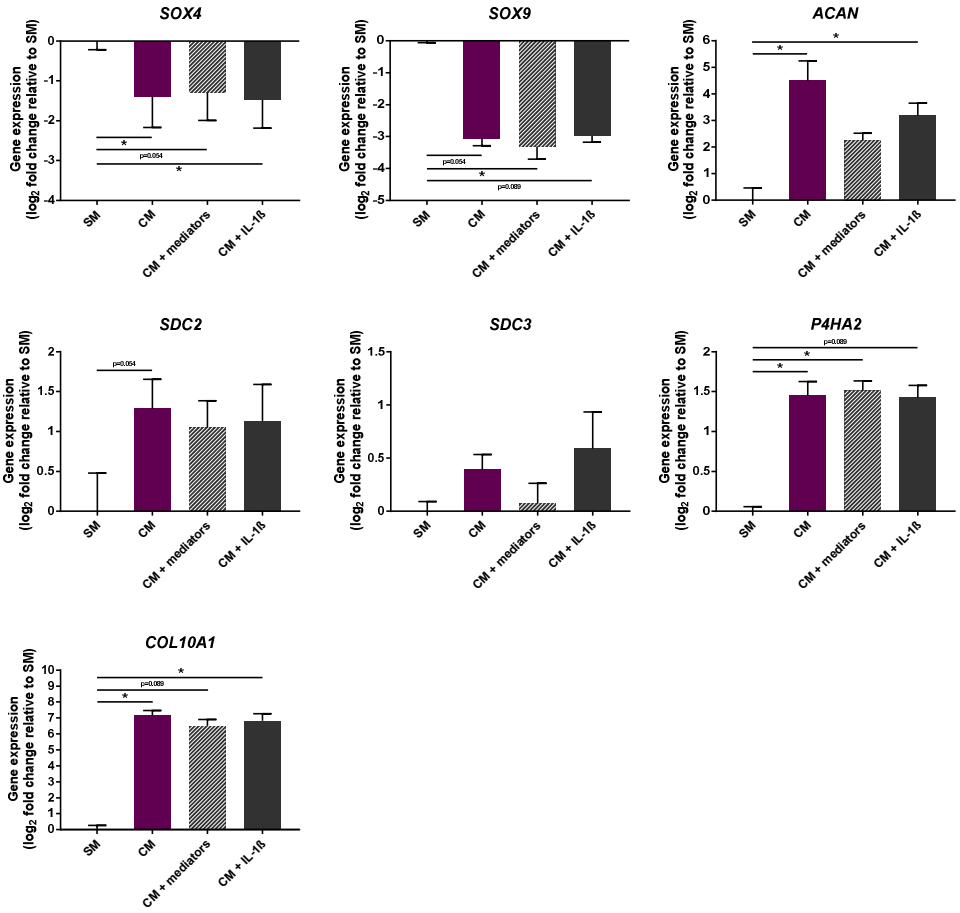


Figure 4.7 Gene expression of chondrogenic markers during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for chondrogenic markers were analysed at day 21 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *ACAN* was reduced in CM treatment conditions containing IL-1β or combined inflammatory mediators. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

4.3.2.3 Osteogenic differentiation markers

Expression of genes encoding for classical osteogenic differentiation markers were analysed to assess the effects of inflammatory mediators on ihBMSCs during their chondrogenic treatment (Figure **4.8** and Figure **4.9**). Although these genes are primarily associated with osteogenic differentiation, the interplay between a variety of genes is crucial for control and differentiation of MSCs towards both osteogenic and chondrogenic lineages, and thus they were included in the analysis during chondrogenic differentiation.

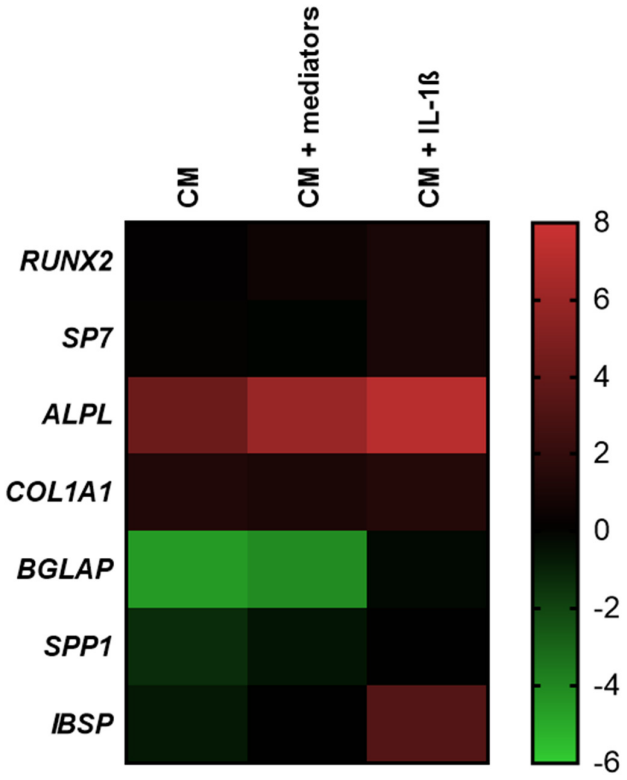


Figure 4.8 Heatmap of osteogenic marker gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of osteogenic markers during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *ALPL*, *RUNX2*, *SP7* and *IBSP* was increased by the presence of IL-1β.

Although expression of *ALPL* was increased in ihBMSCs cultured in CM (4.18 $\log_2$ fold change), this expression was further significantly increased in the presence of IL-1β alone (7.17 $\log_2$ fold change) and in combination with other inflammatory mediators (5.94 $\log_2$ fold change), when compared to SM. Inflammatory mediators did not significantly affect expression of other osteogenic genes; however, trends could be observed. Expression of *RUNX2* was increased in response to IL-1β (0.94 $\log_2$ fold change) and combined inflammatory mediators (0.54 $\log_2$ fold change). Moreover, IL-1β alone increased expression of *SP7* (0.96 $\log_2$ fold change) and *IBSP* (3.21 $\log_2$ fold change), and prevented the significant decrease of *SPP1* observed in cells cultured in CM alone. Expression of *COL1A1* and *BGLAP* were unaffected by the presence of inflammatory mediators, with the former being increased compared to SM and the latter being decreased.

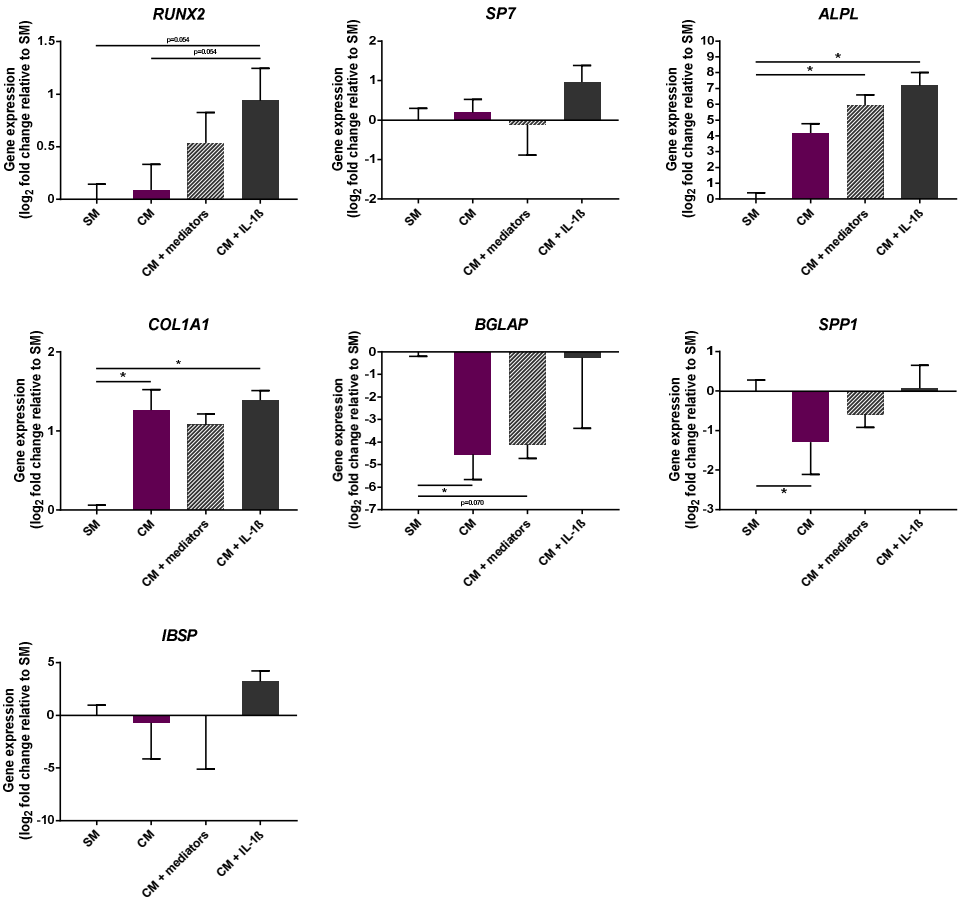


Figure 4.9 Gene expression of osteogenic markers during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for osteogenic markers were analysed at day 21 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The presence of IL-1β (p=0.017) and combined inflammatory mediators (p=0.024) caused a significant increase in *ALPL* expression compared to SM. Although not significant, IL-1β also caused an increase in expression of *RUNX2*, *SP7*, *SPP1* and *IBSP* when compared to CM. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

4.3.2.4 MSC immunomodulatory mediators

Expression of genes encoding for MSC immunomodulatory mediators were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **4.10** and Figure **4.11**).

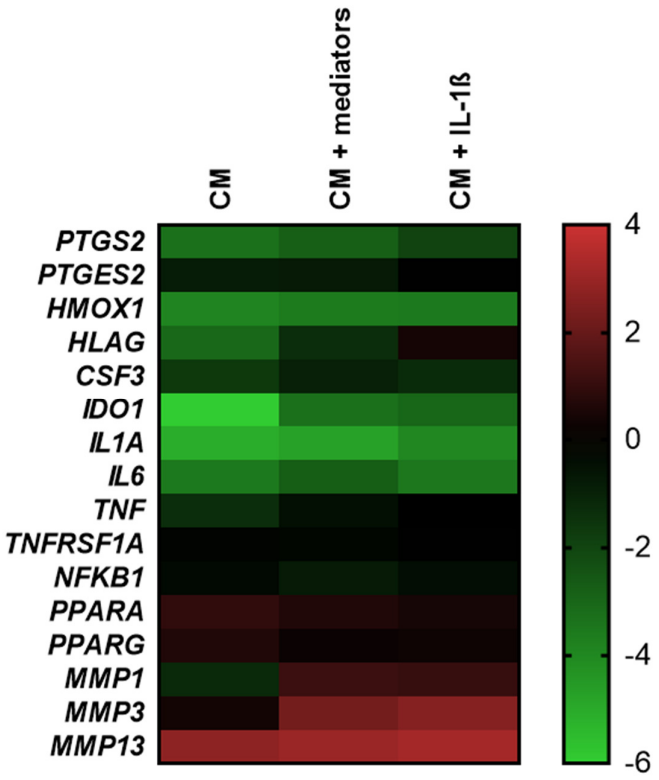


Figure 4.10 Heatmap of immunomodulatory mediator gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of immunomodulatory mediators during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Addition of IL-1β or combined inflammatory mediators to CM increased expression of MMPs.

A number of genes involved in the immunomodulatory capacity of MSCs showed a reduction in expression levels during chondrogenic differentiation. This included *HMOX1, IDO1, IL1A* and *IL6*, and the presence of an inflammatory environment did not affect this expression pattern. In contrast, expression of genes encoding for MMPs were increased with treatment of

inflammatory mediators. Both *MMP1* and *MMP3* expression was affected by the presence of IL-1β, as observed by a 1.07 $\log_2$ fold change from SM in the former and a 2.58 $\log_2$ fold change in the latter. A similar profile was seen during culture with combined inflammatory mediators as this treatment increased expression of *MMP1* (1.15 $\log_2$ fold change) and *MMP3* (2.25 $\log_2$ fold change). Although *MMP13* was detected at high levels during chondrogenic differentiation (2.76 $\log_2$ fold change), there was a small increased trend during exposure to IL-1β alone (3.20 $\log_2$ fold change) and combined inflammatory mediators (2.99 $\log_2$ fold change).

Expression of other immunomodulatory genes were not significantly affected by the process of chondrogenesis, including *PTGS2, PTGES2, CSF3, TNF, TNFRSF1A, HLAG, PPARA, PPARG* and *NFKB1.* Expression of *NOS1, NOS2* and *IFNG* could not be detected in this array.

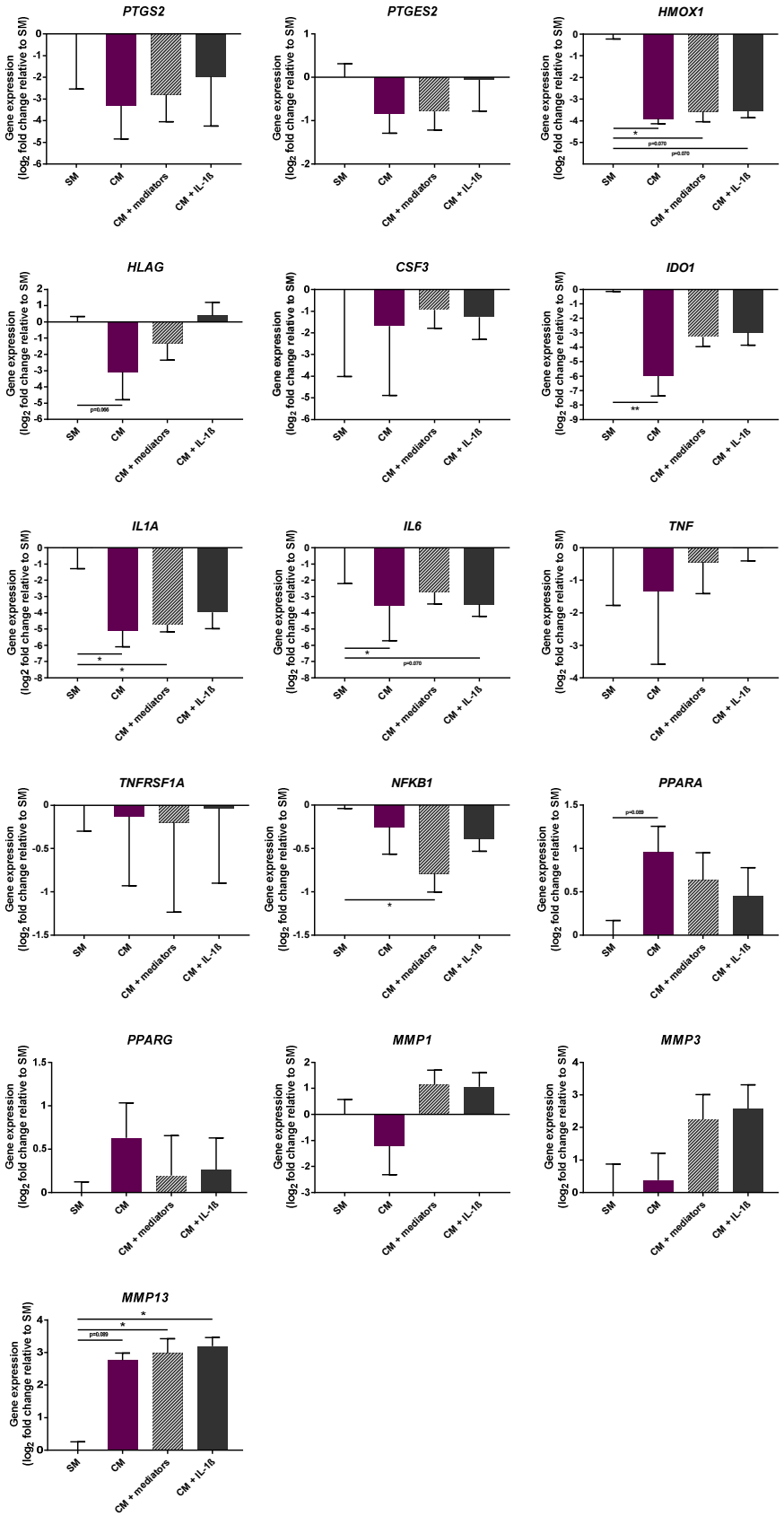

PTGS2
PTGES2
HMOX1
HLAG
CSF3
IDO1
IL1A
IL6
TNF
TNFRSF1A
NFKB1
PPARA
PPARG
MMP1
MMP3
MMP13
Gene expression
(log2 fold change relative to SM)
SM
CM
CM + mediators
CM + IL-1ß

Figure 4.11 Gene expression of immunomodulatory mediators during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for immunomodulatory mediators were analysed at day 21 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Treatment of ihBMSCs with inflammatory mediators increased the expression of *MMP1* and *MMP3,* although this is not statistically significant. n=3 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

4.3.2.5 Growth factors and receptors

Expression of genes encoding for growth factors and their receptors were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **4.12** and Figure **4.13**).

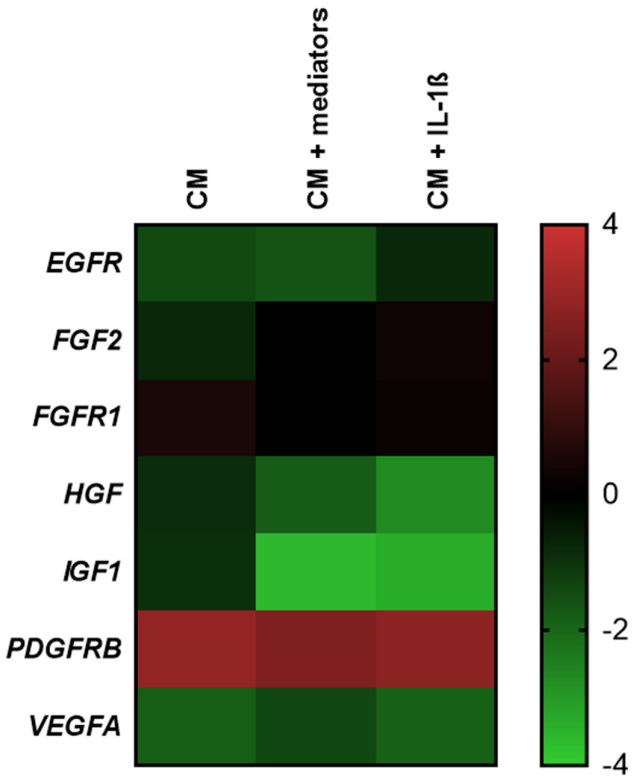


Figure 4.12 Heatmap of growth factor and receptor gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of growth factors and their receptors during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *FGFR1, HGF* and *IGF1* was seen to be decreased in chondrogenic conditions containing inflammatory mediators, whilst *FGF2* expression was increased in these conditions compared to CM alone.

At day 21 of ihBMSC chondrogenic differentiation, expression of *EGFR* and *VEGFA* was decreased, regardless of the presence of inflammatory mediators. The chondrogenic environment also resulted in the decreased expression of other growth factors, including *HGF* and *IGF1*, but their reduction was exacerbated by both the CM + IL-1β and CM + mediators conditions. In contrast, expression of *PDGFRB* was significantly increased during chondrogenic treatment, along with *FGFR1*. However, the presence of inflammatory mediators prevented stimulation of *FGFR1* at day 21. Furthermore, the presence of inflammatory mediators, particularly IL-1β alone, recovered the expression of *FGF2* at this timepoint.

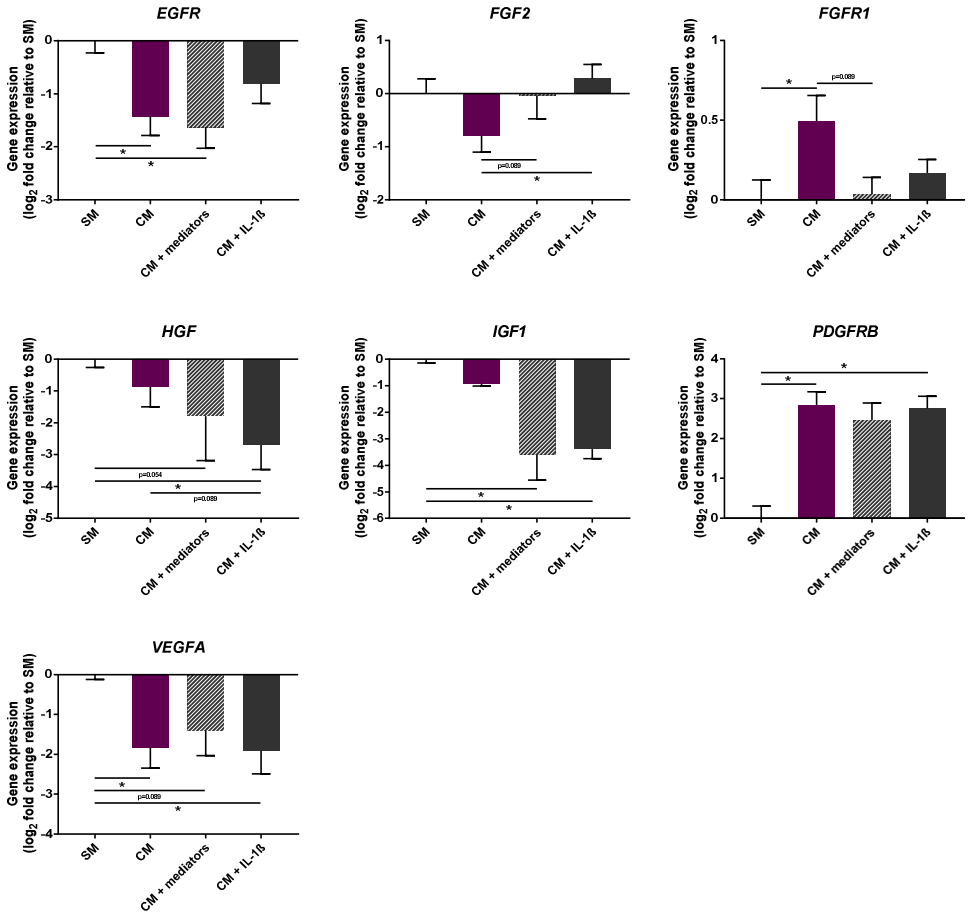


Figure 4.13 Gene expression of growth factors and receptors during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for growth factors and their receptors were analysed at day 21 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The presence of inflammatory mediators exacerbated decreased expression of the growth factors *HGF* and *IGF1*, whilst simultaneously preventing *FGFR1* expression and recovering expression of *FGF2*. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

4.3.2.6 TGF-β/BMP signalling

Expression of genes encoding for members of the TGF-β/BMP signalling pathways were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **4.14** and Figure **4.15**).

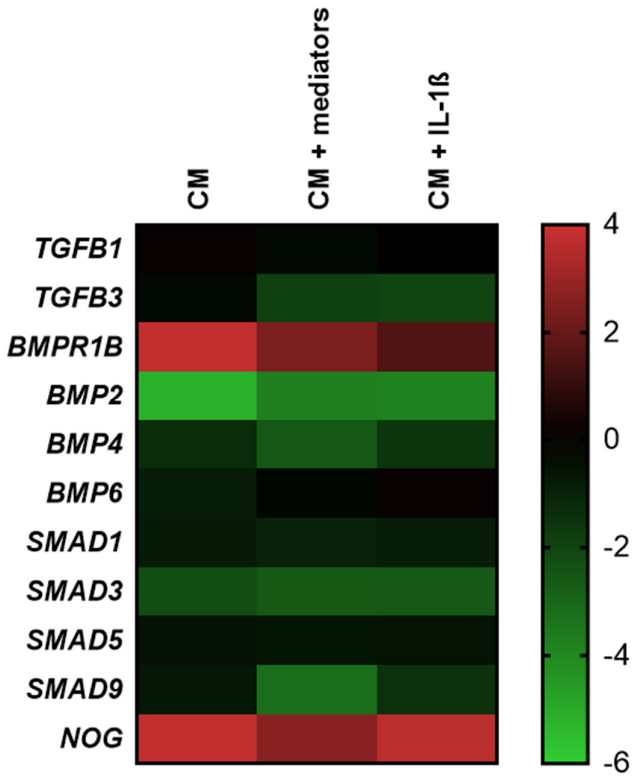


Figure 4.14 Heatmap of TGF-β/BMP signalling gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of TGF-β/BMP signalling molecules during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. The presence of inflammatory mediators decreased expression of *TGFB3*, *BMPR1B* and *NOG* compared to CM.

At day 21 chondrogenic differentiation of ihBMSCs, the expression of Smad proteins was significantly decreased. This included all the Smad genes assessed, including *SMAD1, SMAD3, SMAD5* and *SMAD9.* In a similar way, expression of *BMP2* and *BMP4* expression was decreased under chondrogenic

conditions, whilst *BMP6* expression was unchanged compared to cells cultured in SM. The presence of inflammatory mediators, in the form of IL-1β alone or combined, did not affect the gene expression profile of the cells regarding both Smad and BMP genes. Furthermore, *TGFB1* expression was also unaffected at this stage of chondrogenic differentiation, but the presence of inflammatory mediators significantly reduced expression of *TGFB3.*

Another gene affected by the presence of inflammatory mediators was *BMPR1B*. In CM conditions, expression of this gene was increased with a 3.76 log_2 fold change from SM, but the presence of IL-1β only allowed for a 1.54 log_2 fold increase, whilst combined inflammatory mediators saw a 2.39 log_2 fold increase from SM. The increased expression of *NOG* in CM conditions (3.75 log_2 fold change) was also dampened by the presence of inflammatory mediators, whereby expression was observed as a 2.64 log_2 fold change from SM. This reduction in high expression levels was not observed in CM + IL-1β alone, which displayed a 3.64 log_2 fold increase from SM. Expression of *GDF2 (BMP9)* could not be detected in this array.

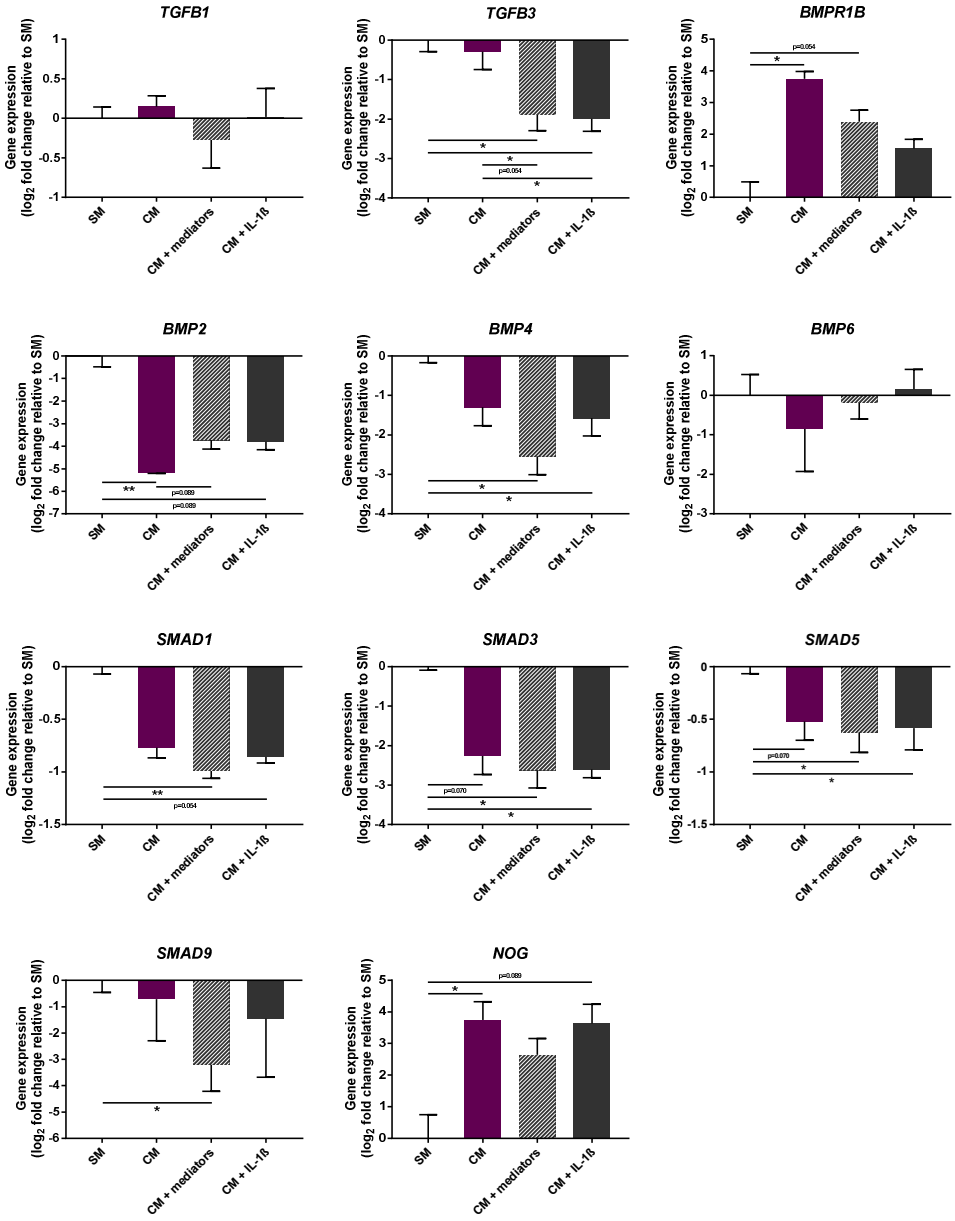


Figure 4.15 Gene expression of TGF-β/BMP signalling molecules during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for TGF-β/BMP signalling molecules were analysed at day 21 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *TGFB3* and *BMPR1B* was reduced by both the CM + IL-1β and CM + mediators treatment groups. n=3 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

4.3.2.7 MAPK signalling

Expression of genes encoding for members of the MAPK signalling pathways were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **4.16** and Figure **4.17**).

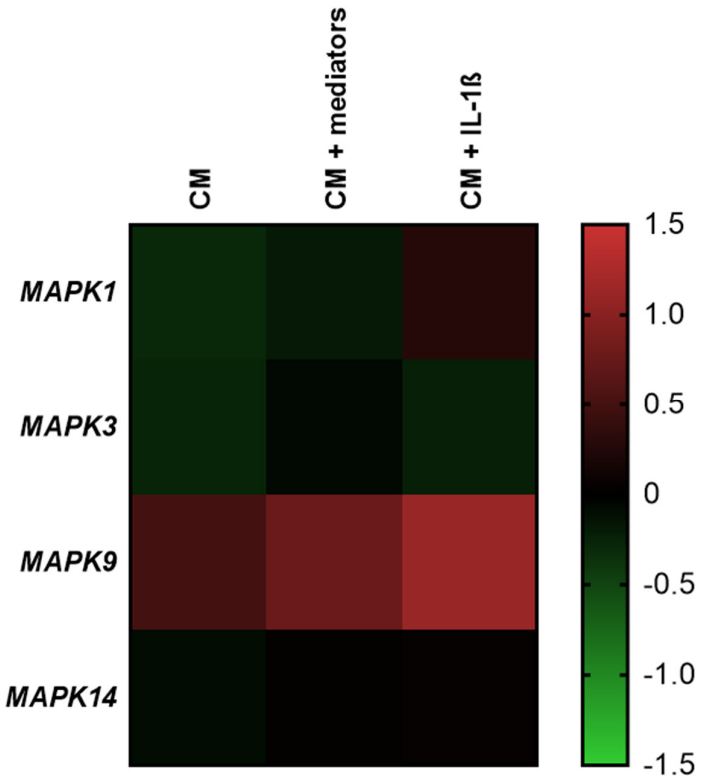


Figure 4.16 Heatmap of MAPK signalling gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of MAPK signalling molecules during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. The presence of IL-1β alone prevented a decrease in expression of *MAPK1*, whilst also increasing expression of *MAPK9*.

Exposure of ihBMSCs to IL-1β during chondrogenic differentiation significantly increased expression of *MAPK1*, which encodes for ERK2, when compared to CM or CM + mediators. Moreover, expression of *MAPK9*, which encodes for c-Jun N-terminal kinase (JNK)-2, was also significantly increased

in cells cultured in CM + mediators (0.77 log_2 fold change) or CM + IL-1β (1.12 log_2 fold change) compared to CM. Expression of *MAPK3* and *MAPK14* was not significantly affected by the chondrogenic differentiation of ihBMSCs.

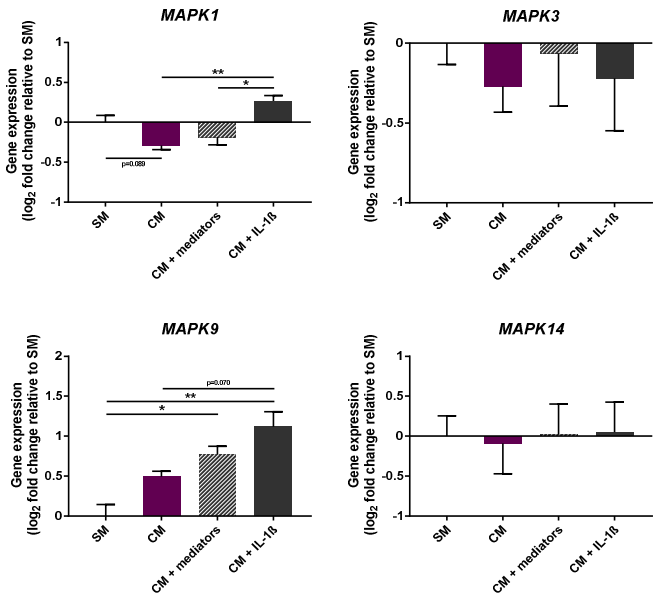


Figure 4.17 Gene expression of MAPK signalling molecules during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for MAPK signalling molecules were analysed at day 21 by TLDAs. Results were reported as log_2 fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *MAPK1* was significantly increased in cells cultured with IL-1β alone, compared to CM (p=0.004) and CM + mediators (p=0.032) IL-1β also significantly increased expression of *MAPK9* compared to SM, when alone (p=0.003) or in combination with other mediators (p=0.042). n=3 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

4.3.2.8 Wnt signalling

Expression of genes encoding for members of the Wnt signalling pathways were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **4.18** and Figure **4.19**).

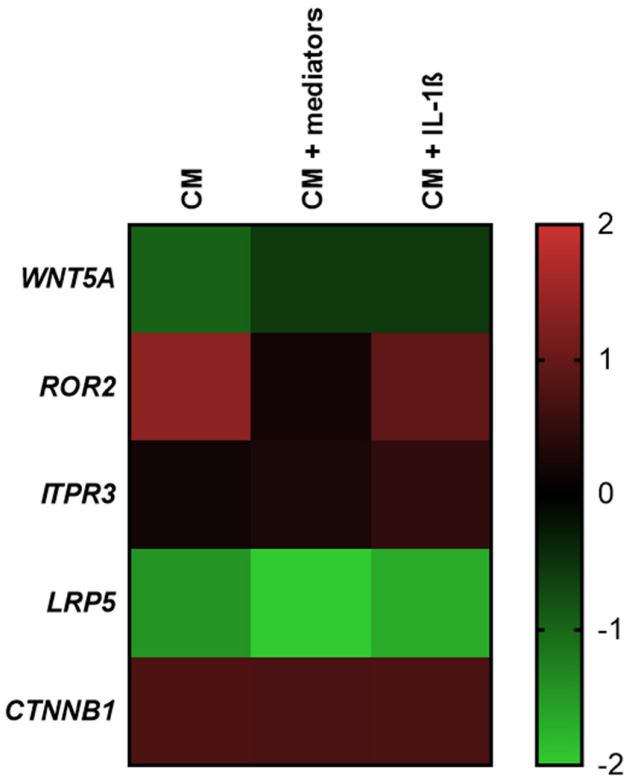


Figure 4.18 Heatmap of Wnt signalling gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of Wnt signalling molecules during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *ROR2* was decreased in cells undergoing chondrogenic differentiation in the presence of inflammatory mediators.

Expression of Wnt signalling molecules during chondrogenesis was unaffected by the presence of inflammatory mediators, except for the non-canonical molecule *ROR2*. Expression of this gene was decreased in cells exposed to combined inflammatory mediators (0.18 $\log_2$ fold change)

compared to those in CM alone (1.36 log_2 fold change). Expression of *WNT5A* and *LRP5* showed a decreased trend in expression in all CM conditions, whilst *CTNNB1* showed an increased trend. Expression of *ITPR3* was unaffected by chondrogenic conditions, and *WNT3A* and *WNT7A* could not be detected in this array.

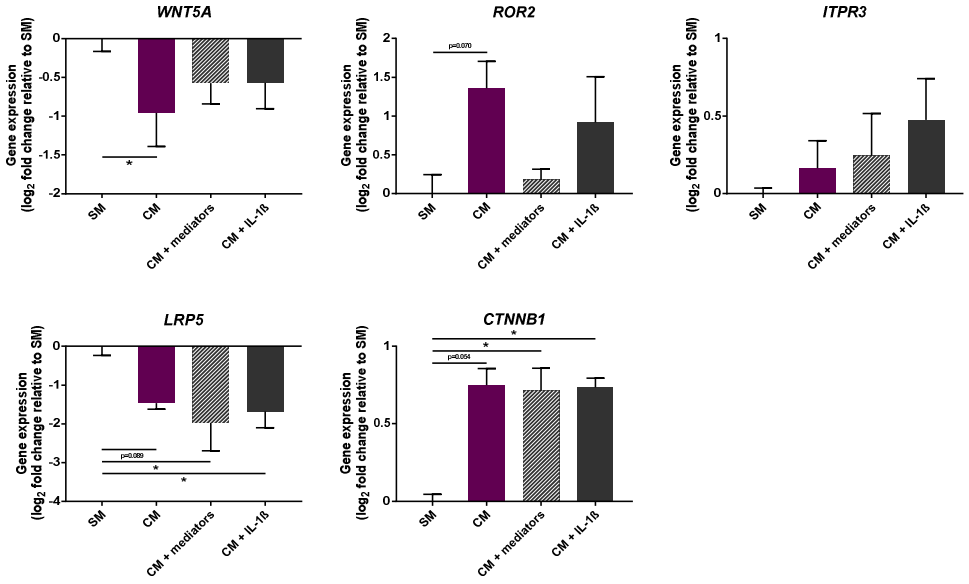


Figure 4.19 Gene expression of Wnt signalling molecules during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for Wnt signalling molecules were analysed at day 21 by TLDAs. Results were reported as log_2 fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *ROR2* was decreased in cells undergoing chondrogenic differentiation in the presence of inflammatory mediators. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

4.3.2.9 Toll like receptor signalling

Expression of genes encoding for TLRs were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **4.20** and Figure **4.21**).

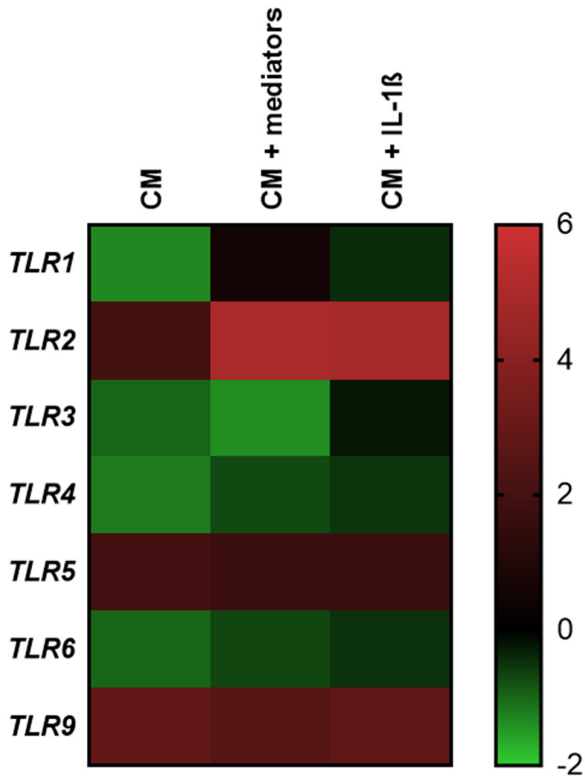


Figure 4.20 Heatmap of TLR signalling gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of TLR signalling molecules during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *TLR2* was significantly increased in cells cultured in CM + mediators or CM + IL-1β.

Expression of *TLR1* was significantly decreased in ihBMSCs cultured in CM with a -1.33 log_2 fold change from SM. However, the presence of inflammatory mediators or IL-1β alone prevented this significant decrease compared to SM. Most strikingly, the expression of *TLR2* was significantly increased in cells exposed to inflammatory mediators (4.95 log_2 fold change) or IL-1β alone (4.82 log_2 fold change). Expression of other TLRs did not show any significant differences between CM conditions, though CM + IL-1β prevented the significant decrease in expression of *TLR3*, *TLR4* and *TLR6*. Expression of *TLR9* and *TLR5* was increased in all CM conditions, though no significance could be observed in the latter.

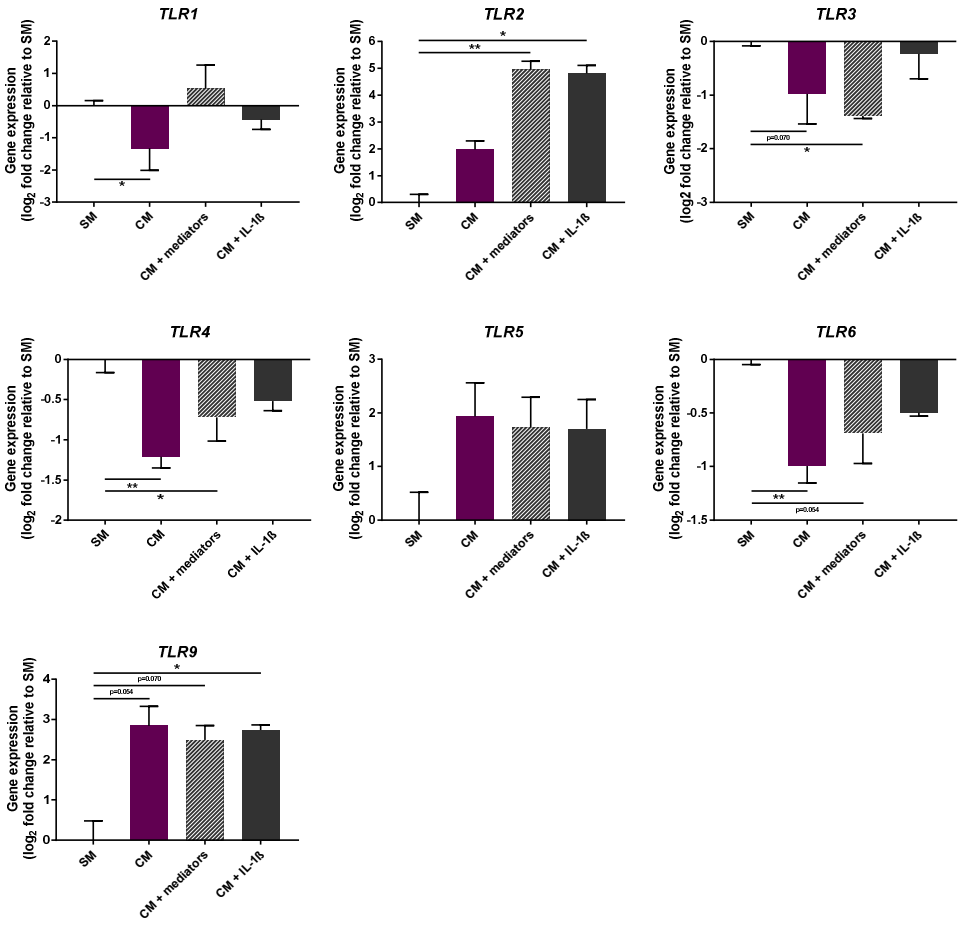


Figure 4.21 Gene expression of TLR signalling molecules during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for TLR signalling molecules were analysed at day 21 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *TLR2* was significantly increased in cells cultured in CM + mediators ($p=0.009$) or CM + IL-1β ($p=0.013$). n=3 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

4.3.2.10 Other signalling pathways

Expression of genes encoding for members of other signalling pathways of interest were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **4.22** and Figure **4.23**).

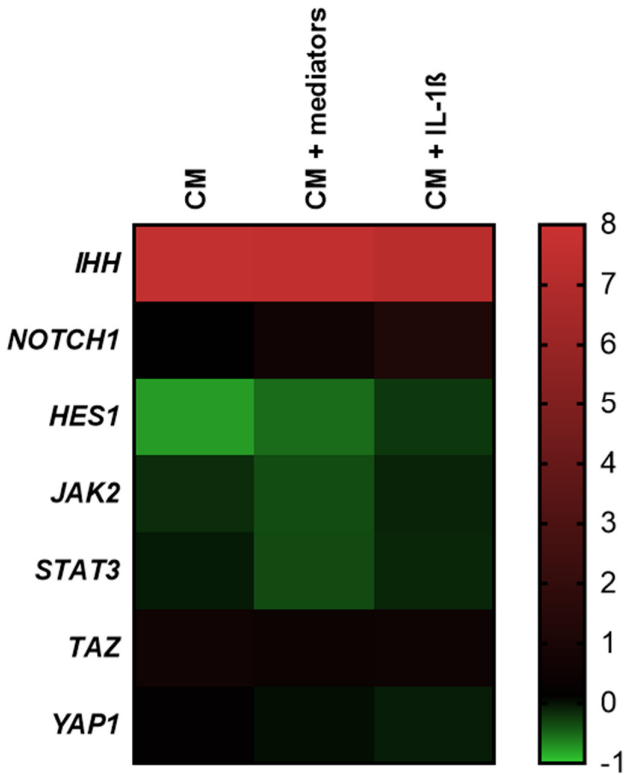


Figure 4.22 Heatmap of other signalling gene expression levels during ihBMSC chondrogenic differentiation

Gene expression levels of other signalling molecules during ihBMSC chondrogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *NOTCH1* signalling was increased in cells cultured with inflammatory mediators.

Gene expression of other cell signalling molecules showed that *IHH* signalling was significantly increased in ihBMSCs after 21 days of chondrogenic differentiation. This was unaffected by the presence of inflammatory mediators, though these conditions did show an increased trend in the expression of *NOTCH1.* Here, combined inflammatory mediators and IL-1β alone increased expression (0.62 and 1.15 $\log_2$ fold change respectively). Moreover, a trend in the expression levels of *HES1* could also be observed, whereby IL-1β did not significantly decrease expression of this gene. Other genes measured were not significantly changed at this timepoint of differentiation, including *JAK2, STAT3, TAZ* and *YAP.* Expression of *SHH* and *NELL* could not be detected in this array.

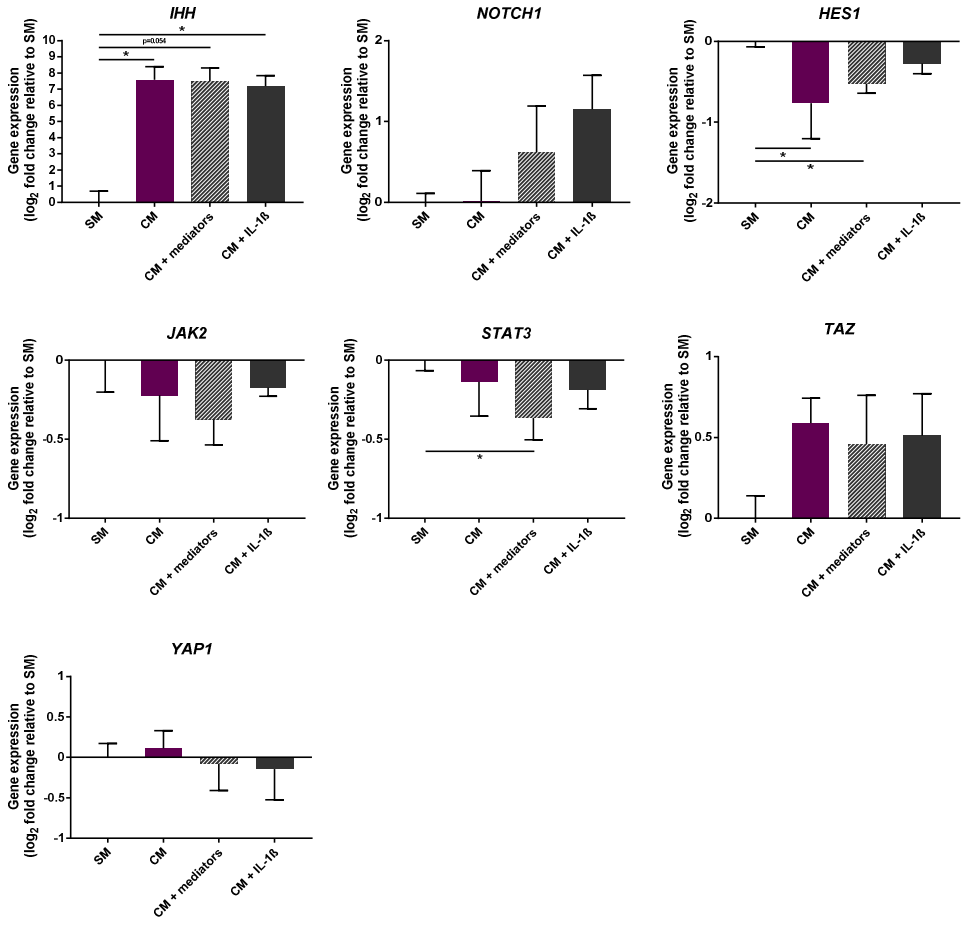


Figure 4.23 Gene expression of other signalling molecules during ihBMSC chondrogenic differentiation

To assess the effects of inflammatory mediators on chondrogenic differentiation of ihBMSCs, the expression of genes encoding for other signalling molecules were analysed at day 21 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Inclusion of inflammatory mediators in CM saw an increased trend in *NOTCH1* expression, whilst IL-1β alone prevented decreased expression of *HES1*. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

4.4 Discussion

Loss of articular cartilage is a hallmark of OA pathogenesis, as resident chondrocytes fail to maintain the homeostatic balance between synthesis and degradation of ECM components (Goldring and Marcu, 2009). This imbalance is exacerbated by the inherent lack of regenerative potential of cartilage due to its avascular and aneural nature (Van Osch *et al.*, 2009), and although endogenous MSCs in the joint have the capacity to undergo chondrogenic differentiation, cartilage is unable to readily recruit these reparative cells (Grogan *et al.*, 2009; Koelling *et al.*, 2009). Therefore, a lot of work has focused on harnessing MSCs for cartilage repair in degenerative diseases such as OA. However, current research does not yield highly successful results, as MSC transplantation reveals limited to no regeneration of articular cartilage (Iijima *et al.*, 2018).

A potential reason for this lack of regeneration is transplantation of cells into an inflammatory environment, as observed in the osteoarthritic joint. Awash with growth factors, cytokines and proteases, MSCs in the osteoarthritic joint can interact with these inflammatory mediators via cell surface receptors, thereby altering the signalling pathways that govern gene transcription. Current research suggests that these interactions result in the activation of signalling cascades driving chondrocyte hypertrophy, a state that promotes endochondral ossification and development of painful osteophytes (Van der Kraan and Van den Berg, 2012).

The mechanisms regulating chondrogenesis in the osteoarthritic joint are not fully elucidated, so this study aimed to address this issue by determining the effects of inflammatory mediators on the chondrogenic differentiation capacity of ihBMSCs *in vitro*. Each of the mediators presented in this study are

found to be upregulated in the osteoarthritic joint at concentrations similar to those tested, allowing for closer *in vivo* representation. Thus far, various experiments in the literature have cultured BMSCs with individual mediators. Although this helps to discriminate between the actions of mediators, any synergistic relationships that might be present in the osteoarthritic joint are lost. Therefore, to gain a clearer picture of MSC chondrogenesis in the osteoarthritic joint, I chose to further these findings through the combination of inflammatory mediators. To measure the effects on chondrogenic differentiation, production of the proteoglycan subunit GAGs was measured, whilst insight into the mechanisms of observed effects was gained through gene expression analysis.

4.4.1 Effects of inflammatory mediators on ihBMSCs during chondrogenic differentiation

The presence of IL-1β or combined inflammatory mediators in CM significantly altered the presence of GAGs in an ihBMSC cell line. Importantly, this data corroborates findings in the literature that show that the pro-inflammatory cytokine IL-1β impedes chondrogenic differentiation of BMSCs (Wehling *et al.*, 2009; Liu *et al.*, 2017). Data presented here extends these findings by showing that the presence of combined inflammatory mediators also reduced the production of GAGs by chondrocytes, though the reduction is less pronounced. To determine whether this is an effect of opposing inflammatory mediators or biological variability of cells, further experimental repeats should be undertaken. Furthermore, inflammatory conditions also decreased the level of nucleic acid detected in the samples. Although the levels of GAGs remained decreased upon normalisation to nucleic acid, the reduction in nucleic acid itself indicated that these inflammatory mediators also affected the number of cells in these conditions. One potential reason for

this is apoptosis of cells, a process that is required for subsequent endochondral ossification *in vivo*. To explore this theory, it would be beneficial to measure GAG and nucleic acid content at multiple timepoints during the chondrogenic differentiation protocol. This would help to gain a better understanding on whether inflammatory mediators reduce proliferation or cause cell death upon advancement of hypertrophy.

Individually, the other tested inflammatory mediators did not significantly affect chondrogenic differentiation of ihBMSCs. This data, therefore, does not corroborate findings presented in the literature.

Produced by OA chondrocytes, the pro-inflammatory chemokine IL-8 is found at elevated levels in the SF of OA patients (Borzi *et al.*, 1999; Pierzchala, Kusz and Hajduk, 2011; Goldring, 2012). This mediator is highly responsive to the mechanical, inflammatory and metabolic stresses that drive OA pathogenesis (Chauffier *et al.*, 2012), and subsequently has been shown to induce hypertrophic differentiation of chondrocytes, combined with increased production of MMP-13 (Merz *et al.*, 2003), chondrocyte apoptosis (Borzi *et al.*, 2002) and loss of proteoglycans (Matsukawa *et al.*, 1995). It has been postulated that IL-8 may exert some of its catabolic effects through increased production of the potent pro-inflammatory cytokine IL-1β (Matsukawa *et al.*, 1995), which directly contributes to cartilage degradation as previously described. It was therefore hypothesised that IL-8 would decrease observed GAG levels in *in vitro* differentiation of ihBMSCs. However, no change in GAG concentration could be detected in cells cultured with IL-8. One reason for this may be a limitation of the *in vitro* assay itself, as cells by day 21 are naturally expressing hypertrophic markers. As this does not represent the quiescent chondrocytes found in healthy cartilage, it may hide the catabolic effects of IL-8. In a similar way, another pro-inflammatory

chemokine that has been shown to promote chondrocyte apoptosis and increased expression of *MMP-3* and *MMP-13*, is MCP-1 (Xu *et al.*, 2015). This chemokine is of particular interest as it functions to recruit MSCs to a site of injury, but inhibits them from undergoing chondrogenesis *in vitro* (Harris *et al.*, 2013). Like IL-8, culture of ihBMSCs with MCP-1 in this experiment did not affect the levels of GAGs detected at day 21, contradicting findings that this chemokine inhibits chondrogenesis.

The cytokine IL-10 exerts some of its anti-inflammatory effects through inhibition of pro-inflammatory cytokine production and stimulation of B cells. In OA conditions the presence of IL-10 has been shown to not only prevent IL-1β mediated cartilage degradation, but also to stimulate proteoglycan synthesis (van Roon *et al.*, 1996), highlighting important anabolic effects that could be exploited in OA therapy. However, the addition of IL-10 to ihBMSCs undergoing chondrogenic differentiation did not affect the levels of GAGs in this study, nor did it counteract the effects of IL-1β when combined with this mediator. The expression of the IL-10 receptor, *IL10RB,* that allows IL-10 to interact with cells is known to be expressed in human chondrocytes and was detected in through these arrays. However, IL-10 did not appear to alter the expression of genes associated with chondrocyte hypertrophy in these experiments. Interestingly, Henrotin *et al.,* (2000) showed that IL-10 expression was undetectable in the absence of IL-1β, indicating that a synergistic relationship is essential for its anabolic effects, and may explain why no effect was observed during individual exposure.

Finally, the role of NGF in OA pathophysiology has been well established in the mediation of pain, and is found to be elevated in osteoarthritic SF (Miller, Miller and Malfait, 2014). However, the effect of NGF on chondrogenesis has been relatively unstudied. Recent work by Lu *et al.,* (2017) showed that NGF

upregulates expression of cartilage-specific markers during differentiation of rabbit BMSCs, without promoting chondrocyte hypertrophy. Data in this project did not confirm these findings in ihBMSCs that were cultured with NGF, as no change in GAG production could be detected compared to standard chondrogenic differentiation.

As suggested, limitations of the *in vitro* differentiation system may be masking more subtle effects of inflammatory mediators during chondrogenesis. Although it successfully recapitulates the sequence of events leading to chondrogenic differentiation *in vivo*, this process harnesses the intrinsic nature of MSCs to progress towards endochondral ossification rather than remaining in the stable phase seen in healthy cartilage (Chen *et al.*, 2015). Although no *in vitro* protocols are currently able to completely prevent initiation of a hypertrophic phenotype, adoption of a micromass culture system may help to minimise the impact of this, and thus may be useful for future work (Zhang *et al.*, 2010).

4.4.2 Effects of inflammatory mediators on gene expression of ihBMSCs during chondrogenic differentiation

To gain insight into the mechanisms underpinning IL-1β mediated chondrogenic differentiation during ihBMSC chondrogenic differentiation, gene expression analysis was undertaken. This allowed for investigation into a variety of signalling pathways involved in normal cartilage homeostasis, and highlighted that growth factors, proteases and members of TLR and MAPK signalling were affected by the presence of inflammatory mediators. Importantly, the expression of hypertrophic markers, such *COL10A1*, *MMP13*, *IHH* and *RUNX2* (Van der Kraan and Van den Berg, 2012), were also increased. Furthermore, although the gene expression of *IL1B* itself was not

seen to be increased in these cells, the gene expression of the IL-1β receptor, *IL1R1*, was significantly increased in all conditions cultured with chondrogenic medium. Despite the presence of inflammatory mediators not affecting the expression levels of this receptor it is important to note that the general increase during chondrogenesis means that these newly formed chondrocytes will be more sensitive to the presence of IL-1β in the medium, in a similar way to that seen in the osteoarthritic microenvironment.

Importantly, gene analysis experiments were normalised for cDNA input, therefore gene expression results were not influenced by the decreased level of nucleic acid observed in the DMMB assay.

4.4.2.1 Differentiation markers

Measurement of differentiation markers at day 21 of ihBMSC chondrogenesis revealed that expression of *ACAN* was decreased in cells exposed to inflammatory mediators, supporting findings that IL-1β reduces *ACAN* expression (Pfander *et al.*, 2004). Interestingly, although the levels of GAGs (measured in the DMMB assay) were not as significantly reduced in the presence of combined inflammatory mediators compared to IL-1β alone, the decreased expression of *ACAN* was more pronounced when inflammatory mediators were combined. This inconsistency may be due to the assays measuring different outputs (gene vs. protein), but may also result from biological variability of ihBMSCs *in vitro,* so further replicates would strengthen this dataset. Moreover, results from the DMMB assay suggested that a decreased number of cells in inflammatory conditions was partially contributing to the decreased levels of GAGs, however this measure of gene expression demonstrates that a reduction of GAG synthesis is also associated with lower proteoglycan gene expression.

Expression of other differentiation markers in this study are indicative of a hypertrophic phenotype. This is one of the greatest challenges when considering MSCs for cartilage repair, as chondrocytes derived from healthy hyaline articular cartilage represent a stable phenotype that does not undergo terminal differentiation (Mariani, Pulsatelli and Facchini, 2014). As described, many *in vitro* protocols induce features characteristic of fibrocartilage and chondrocyte hypertrophy (Zhang *et al.*, 2010; Chen *et al.*, 2015), as may be the case here. For example, expression of both *COL1A1* and *COL10A1*, features of fibrocartilage and hypertrophy respectively, were increased in all ihBMSCs undergoing chondrogenic differentiation at day 21. However, the addition of inflammatory mediators further increased the expression of other hypertrophy markers, such as *RUNX2*, *ALPL* and *IBSP*.

The role of RUNX2 in endochondral ossification is well established, as deletion of *Runx2* in mouse chondrocytes results in failure of endochondral ossification and lethality at birth (Chen *et al.*, 2014). Unlike SOX9 which is important in early chondrogenesis (Akiyama *et al.*, 2002), this transcription factor is considered an important player in promotion of chondrocyte hypertrophy and subsequent endochondral ossification (Chen *et al.*, 2014). The fact that the IL-1β condition in this experiment showed increased levels of *RUNX2* expression indicates that IL-1β may be activating signalling pathways that converge at this transcription factor, thereby initiating the process of ossification. Moreover, expression of *ALPL*, which is also involved in ossification, was increased in the presence of inflammatory mediators. Finally, *IBSP*, which encodes for bone sialoprotein (BSP), is another known marker of hypertrophic chondrocytes and is seen to be involved in mineralisation and neovascularistion of cartilage tissue (Pesesse *et al.*, 2014). Unlike the work by Pesesse *et al.*, (2014), the present work was able to show that IL-1β can upregulate expression of this gene.

Each of these described hypertrophy markers were increased in the presence of IL-1β alone, but their expression was seen to be dampened or lost by the presence of other inflammatory mediators. This indicates that other inflammatory mediators in the medium might be limiting the effects of IL-1β. It would therefore be useful to identify if this is the result of one particular mediator, which could be further investigated as a therapeutic strategy. This data also fits with the observation that levels of GAGs were more significantly reduced in IL-1β alone, and suggests that IL-1β in the osteoarthritic joint might not produce as potent effects as those seen in *in vitro* studies.

4.4.2.2 Mediators of cartilage degradation

Importantly, exposure of ihBMSCs to inflammatory mediators during chondrogenic differentiation significantly increased the expression of proteases that are known to contribute to OA cartilage destruction. MMP-13 is selectively secreted by hypertrophic chondrocytes as it is required for breakdown of ECM, which allows vascular and cellular invasion during endochondral ossification. As it is a marker of hypertrophy, it suggests that the differentiated ihBMSCs at 21 days of chondrogenic differentiation displayed high expression levels of *MMP13*. The presence of inflammatory mediators did not alter these levels. However, they did stimulate expression of other genes encoding for MMPs that were not increased during standard chondrogenic differentiation. The enhanced expression of both *MMP1* and *MMP3* suggests that ihBMSC-derived chondrocytes were directed by an inflammatory environment to produce factors that contribute to the progression of OA pathophysiology. This corroborates previous findings in the literature, and provides further evidence that the presence of other inflammatory mediators might not avert IL-1β driven expression of cartilage degrading enzymes; this includes the anti-inflammatory cytokine IL-10.

4.4.2.3 Signalling pathways

Despite the possible limitation of this *in vitro* protocol, it was important to identify that IL-1β and a combination of inflammatory mediators drives the process of terminal differentiation beyond that seen in CM alone. To identify how the described differentiation markers and mediators of cartilage degradation are induced, it is crucial to identify which signalling pathways are up- or down-regulated in the presence of an inflammatory environment.

Research into chondrocyte signalling frequently highlights the role of TGF-β/BMP signalling in both cartilage homeostasis and dysregulation (Mariani, Pulsatelli and Facchini, 2014). However, gene expression of ihBMSCs undergoing chondrogenic differentiation does not implicate canonical TGF-β/BMP signalling pathways (Smad-dependent) in the observed changes to chondrocyte hypertrophy and GAG production in this study. Instead, the Smad and BMP genes tested were either significantly decreased or unchanged during chondrogenic treatment, with or without inflammatory mediators. Interestingly, expression of the BMP receptor, *BMPR1B* was significantly increased in ihBMSCs cultured in CM, but this change was lost following exposure to inflammatory mediators, particularly IL-1β alone. Contradictory findings have been presented in the literature, whereby BMPs such as BMP-2 have been implicated in both induction of chondrocyte hypertrophy and cartilage protection; the latter being through a simultaneous increase of ECM protein synthesis and decrease of protease expression (van der Kraan, Blaney Davidson and van den Berg, 2010). The results presented here suggest that the postulated hypertrophic effects of BMPs are not observed during *in vitro* ihBMSC chondrogenic differentiation. Instead, the increased hypertrophic markers in chondrogenic cultures exposed to inflammatory mediators was observed in conjunction with lower expression levels of *BMPR1B*, suggesting

that these cells might be less receptive to the presence of BMPs, and therefore giving more credence to the hypothesis that BMPs act to preserve cartilage integrity. Although this information cannot be directly ascertained from this data, as cells expressing high levels of *BMPR1B* were still displaying features of hypertrophy, it does suggest that the therapeutic use of protective BMPs, such as the anabolic BMP-7 (Badlani *et al.*, 2009), could be limited if MSC-derived chondrocytes have been driven towards hypertrophy by an inflammatory environment.

Although no changes to Smad gene expression could be observed in this study, alterations to members of the non-canonical TGF-β/BMP signalling (Smad-independent) pathway were detected. Here the presence of IL-1β during ihBMSC chondrogenesis increased the expression of *MAPK1*, which encodes for ERK2, compared to CM alone. This work corroborates findings by Wang *et al.*, (2011) who showed that IL-1β activates the MAPK/ERK pathway in human chondrocytes, which directly contributes to increased expression of *MMP3* and *MMP13* and decreased expression of *ACAN.* This work extended these findings by showing that a physiologically relevant concentration of IL-1β causes the same increased effect on *MAPK1* expression as a supraphysiological concentration, however this was counteracted by the presence of other inflammatory mediators. This may begin to highlight pathway activation differences between variable inflammatory environments, although the narrow differences between expression changes indicate that it may constitute a false positive result. To investigate this further it would be useful to assess protein expression levels. Another gene affected by the presence of IL-1β was *MAPK9*, which encodes for JNK2. In a similar way, activation of JNK is associated with increased expression of MMPs in chondrocytes and may denote a mechanism for regulation of MMP expression in this study (Mariani, Pulsatelli and Facchini, 2014).

Other genes affected by the presence of inflammatory mediators during chondrogenesis include those for the growth factors *TGFB3*, *IGF1* and *FGF2*. TGF-β3 is involved in the anabolic actions of chondrocytes, contributing to increased synthesis of ECM proteins (Tang *et al.*, 2009). Moreover, IGF-1 has been linked to both the stabilisation of chondrocytes in their permanent, quiescent state and stimulation of proteoglycan synthesis (Bohme *et al.*, 1992). In this study, expression of both *TGFB3* and *IGF1* was decreased in ihBMSCs that had undergone chondrogenic differentiation for 21 days. However, this expression was decreased further in ihBMSCs cultured with inflammatory mediators, suggesting that a lack of TGF-β3 and IGF-1 is conducive to increased hypertrophy. In contrast, *FGF2* expression has been linked to many catabolic events in human chondrocytes, including upregulation of MMPs, stimulation of pro-inflammatory cytokines and antagonism of anabolic factors, such as BMP-7 (Li *et al.*, 2012; Mariani, Pulsatelli and Facchini, 2014). In this study a decrease in *FGF2* expression under chondrogenic conditions was prevented by the presence of inflammatory mediators, whilst MMP and pro-inflammatory cytokine expression was increased, thereby substantiating the findings that this growth factor may be involved in each of these catabolic processes.

Finally, expression of *TLR2* was increased by the presence of inflammatory mediators in this study, simultaneously affirming findings in the literature that IL-1β upregulates *TLR2* expression (Su *et al.*, 2005), and proving that this effect is not altered by the actions of other inflammatory mediators. Chondrocytes derived from OA cartilage have been shown to express elevated levels of TLRs, which increase further in conjunction with the severity of disease. A crucial component of the innate immune response, TLRs generate signalling cascades that result in the increased secretion of inflammatory

mediators and proteases. Therefore, it could be postulated that the increased *TLR2* expression observed in this study augmented expression of MMPs.

4.4.3 Conclusions

In the osteoarthritic joint, chondrocytes progress from their stable phenotype into a state of hypertrophy, which leads to increased secretion of cartilage degrading proteases, and stimulation of endochondral ossification processes that have been linked to development of painful osteophytes (Van der Kraan and Van den Berg, 2012). As the development of chondrocyte hypertrophy has been consistently linked to the inflammatory environment, it is therefore crucial to consider the effects of this when developing MSC therapeutic options. This will help to prevent transplanted cells from contributing to disease progression, and instead allow them to exert therapeutic effects that delay, prevent, or even reverse joint damage.

Data presented here shows that the exposure of ihBMSCs to IL-1β, a pro-inflammatory cytokine increased in the osteoarthritic joint, significantly altered the process of chondrogenic differentiation *in vitro*, as measured by decreased levels of GAGs. These effects were observed in the presence of IL-1β alone, or in combination with other inflammatory mediators (IL-8, IL-10, MCP-1 and NGF), though the effect was reduced in the latter.

Gene expression analysis revealed that this decreased quantification of GAGs is linked to increased chondrocyte hypertrophy markers, as shown by the increased expression of genes encoding for RUNX2, ALP and BSP in inflammatory chondrogenic conditions. In parallel to this was decreased expression of aggrecan and increased expression of proteases (MMPs), representing two of the key features driving OA cartilage breakdown. To

assess how IL-1β caused this change in chondrocyte phenotype, analysis of cell signalling pathways revealed the possible role of TLR and MAPK signalling in these cells. In contrast, the negative role of growth factors, that normally contribute to cartilage maintenance in the healthy joint, was illustrated through decreased expression in the inflammatory environment.

Although these *in vitro* conditions do not represent the complete complexity of the osteoarthritic joint, and the protocol is limited by inherent progression into the hypertrophic stage, it does begin to give insight into the effects of inflammatory mediators on MSCs during OA. The identification of pathways affected by these inflammatory mediators is important not only to gain a clearer insight into the pathogenesis of OA, but also to determine how MSCs could be used for successful targeted therapy. A summary of the key changes in ihBMSC gene expression during chondrogenic differentiation can be observed in Table **4.1**.

	CM	CM + mediators	CM + IL-1β
Gene	Fold change (log_2)	Fold change (log_2)	Fold change (log_2)
Chondrogenic differentiation and hypertrophy markers			
ACAN	4.50	2.26	3.19
RUNX2	0.09	0.54	0.94
ALPL	4.18	5.94	7.17
IBSP	-0.71	-0.05	3.21
IHH	7.57	7.51	7.19
Cartilage degradation mediators			
MMP13	2.76	2.99	3.20
MMP1	-1.20	1.15	1.07
MMP3	0.38	2.25	2.58
TGFβ/BMP signalling			
BMPR1B	3.76	2.39	1.54
MAPK1	-0.29	-0.19	0.27
MAPK9	0.49	0.77	1.12
Growth factors			
TGFB3	-0.29	-1.90	-2.00
IGF1	-0.92	-3.60	-3.39
FGF2	-0.79	-0.03	0.28
Toll like receptor signalling			
TLR2	1.98	4.95	4.82

Table 4.1 Key changes in ihBMSC gene expression during chondrogenic differentiation

Exposure of ihBMSCs to combined inflammatory mediators and IL-1β alone during chondrogenic differentiation resulted in significant changes to gene expression. Shown as the log_2 fold change from SM, genes encoding for chondrocyte hypertrophy markers, cartilage degrading enzymes, MAPKs and TLR-2 were significantly increased following exposure to these conditions. In contrast, the expression of *ACAN* and genes encoding for anabolic growth factors was significantly decreased.

Chapter 5 Analysis of the MSC osteogenic response after exposure to inflammatory mediators

5.1 Introduction

5.1.1 Bone remodelling in the healthy joint

5.1.1.1 Bone remodelling process

Bone is a metabolically active organ that constitutes the skeleton and, besides its role in locomotion, also functions as the structural support for soft tissues and internal organs, a storage site for minerals such as calcium and phosphate, and a reservoir for HSCs and MSCs in the bone marrow (Florencio-Silva *et al.*, 2015). In order to maintain skeletal integrity and function, a tightly controlled mechanism of bone remodelling is employed, whereby bone resorption and formation are finely balanced (Hadjidakis and Androulakis, 2006). In healthy adult bone, this coupling of resorption and formation allows adaptation to mechanical load and stress, repairing damage such as microfractures, as well as maintenance of bone volume and calcium homeostasis (Yamashita, Takahashi and Udagawa, 2012).

Bone remodelling can be defined by a series of highly coordinated, consecutive phases: activation, resorption, reversal, formation and termination (Raggatt and Partridge, 2010). For successful remodelling, and hence preservation of bone mass, this process of events is reliant on the precise spatio-temporal activation of multiple cells and signalling pathways in response to the microenvironment. Thus, an imbalance in this system can result in diseases such as osteoporosis and osteoarthritis (Neve, Corrado and Cantatore, 2011; Burr and Gallant, 2012). Two of the key cells responsible in

bone remodelling are osteoclasts and osteoblasts, for bone resorption and formation respectively (Hadjidakis and Androulakis, 2006).

Derived from the macrophage/monocyte lineage, pre-osteoclasts react to changes in the bone microenvironment, signalling the requirement and initiation of bone turnover. Following this activation, pre-osteoclasts fuse at required sites and differentiate towards multinucleated osteoclasts, in a process called osteoclastogenesis (Neve, Corrado and Cantatore, 2011; Crane and Cao, 2014). This allows them to adhere to and dissolve bone through acidification pathways during the resorption phase (Rousselle and Heymann, 2002). In order to avoid excessive resorption, osteoclasts undergo apoptosis, and reversal cells, of unknown phenotype, clean up the organic matrix that has been released (Abdelgawad *et al.*, 2016). It is postulated that these reversal cells, combined with the release of growth factors, cytokines and other proteins from the degraded bone matrix, are critical for the coupling of bone resorption and formation, as the functions of these released factors include MSC recruitment and differentiation, for the replacement of resorbed bone (Mohan and Baylink, 1991). Initially MSCs committed to the osteogenic lineage differentiate into proliferating pre-osteoblasts, before their full maturation into osteoblasts (a process termed osteogenesis). These cells are responsible for the synthesis of new bone matrix, as well as subsequent promotion of its mineralisation (Caplan, 1991; Pittenger, 1999). Both of these steps are regulated through the expression of proteins, including alkaline phosphatase (ALP) and type I collagen for matrix synthesis, and osteocalcin, osteopontin and osteonectin for matrix mineralisation (Eriksen, 2010). Upon mineralisation, termination of the bone formation phase is observed by apoptosis or transformation of osteoblasts into bone lining cells or osteocytes, the latter of which constitutes 90% to 95% of adult bone tissue (Schaffler and

Kennedy, 2012). An overview of this bone remodelling process can be seen in Figure **5.1**.

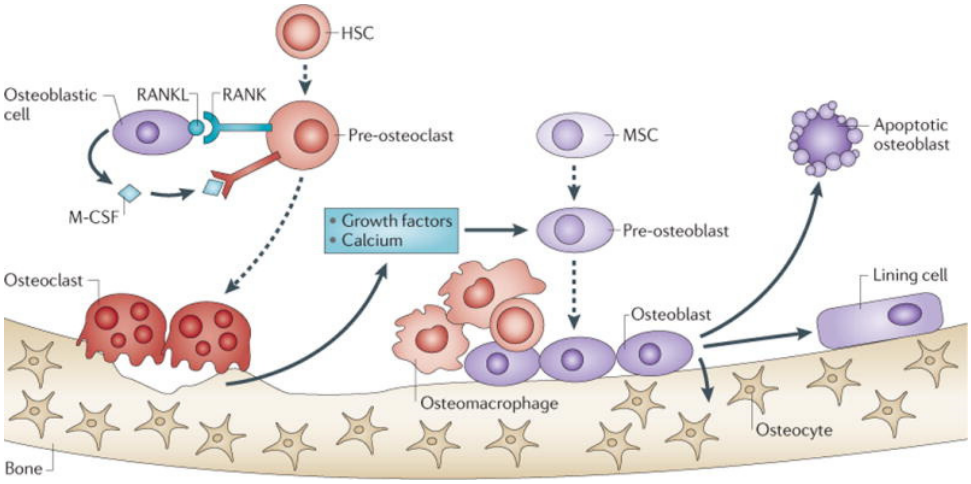


Figure 5.1 Process of bone remodelling in the healthy joint

Bone remodelling in the healthy joint relies on the tight regulation of both bone resorption by osteoclasts and subsequent bone formation by osteoblasts. Release of growth factors and other proteins from the bone matrix stimulates the differentiation of MSCs into osteoblasts in order to replace the resorbed bone and regulate the process of osteoclastogenesis (Weilbaecher, Guise and McCauley, 2011).

Although the main functions of MSC derived osteoblasts lay in the synthesis and mineralisation of bone, as described, their role also extends to the regulation of osteoclastogenesis (Neve, Corrado and Cantatore, 2011). This occurs through various mechanisms, with the most widely studied being the OPG/RANKL/RANK pathway, in which RANKL (receptor activator of nuclear factor kappa-B ligand) expressed on the surface of pre-osteoblasts binds to pre-osteoclasts via RANK (receptor activator of nuclear factor kappa-B), stimulating their maturation into osteoclasts (Simonet *et al.*, 1997; Khosla, 2001). This process is overseen by OPG (osteoprotegerin), which can block the

activity of RANKL, and has led to the hypothesis that the ratio of these two mediators controls osteoclastogenesis (Hadjidakis and Androulakis, 2006). Other mechanisms independent to this includes the production of WNT5A by osteoblasts, which upregulates RANK expression to enhance osteoclast differentiation (Yamashita, Takahashi and Udagawa, 2012). Moreover, other local factors can modulate this system, including cytokines such as TNF-α and IL-10 that can directly increase RANKL expression (Hadjidakis and Androulakis, 2006). Taken together, this information displays the important and complex interactions that govern bone remodelling, and starts to uncover some of the signalling pathways involved in this process. Some of the most widely studied pathways will now be discussed in further detail.

5.1.1.2 Signalling pathways in bone remodelling

As previously described, bone homeostasis relies on precise regulatory mechanisms between osteoclast and osteoblast activity, including the termination of osteoclast bone resorption and recruitment of MSCs for osteogenic differentiation into osteoblasts (osteogenesis). As MSCs can differentiate into other mesenchymal cell lineages, a range of growth factors, cytokines and other proteins in the microenvironment are essential for determining their commitment into the osteoblast lineage (James, 2013). These factors may be stored and secreted from the bone matrix or produced locally in the joint in response to osteoclast bone resorption. Alternatively, they may be systemic and arrive through the vasculature via angiogenesis, as blood vessels are in close proximity to bone remodelling sites (Florencio-Silva *et al.*, 2015).

As growth factors and cytokines are hydrophilic molecules unable to penetrate the cell membrane, they exert their effects by binding to receptors

on the surface of target cells. This interaction activates specific intracellular signalling cascades, that ultimately target and stimulate transcription factors involved in the regulation of osteogenesis (Hayrapetyan, Jansen and van den Beucken, 2015). The targeted transcription factors include those belonging to the runt-domain gene family, such as RUNX2 (also called Core-binding factor alpha, CBFA1) (Yamaguchi, Komori and Suda, 2009) that is often described as the master transcription factor of osteogenesis. In fact, *Runx2*$^{-/-}$ mice exhibit a complete lack of bone formation due to the arrest of osteoblast maturation at an early stage of differentiation (Komori *et al.*, 1997). Another transcription factor that runs downstream of RUNX2 is Osterix (Osx). Important in osteoblast commitment, knockdown of this gene in mice results in defective bone formation (Huang and Olsen, 2015), though its effects are reliant on the presence of other mediators, such as BMP-6 (Zhu *et al.*, 2012). Many of these signalling pathways governing transcription, including TGF-β/BMP and Wnt, are well defined for a variety of cellular processes, and their specific roles in the process of bone remodelling are coming to light (Hayrapetyan, Jansen and van den Beucken, 2015). For example, it is well regarded that the systemic factor parathyroid hormone, is a key orchestrator of the signalling pathways driving MSC migration and differentiation (Crane and Cao, 2014).

TGF-β/BMP signalling pathways are imperative across a broad range of cellular processes, including bone remodelling. Members of the TGF-β superfamily (e.g. TGF-β, BMPs) are found in abundance in the bone matrix and are released and activated by osteoclasts to induce migration of MSCs to resorption pits (Crane and Cao, 2014). Moreover, they also stimulate the production of other growth factors, like FGF2, that positively influence osteoblast differentiation (Sobue *et al.*, 2002). Signal transduction of the TGF-β/BMP pathway can be either canonical (Smad dependent) or non-canonical (Smad independent), the latter of which is through the p38 MAPK pathway.

The MAPK signalling cascade has a distinct role in regulating osteogenesis, as it is involved in both ALP and osteocalcin expression in osteoblasts (Hayrapetyan, Jansen and van den Beucken, 2015). The MAPK pathway also transduces signals from FGF2 in order to phosphorylate and activate RUNX2, and this growth factors importance has been highlighted by *Fgf2*$^{-/-}$ mice that are shown to have decreased bone mass (Hayrapetyan, Jansen and van den Beucken, 2015). However, it should be noted that the temporal activity of these signalling pathways may be the crucial element for successful osteogenesis, as although both TGF-β and FGF2 have been shown to induce osteoblast proliferation, they also seem to inhibit both ALP activity and osteoblast mineralisation (Bosetti *et al.*, 2007).

Other growth factors found in the bone remodelling microenvironment include IGF-1 and platelet derived growth factor (PDGF) (Neve, Corrado and Cantatore, 2011). IGF-1 is known to activate MSC differentiation into the osteoblast lineage (Xian *et al.*, 2012), and is also a potent chemotactic factor that may contribute to osteogenesis through the recruitment of osteoblasts to resorption sites (Nakasaki *et al.*, 2008). Similarly, PDGF also functions at sites of resorption to mobilise MSCs (Caplan and Correa, 2011a). Receptors for each of the described growth factors have been identified on osteoblasts themselves, indicating that these cells regulate activity in an autocrine manner, as well as exerting paracrine effects (Hadjidakis and Androulakis, 2006).

Another important signalling pathway involved in bone remodelling is the Wnt signalling pathway, which exhibits both pro-osteogenic and anti-adipogenic activity (James, 2013). Activated in MSCs and osteoblasts, it has been found to be important in differentiation and proliferation respectively, exerting its effects through canonical (β-catenin dependent) or non-canonical

(β-catenin independent) routes (Kim *et al.*, 2015). The role of Wnt signalling in bone formation was first highlighted by low density lipoprotein receptor-related protein (LRP)-5 gene mutational studies showing that reduced bone mass is a consequence of loss of function mutations (Gong *et al.*, 2001). In contrast, *LRP5* gain of function mutations leads to a high bone mass phenotype (Boyden *et al.*, 2002; Little *et al.*, 2002). As LRP5 is a key component of the transmembrane complex (Frizzled/LRP5/LRP6) that binds Wnt ligands for stimulation of the canonical Wnt signalling cascade, research into the β-catenin dependent pathway took precedence. Subsequently, confirmation of its role in bone formation has been repeatedly presented in the literature, as β-catenin associates with transcription factors, such as RUNX2 and Osx, that determine MSC commitment towards the osteoblast lineage (Kobayashi *et al.*, 2016). Its role in bone remodelling is not limited to osteoblast differentiation, however, as it has been shown to indirectly repress osteoclast differentiation, and thus bone resorption, by the secretion of OPG (Baron and Kneissel, 2013). More recently, evidence for the role of non-canonical Wnt signalling in bone remodelling is emerging and, although less well studied, the β-catenin independent pathways have an important function in promotion of both bone formation and resorption. In particular, WNT5A has been shown to activate non-canonical Wnt/Ca^{2+} and Wnt/planar cell polarity pathways to promote osteoblast differentiation (Kobayashi *et al.*, 2016). Furthermore, *WNT5A* knockdown results in suppression of osteoblast markers (Sonomoto *et al.*, 2012). Unlike ligands that stimulate the canonical Wnt signalling pathway, WNT5A has also been shown to increase the expression of RANK on pre-osteoclasts, thereby inducing osteoclastogenesis (Maeda *et al.*, 2012; Kobayashi *et al.*, 2016).

Roles of other signalling pathways in bone remodelling are continuously being uncovered. This includes, but is not limited to, Hedgehog signalling,

whereby IHH has an essential function in promoting osteoblast differentiation (Yang *et al.*, 2015), and Notch signalling, which conversely is important in suppression of osteoblast differentiation (Regan and Long, 2014). Moreover, expression of toll like receptors (TLRs) has been linked to enhanced osteogenic differentiation (Shirjang *et al.*, 2017). This brief overview begins to reveal the multitude of signalling pathways that govern the complex system of homeostatic bone remodelling. With this in mind, it is unsurprising to learn that significant shifts in the microenvironment, as seen in diseased states, alters the process and role of bone remodelling.

5.1.2 Bone remodelling in the osteoarthritic joint

5.1.2.1 Evidence of altered bone remodelling in OA

Early analysis of osteoarthritic bone provided the first indications of abnormal bone remodelling in OA, highlighting the fact matrix deposition exceeds mineralisation (Batra and Charnley, 1969; Reimann, Mankin and Trahan, 1977). However, due to the initial focus of OA pathogenesis on cartilage degradation, the role of subchondral bone in OA was largely overlooked. Since then, these findings have been replicated in femoral heads to show that bone located in the subchondral zone of osteoarthritic joints simultaneously exhibit increased bone collagen metabolism and decreased collagen mineralisation, compared to healthy joints (Grynpas *et al.*, 1991). Therefore, despite the bone sclerosis and thickening observed in OA, a decrease in bone density through hypomineralisation means that the quality of bone is reduced (Li and Aspden, 1997). Moreover, this combined increase in bone volume and hypomineralisation is primarily observed at high load bearing sites. As the mechanical load properties on articular cartilage are therefore altered, this aberrant bone remodelling is postulated to contribute to cartilage degradation (Zhen *et al.*, 2013). One mechanism that this may occur through is an increased

incidence of microfractures initiating reparative bone remodelling events by decreasing the OPG:RANKL ratio, as observed in animal models (Burr and Gallant, 2012).

Evidence has now revealed that the rate of altered bone remodelling in OA changes over the course of the disease. Early OA is characterised by an increase in bone remodelling and vascularity, whereas late OA displays the characteristic phenotypes described in the above studies due to an imbalance between bone resorption and formation; the former being reduced whilst the latter is increased (Findlay and Atkins, 2014). This drive towards bone formation in late OA could be linked to the functional capacity of MSCs at this stage of disease, as they possess substantially reduced chondrogenic and adipogenic differentiation potential, but increased ability to undergo osteogenesis (Murphy *et al.*, 2002). With this in mind, it is interesting to note that a clinical feature of OA is the formation of vascularised, bony outgrowths called osteophytes, which are thought to result from the inappropriate recruitment and activation of MSCs (van der Kraan and van den Berg, 2007) (Figure **5.2**). Controversy surrounding their purpose is present, with some studies implicating their role in stabilisation of the joint to protect cartilage from further degradation (Brandt, 1999), whilst others illustrate their role in OA pain (Lanyon *et al.*, 1998), in part due to the positive correlation between cartilage destruction and osteophyte formation (Van Osch *et al.*, 1996).

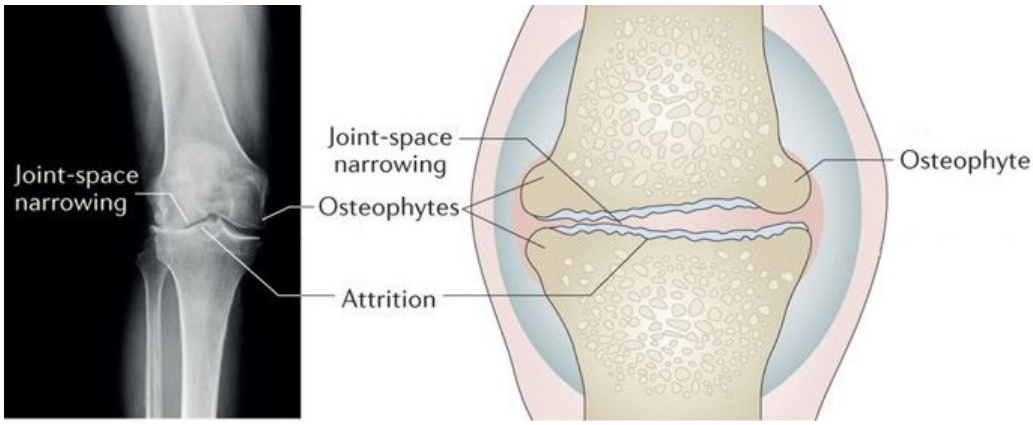


Figure 5.2 Location of osteophytes in the knee OA joint

Bony outgrowths, called osteophytes, are a radiographic feature of advanced OA, along with joint space narrowing and bone attrition. They are located at the margin of the joint and are thought to result from inappropriate recruitment and activation of MSCs (Adapted from Goldring and Goldring, 2016).

As well as a change in phenotype, osteoblasts derived from OA bone also display differential gene expression compared to osteoblasts from osteoporotic or healthy bone. For example, expression of the osteogenic differentiation markers ALP and osteocalcin are both found to be increased in OA osteoblasts (Couchourel *et al.*, 2009). Furthermore, the ratio of calcium: phosphate in osteoblast mineralisation exhibits much greater variability in OA bone (Kumarasinghe *et al.*, 2012). These changes in gene expression can help to understand phenotypic alterations, but a clearer understanding into the ligands and signalling pathways directing this shift in gene expression is required to understand the process of altered bone remodelling in OA.

5.1.2.2 Process of altered bone remodelling in OA

Ectopic bone formation can be observed in diseases with high inflammatory cytokines (Collison, 2018), and reduced by anti-inflammatory drugs (Fransen and Neal, 2004), illustrating the importance of the microenvironment for the success of homeostatic bone remodelling. As the OA joint is characterised by

infiltration of cells and mediators of inflammation, the concentration changes of growth factors and cytokines that promote balanced bone remodelling in the healthy joint are now implicated in aberrant bone remodelling. They subsequently interact with osteoclasts, osteoblasts and MSCs in the joint, controlling their activity through induction of signalling pathways. One way is through altered expression of OPG and RANKL, however the specific roles of both signalling pathways and their ligands in this process remains unclear (Maruotti, Corrado and Cantatore, 2017).

It is known that one group of ligands linked to altered bone remodelling in OA are members of the TGF-β superfamily, which in the healthy joint are essential for the migration of MSCs to bone resorption sites, and for indirect stimulation of factors involved in MSC differentiation (Crane and Cao, 2014). Therefore, it is unsurprising that alterations in their local concentrations have been linked to aberrant bone remodelling. In OA, osteoblasts display increased production and activation of TGF-β, contributing to synovial thickening and formation of osteophytes (Scharstuhl *et al.*, 2002, 2003), whilst targeted inhibition of TGF-β in mouse and rat models attenuates OA development (Zhen *et al.*, 2013). To promote an OA phenotype, increased levels of TGF-β is likely to primarily exert its effects through increased recruitment of MSCs. In clinical and animal models of OA, an increase in TGF-β levels, and thus increased accumulation of MSCs, can be observed in the joint. In the healthy joint tissue these MSCs are found at resorption sites, but the altered microenvironment of OA leads to osteoblast commitment of MSCs in bone marrow cavities, resulting in the formation of bone marrow lesions (Zhen *et al.*, 2013). These lesions are characterised by poorly mineralised bone, and are associated with increased pain and cartilage damage (Dore *et al.*, 2010).

Other pro-inflammatory cytokines found in the osteoarthritic joint include IL-1β, which increases human MSC osteogenesis *in vitro* via the Wnt-5a/receptor tyrosine kinase-like orphan receptor 2 pathway (Sonomoto *et al.*, 2012), MAPK pathway (Ferreira *et al.*, 2013), NF-κB pathway and BMP-2 pathway (Mao *et al.*, 2016). In addition, this mediator has also been proven to promote bone loss through activation of osteoclastogenesis (Kim *et al.*, 2009).

Although these findings validate the importance of the microenvironment on bone remodelling, poor understanding of the signalling pathways governing these effects prevents development of targeted treatments for OA. Therefore, to gain a better understanding of deregulated bone remodelling, a closer look at the spatio-temporal activation of signalling pathways by ligands present in the osteoarthritic joint needs to be undertaken.

5.1.3 Osteogenic differentiation of MSCs *in vitro*

To initiate and assess the differentiation capacity of MSCs *in vitro*, inclusion of lineage specific growth factors and chemicals such as ascorbic acid, β-glycerophosphate and dexamethasone in serum rich culture medium can be adopted (Pittenger *et al.*, 1999). The differentiation response can subsequently be measured through a number of techniques, including quantitative assessment of alkaline phosphatase activity (Sabokar *et al.*, 1994) and semi-quantitative assessment of mineral deposition via Alizarin Red S staining (Gregory *et al.*, 2004). Moreover, the genetic and proteomic profile of the MSCs can be analysed to highlight the expression of genes and proteins associated with the osteoblast phenotype as opposed to the MSC phenotype.

Following proliferation of MSCs, the early stages of *in vitro* differentiation are characterised by the expression of transcription factors RUNX2 and Osx as

they drive MSC commitment towards the osteoblast lineage (Birmingham *et al.*, 2012). Proliferation of the cells declines as they begin to differentiate, and expression of ALP activity is induced up until around day 14 (Aubin, 2001), along with expression of collagen type 1 (*COL1A1*) that provides the matrix for mineral deposition (Quarles *et al.*, 1992). Following this, expression of late markers such as osteocalcin and osteopontin can be observed in conjunction with the deposition of calcium and phosphate (Huang *et al.*, 2007) (Figure **5.3**).

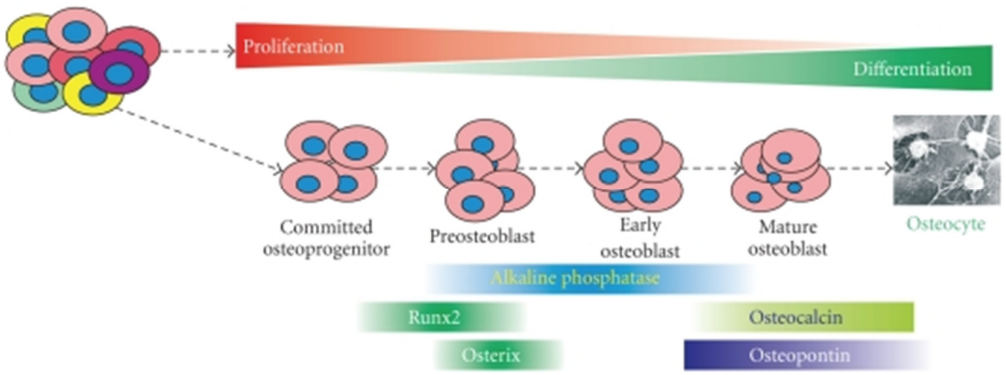


Figure 5.3 Key differentiation markers in osteogenesis

Osteogenic differentiation of MSCs can be characterised by a number of key markers. During early differentiation, committed osteoprogenitors proliferate before differentiating into pre-osteoblasts. Expression of transcription factors RUNX2 and Osx can be observed during this stage as proliferation begins to fall. ALP activity can also be measured as cells differentiate further into early osteoblasts. Following this, maturation of osteoblasts and mineralisation occurs as measured by expression of late markers osteocalcin and osteopontin (Adapted from Wagner *et al.*, 2010).

5.1.4 Aims and Hypotheses

The general aim of the *in vitro* study presented in this chapter was to identify the effect of inflammatory mediators, found to be upregulated in osteoarthritic tissue, on the osteogenic differentiation capacity of BMSCs.

To achieve this, BMSCs were cultured in osteogenic medium supplemented with inflammatory mediators and the ALP activity and mineral deposition was measured to establish any change to the osteogenic process. Due to a wealth of conflicting data in the literature, potentially due to differences in cell types and species, this work was performed using a murine BMSC cell line, an ihBMSC cell line and human primary BMSCs. Use of these different cell types would allow for the confirmation of species differences and therefore help to identify the effects that are more likely to be observed in human clinical trials. Following this, any changes to an ihBMSC cell line were evaluated by TaqMan Low Density Arrays (TLDAs) in order to identify changes to signalling pathways governing differentiation.

Based on the existing literature, it was hypothesised that the inclusion of pro-inflammatory mediators and growth factors (IL-1β, IL-8, MCP-1, NGF) in osteogenic medium would increase the rate of osteogenesis in human BMSCs through upregulation of non-canonical TGF-β/BMP and Wnt signalling pathways, as well as upregulation of pro-inflammatory cytokines that create a positive feedback loop (Sonomoto *et al.*, 2012; Xie *et al.*, 2016; Yasui *et al.*, 2017). In contrast, it was hypothesised that pro-inflammatory mediators would decrease the rate of osteogenesis in murine BMSCs (Lacey *et al.*, 2009), confirming effects of species differences. It was also hypothesised that anti-inflammatory mediators (IL-10) would slow the rate of osteogenesis BMSCs (Van Vlasselaer *et al.*, 1994).

5.2 Methods

For more detailed methods describing MSC culture and mediator exposure please see Chapter 2 Materials and Methods.

5.2.1 Osteogenic Differentiation

Osteogenic differentiation was induced in adherent murine BMSCs, ihBMSCs and human primary BMSCs cultured in osteogenic medium (OM) over a period of 21 days. OM consisted of standard medium (SM) supplemented with 100nM dexamethasone (Sigma-Aldrich), 0.05mM L-ascorbic acid-2-phosphate (Sigma-Aldrich) and 10mM β-glycerophosphate (Sigma-Aldrich) (Jaiswal *et al.*, 1997; Rashidi *et al.*, 2012). ihBMSCs and human primary BMSCs were seeded at $4x10^3$cells/cm^2 in SM in a 48-well plate or T25 flask (ThermoFisher), and incubated for 24 hours at 37°C, 5% CO_2 to allow for attachment. Murine BMSCs were seeded at $1x10^4$cells/cm^2 in SM in a 48-well plate (ThermoFisher), with the same incubation protocol followed. After this time, the medium was replaced with OM with or without inflammatory mediators. The medium was replaced three times a week for 7, 10, 14 and 21 days, and the differentiation status assessed by alkaline phosphatase activity, alizarin red staining and gene expression levels gained through TLDAs. Figure **5.4** provides a schematic of the experimental design.



Figure 5.4 Schematic of the osteogenic experimental design

5.2.2 Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) is an early marker for osteogenic differentiation and was measured using sigmaFAST™ p-Nitrophenyl phosphate tablets (Sigma-Aldrich). The ALP assay was performed according to manufacturer's instructions. Briefly, wells were washed twice with PBS before 300µl of p-Nitrophenyl substrate was added to each well. Samples were immediately transferred to an Infinite 200 microplate reader at 37°C, and absorbance was read at 405nm every 5 minutes until exhaustion of the reaction substrate occurred. For subsequent analysis, the slope of the reaction was obtained to

derive the rate of ALP activity (μM/min). This data was plotted as the fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons.

5.2.3 Alizarin Red S Staining

Alizarin Red S staining (Sigma-Aldrich) was used to measure mineralisation through the staining of calcium phosphate deposits. Fixed cells were washed twice with PBS before addition of a 1% (w/v) Alizarin Red solution (300μl). The plate was incubated for 10 minutes at RT and the cells were washed with sterile distilled water to remove excess stain. To observe mineralisation, brightfield images were then acquired using an Eclipse TS100 inverted microscope (Nikon). To quantify mineralisation, 200μl of de-staining solution (20% (v/v) methanol, 10% (v/v) acetic acid, 70% distilled water) was added to each well and incubated for 15 minutes with gentle agitation, after which samples were transferred to a 96 well plate and read on an Infinite 200 microplate reader at 405nm. For subsequent analysis, the data was plotted as the fold change from OM and a Kruskal Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons.

5.2.4 TaqMan Low Density Arrays

TLDAs (ThermoFisher) were used to measure changes in gene expression of ihBMSCs during osteogenic differentiation. For more detailed methods on cDNA preparation and validation, and TLDAs please see Chapter 2 Materials and Methods.

Briefly, ihBMSCs were cultured in SM, OM, OM + mediators or OM + IL-1β for 10 days, after which time cells were washed twice with PBS and resuspended in Tri-reagent (Sigma Aldrich). RNA was subsequently extracted from the cells using a RNeasy Micro Kit (Qiagen), according to manufacturer's instructions. The RNA concentration and purity were then assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA) and, following this, cDNA was synthesised from 2ug of total RNA using a High-Capacity RNA-to-cDNA Kit (ThermoFisher), according to manufacturer's instructions. Successful cDNA synthesis was confirmed by RT-PCR and gel electrophoresis, upon which 384-well microfluid TaqMan array cards were loaded with cDNA and run on the 7900HT Fast-Real Time PCR System using SDS RQ Manager Software (Applied Biosystems). The generated Ct values were normalised to the geometric mean of glucuronidase beta (*GUSB*) and TATA-box binding protein (*TBP*), which were identified as the most stable reference genes. The data was then corrected for baseline and the $\log_2$ fold change from SM plotted using GraphPad Prism 7 (GraphPad Software, San Diego, CA). A Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's test for post hoc multiple comparisons.

5.3 Results

5.3.1 Response of murine BMSCs to inflammatory mediators during osteogenic differentiation

Initial studies investigated the effects of inflammatory mediators (IL-1β, IL-8, IL-10, MCP-1, NGF) on murine BMSCs during osteogenic differentiation. Each of these mediators were either added individually to OM or in combination, the latter of which was chosen to better replicate the osteoarthritic microenvironment, where a range of both pro- and anti-inflammatory mediators are present. Effects of these treatments were assessed through the measurement of ALP activity and mineral deposition after 7 and 10 days.

Early osteogenic differentiation is characterised by the transcription and protein expression of ALP, leading it to act as the primary osteoprogenitor marker during *in vitro* differentiation. In this study there was a significant increase in ALP activity ($p=0.005$) between cells that had been cultured in SM and those cultured in OM, confirming successful differentiation of these cells. Interestingly, exposure to OM + mediators ($p=0.033$) and OM + IL-1β ($p=0.050$) significantly reduced the ALP activity of these cells compared to OM alone, though ALP activity can still be detected. None of the other individually added inflammatory mediators significantly altered the ALP activity of the cells compared to OM alone (Figure **5.5**).

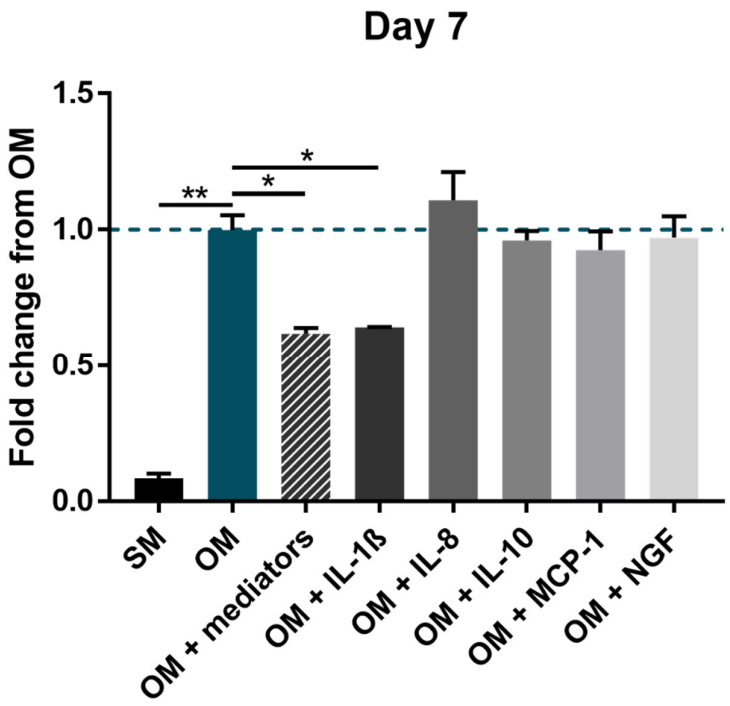


Figure 5.5 ALP activity in murine BMSCs during osteogenic differentiation after exposure to inflammatory mediators

ALP activity (μM/min) was quantitatively measured in murine BMSCs after exposure to inflammatory mediators for 7 days. Results were plotted as fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The presence of inflammatory mediators in OM significantly decreased ($p=0.033$) ALP activity compared to OM alone, as did the presence of IL-1β in OM ($p=0.050$). No change in ALP activity could be observed from the addition of other individual inflammatory mediators in OM. n=3 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

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As osteogenic differentiation progresses, terminal differentiation and matrix mineralisation occurs, as observed by the deposition of calcium and phosphate. After 7 and 10 days exposure, cells cultured in OM showed successful mineral deposition compared to SM. However, there was a significant decrease in detected calcium at day 7 compared to OM when cells were treated with either OM + mediators ($p=0.044$) or OM + IL-1β ($p=0.050$). This decrease was also observed at day 10, though it was not significant at this timepoint, indicating that the treatments delayed mineral deposition and slowed down the ability of the murine BMSCs to take on the required features of the differentiation process. None of the other individually added inflammatory mediators appeared to significantly alter levels of mineral deposition compared to OM (Figure **5.6**).

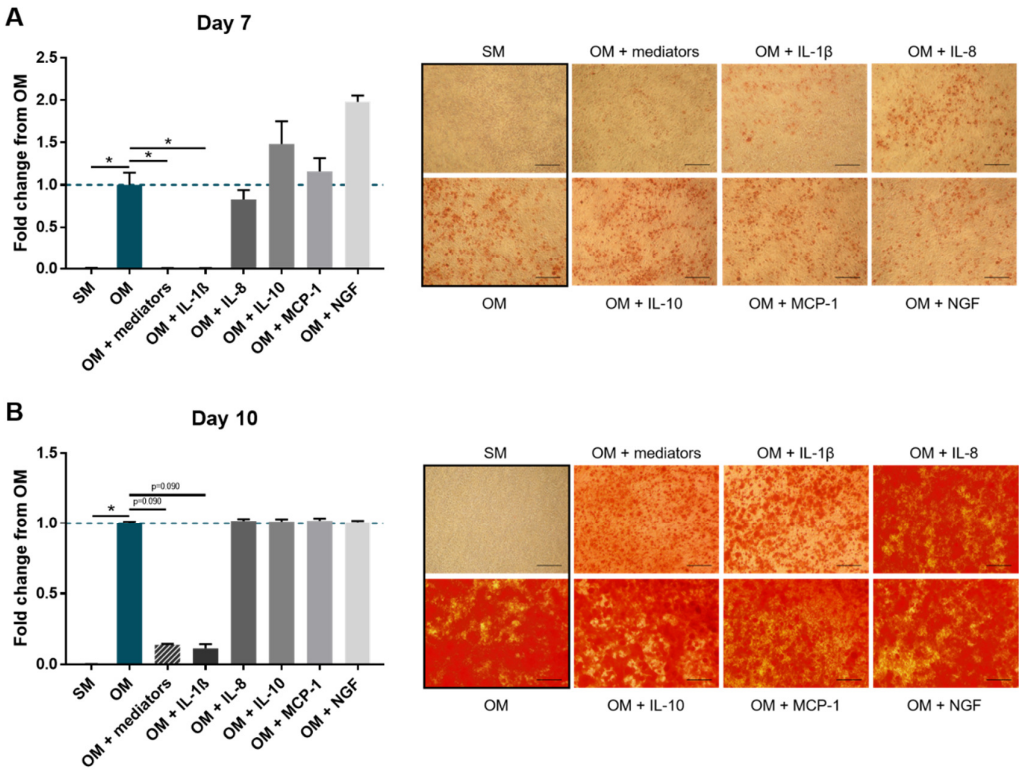


Figure 5.6 Levels of mineral deposition during osteogenic differentiation of murine BMSCs after exposure to inflammatory mediators

Mineral deposition during osteogenic differentiation of murine BMSCs was semi-quantitatively measured by Alizarin Red S staining after exposure to inflammatory mediators for 7 (**A**) and 10 (**B**) days. Results were plotted as fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. A significant decrease in mineral deposition could be observed at day 7 in the OM + mediators ($p=0.044$) and OM + IL-1β ($p=0.050$) conditions compared to OM alone (**A**), though deposition could still be detected. This decrease in mineral deposition could also be observed at day 10 in both the OM + mediators and OM + IL-1β conditions, though it was no longer significant ($p=0.090$) (**B**). No change in mineral deposition could be observed from the addition of other individual inflammatory mediators in OM. n=4 biological replicates, * $p<0.05$. Error bars represent SEM. Scale bars = 150μM.

5.3.2 Response of ihBMSCs to inflammatory mediators during osteogenic differentiation

Following treatment of murine BMSCs, ihBMSCs from an immortalised cell line were investigated to evaluate the effects of inflammatory mediators (IL-1β, IL-8, IL-10, MCP-1, NGF) on osteogenic differentiation. As the *in vitro* differentiation of ihBMSCs has a longer temporal profile compared to murine BMSCs, ALP activity was assessed after 7 and 10 days, whilst mineral deposition was measured after 14 and 21 days.

In this study, there was an increase in ALP activity between cells that had been cultured in SM and those cultured in OM, confirming successful differentiation of cells. Further to this, there was also a significant increase in ALP activity at both day 7 ($p=0.046$) and day 10 ($p=0.016$) when cells had been exposed to OM + mediators. ALP activity was also significantly increased at day 10 differentiation by the presence of IL-1β in OM ($p=0.005$). None of the other individually added inflammatory mediators significantly altered the ALP activity of the cells compared to OM alone (Figure **5.7**).

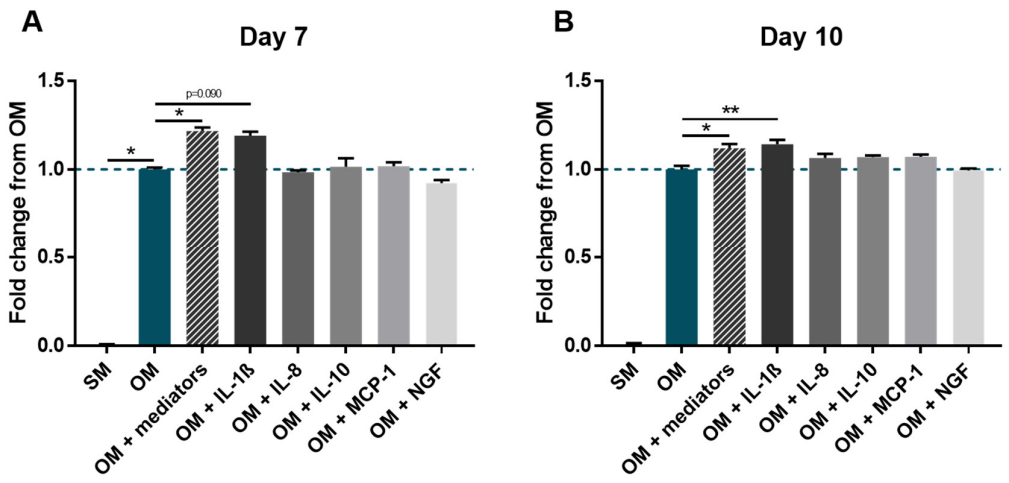


Figure 5.7 ALP activity in ihBMSCs during osteogenic differentiation after exposure to inflammatory mediators

ALP activity (μM/min) was quantitatively measured in ihBMSCs after exposure to inflammatory mediators for 7 (**A**) and 10 (**B**) days. Results were plotted as fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The presence of inflammatory mediators in OM significantly increased (p=0.032) ALP activity compared to OM alone at day 7 (p=0.046) and day 10 (p=0.016). The presence of IL-1β in OM also increased ALP activity by day 10 (p=0.005). No change in ALP activity could be observed from the addition of other individual inflammatory mediators in OM. n=4 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

Following assessment of ALP activity, levels of mineral deposition were measured at day 14 and 21 by Alizarin Red S staining. This measurement further confirmed that treatment of OM with combined mediators or IL-1β alone affected osteogenesis, as there was significantly greater deposition observed in these conditions at day 14 (OM + mediators $p=0.036$; OM + IL-1β $p=0.023$). By day 21, mineral deposition levels were similar across all treatment types in which cells were cultured with OM. None of the other individually added inflammatory mediators significantly altered levels of mineral deposition compared to OM (Figure **5.8**).

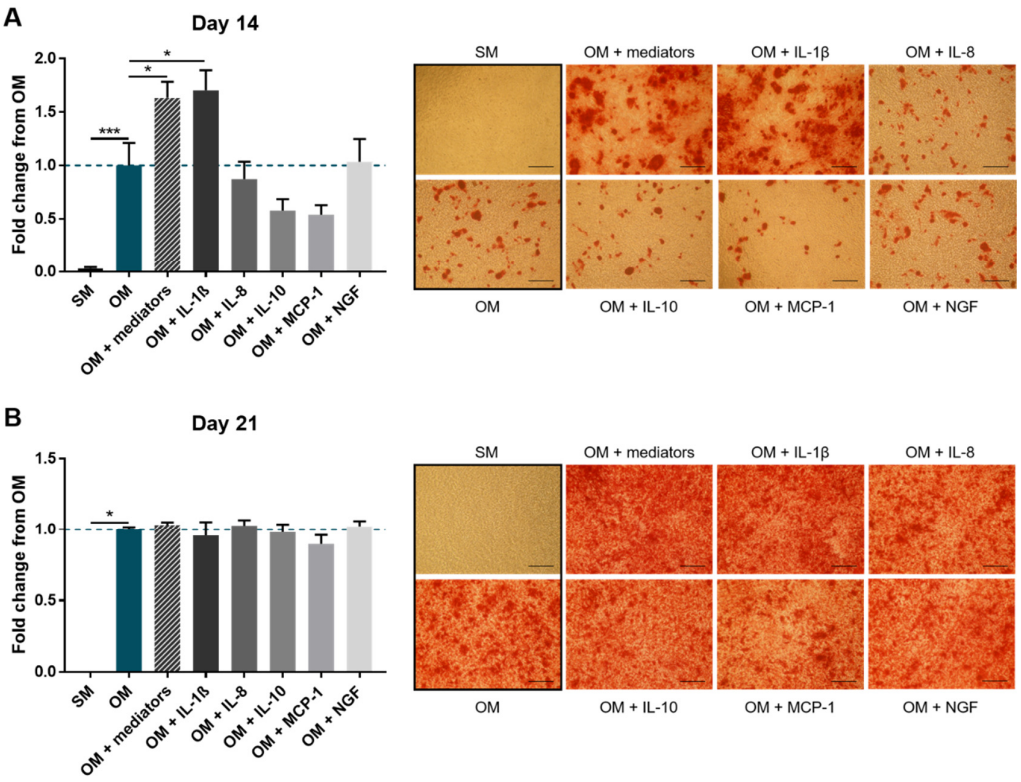


Figure 5.8 Levels of mineral deposition during osteogenic differentiation of ihBMSCs after exposure to inflammatory mediators

Mineral deposition during osteogenic differentiation of ihBMSCs was semi-quantitatively measured by Alizarin Red S staining after exposure to inflammatory mediators for 14 (**A**) and 21 (**B**) days. Results were plotted as fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. A significant increase in mineral deposition could be observed at day 14 in the OM + mediators ($p=0.036$) and OM + IL-1β ($p=0.023$) conditions compared to OM alone (**A**). This increase in mineral deposition could not be observed at day 21, where all OM treatment groups showed similar levels of mineral deposition (**B**). No change in mineral deposition could be observed from the addition of other individual inflammatory mediators in OM. n=3 biological replicates, * $p<0.05$, ** $p<0.005$, *** $p<0.001$. Error bars represent SEM. Scale bars = 150μM.

5.3.3 Effects of IL-1β on ihBMSC osteogenesis

As IL-1β was shown to stimulate an earlier osteogenic response in ihBMSCs, the potential dose response relationship of this mediator was investigated. In these experiments, ihBMSCs were exposed to 1pg/ml, 10pg/ml, 100pg/ml or 1000pg/ml IL-1β for 21 days. Similar to the previous experiment, ALP activity was measured at day 7 and 10, whilst mineral deposition was assessed at day 14 and 21. An additional timepoint at day 10 was added for mineral deposition to see if mineralisation had already been initiated by this early timepoint.

ihBMSCs cultured in OM had increased ALP activity compared to those cultured in SM, confirming that successful differentiation had occurred. Inclusion of IL-1β in OM significantly increased the ALP activity of cells at day 7 at concentrations above 10pg/ml (10pg/ml p=0.040; 100pg/ml p=0.036; 1000pg/ml = 0.004). However, there was no significant difference observed between these higher doses. By day 10 of treatment with 1000pg/ml IL-1β, ALP activity was not increased, but a continued increase could be observed in groups treated with 10pg/ml (p=0.016) and 100pg/ml (p=0.008) IL-1β (B). Treatment with 1pg/ml IL-1β did not significantly affect ALP activity compared to OM alone (Figure **5.9**).

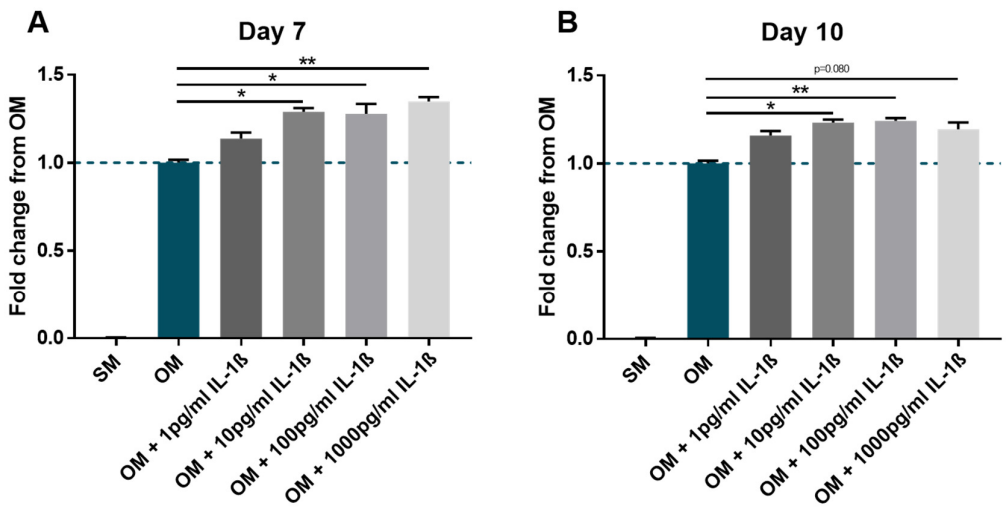


Figure 5.9 ALP activity in ihBMSCs during osteogenic differentiation after exposure to IL-1β

ALP activity (μM/min) was quantitatively measured in ihBMSCs after exposure to IL-1β for 7 (**A**) and 10 (**B**) days. Results were plotted as fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Inclusion of IL-1β in OM at concentrations above 10pg/ml significantly increased ALP activity compared to OM alone at day 7 (10pg/ml p=0.040; 100pg/ml p=0.036; 1000pg/ml = 0.004). At day 10 ALP activity of 10pg/ml (p=0.016) and 100pg/ml (p=0.008) IL-1β treatment groups remained significantly increased compared to OM, but the 1000pg/ml IL-1β group did not. No change in ALP activity could be observed from the addition of 10pg/ml IL-1β in OM at either timepoint. n=4 (technical replicates),

* $p<0.05$, ** $p<0.005$. Error bars represent SEM.

Measurement of mineral deposition also showed that exposure of ihBMSCs to varying concentrations of IL-1β significantly affected this measure of osteogenesis. Although no significant change in mineral deposition could be detected by day 10 between OM and IL-1β treatment groups following stain extraction, a representative panel of images indicated that mineral deposition had begun in discrete areas of cultures exposed to IL-1β (**A**). Following 14 days exposure, the 100pg/ml ($p=0.011$) and 1000pg/ml ($p=0.028$) IL-1β treatment groups caused a significant increase in mineral deposition compared to OM alone (**B**), highlighting a dose response relationship. In contrast, by day 21 a significant decrease in mineral deposition could be observed in cells cultured in 1000pg/ml IL-1β ($p=0.030$), whilst no other treatment groups showed an effect (**C**). The lower concentrations of IL-1β (1pg/ml and 10pg/ml) did not significantly alter mineral deposition at any timepoint, though it should be noted that over a 2-fold change was still measured in these treatment groups at day 14 (Figure **5.10**).

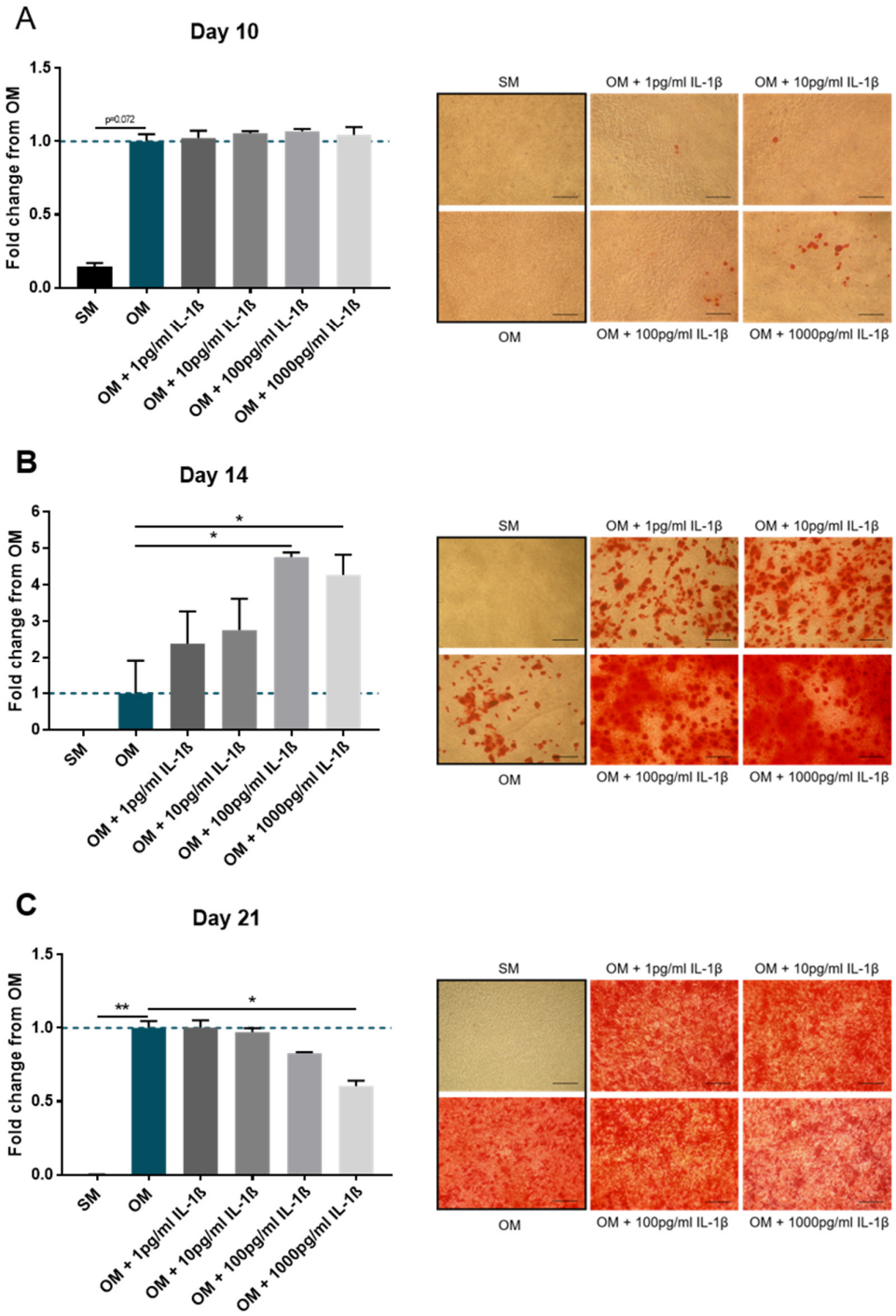


Figure 5.10 Levels of mineral deposition during osteogenic differentiation of ihBMSCs after exposure to IL-1β

Mineral deposition during osteogenic differentiation of ihBMSCs was semi-quantitatively measured by Alizarin Red S staining after exposure to IL-1β for 10 (**A**), 14 (**B**) and 21 (**C**) days.

Results were plotted as fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. No change in mineral deposition could be quantified at day 10, though a panel of representative images displays that mineral deposition had started in all cells cultured in OM + IL-1β (**A**). A significant increase in mineral deposition could be observed at day 14 in cells exposed to higher doses of IL-1β (100pg/ml p=0.011; 1000pg/ml p=0.028) (**B**). At day 21 this increase in mineral deposition could not be observed, and 1000pg/ml IL-1β instead caused a significant decrease in deposition (p=0.030) (**C**). No statistically significant change in mineral deposition could be observed at any timepoint from the addition 1pg/ml or 10pg/ml IL-1β compared to OM. n=4 (technical replicates), * $p<0.05$, ** $p<0.005$. Error bars represent SEM. Scale bars = 150μM.

Data from previously described experiments showed that the OM + mediators treatment group stimulated earlier osteogenic differentiation of ihBMSCs in the same way as IL-1β alone (Section 5.3.2). To analyse whether IL-1β was driving this effect, ihBMSCs were cultured in OM containing combined inflammatory mediators except IL-1β. ALP activity was assessed on day 7, whilst mineral deposition was measured on day 14 and 21.

In this study, only an increase in ihBMSC ALP activity following OM + IL-1β exposure was replicated (p=0.031). In contrast, treatment with inflammatory mediators without the presence of IL-1β did not alter activity levels compared to OM (Figure **5.11**).

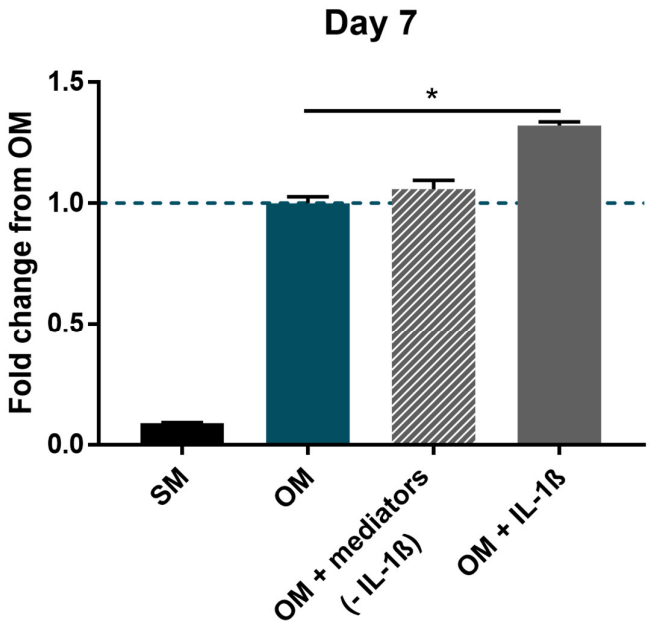


Figure 5.11 ALP activity in ihBMSCs during osteogenic differentiation after exposure to inflammatory mediators minus IL-1β

ALP activity (μM/min) was quantitatively measured in ihBMSCs after exposure to IL-1β or inflammatory mediators (except IL-1β) for 7 days. Results were plotted as fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. A significant increase in ALP activity could be seen in OM + IL-1β ($p=0.031$) compared to OM, but no difference was present in OM treatment groups lacking IL-1β. n=4 (technical replicates), * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

Measurement of mineral deposition further confirmed this effect, as treatment of ihBMSCs with inflammatory mediators (except IL-1β) did not alter the rate of mineral deposition compared to OM, whereas an increase in the OM + IL-1β treatment group could be seen at day 14 ($p=0.119$) and day 21 ($p=0.042$) (Figure **5.12**). Moreover, these effects of IL-1β were reliant on the OM environment, as SM + IL-1β did induce osteogenic differentiation of ihBMSCs (data not shown).

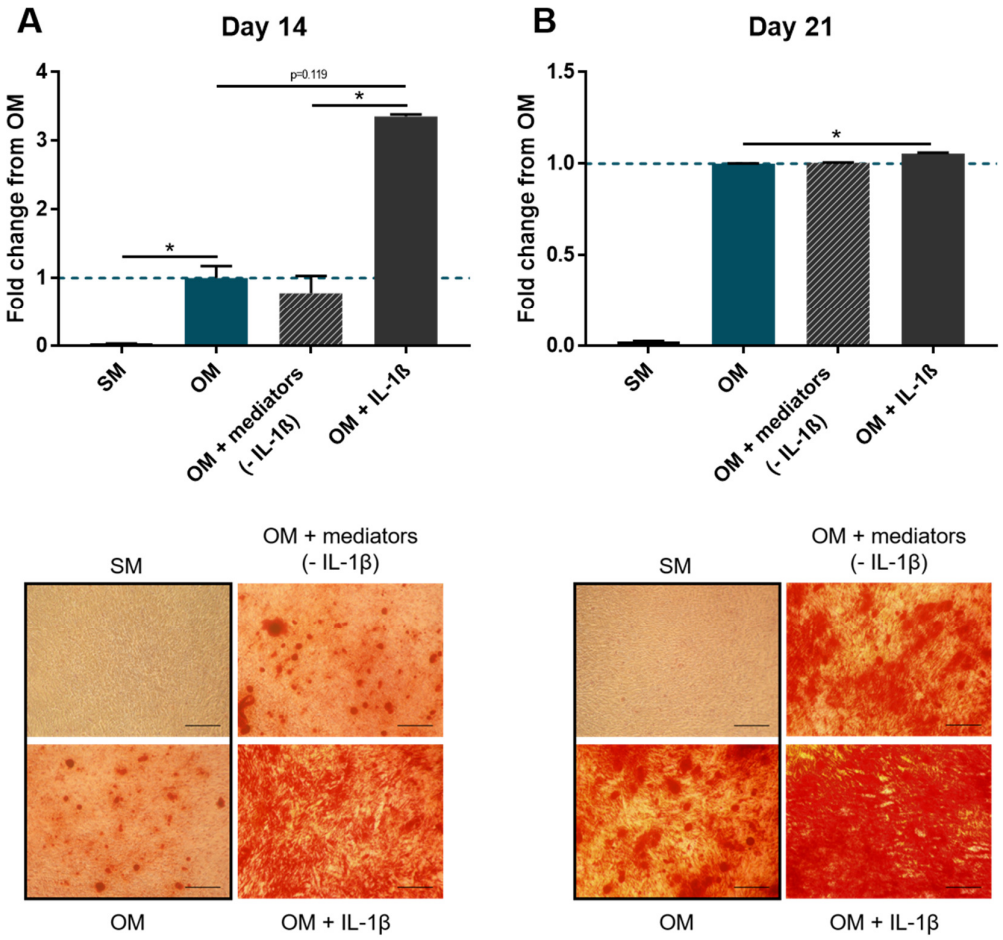


Figure 5.12 Levels of mineral deposition during osteogenic differentiation of ihBMSCs after exposure to inflammatory mediators minus IL-1β

Mineral deposition during osteogenic differentiation of ihBMSCs was semi-quantitatively measured by Alizarin Red S staining after exposure to IL-1β or inflammatory mediators (except IL-1β) for 14 (**A**) and 21 (**B**) days. Results were plotted as fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. At day 14, OM + IL-1β increased mineral deposition compared to OM conditions without IL-1β (OM p=0.119; OM + mediators p=0.045). At day 21, OM + IL-1β significantly increased mineral deposition compared to OM (p=0.0415). n=4 (day 14, technical replicates), n=3 (day 21, technical replicates), * $p<0.05$. Error bars represent SEM. Scale bars = 150μM.

5.3.4 Response of human primary BMSCs to inflammatory mediators during osteogenic differentiation

The results presented so far in this chapter identified that there are species differences between the effects of inflammatory mediators on BMSC osteogenic differentiation. To further the investigation on ihBMSCs, a human primary BMSC culture was utilised. Following the osteogenic differentiation protocol, human primary BMSCs were cultured in the presence of inflammatory mediators (IL-1β, IL-8, IL-10, MCP-1, NGF) for 11 days, after which mineral deposition was measured. The same observed effect was seen in the human primary BMSCs as it was in the ihBMSC cell line, whereby OM + mediators and OM + IL-1β increased mineral deposition compared to OM, however this was not significant with the statistical test used (Figure **5.13**).

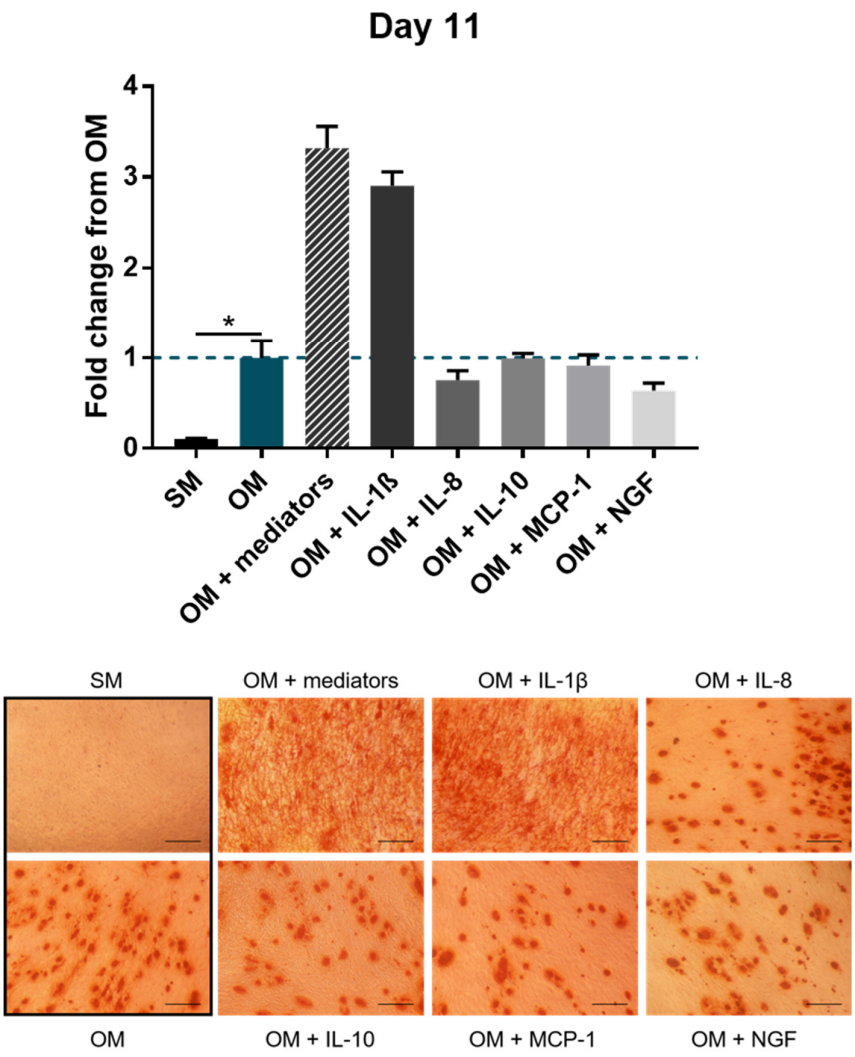


Figure 5.13 Levels of mineral deposition during osteogenic differentiation of human primary BMSCs after exposure to inflammatory mediators

Mineral deposition during osteogenic differentiation of human primary BMSCs was semi-quantitatively measured by Alizarin Red S staining after exposure to inflammatory mediators for 11 days. Results were plotted as fold change from OM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Both the OM + mediators and OM + IL-1β conditions increased the level of mineral deposition compared to OM. No change in mineral deposition could be observed from the addition of other individual inflammatory mediators in OM. n=3 technical replicates, * $p<0.05$. Error bars represent SEM. Scale bars = 150μM.

5.3.5 Effects of inflammatory mediators on ihBMSC gene expression during osteogenic differentiation

Results from the ihBMSC osteogenic differentiation experiments (in section 5.3.2) showed that the addition of IL-1β to OM induced a shift in the temporal differentiation pattern of these cells, as evidenced by increased ALP activity and mineral deposition at earlier timepoints. This shift was observed in OM treatment groups containing IL-1β alone, or in combination with other inflammatory mediators (IL-1β, IL-8, IL-10, MCP-1, NGF). To gain insight into the mechanisms governing this phenomenon, the gene expression profile of these cells was analysed at day 10 of the differentiation protocol. This timepoint was chosen based on evidence that mineral deposition could be detected in cells exposed to IL-1β, but not yet in cells cultured in OM alone (Figure **5.10**). The genes analysed in this study are described in Table **2.3**.

5.3.5.1 Inflammatory mediators and their receptors

Expression of genes encoding for the inflammatory mediators used in this study were analysed, along with their receptors, to assess the autocrine effects of these mediators on MSCs, as well as the effect of osteogenesis itself (Figure **5.14** and Figure **5.15**).

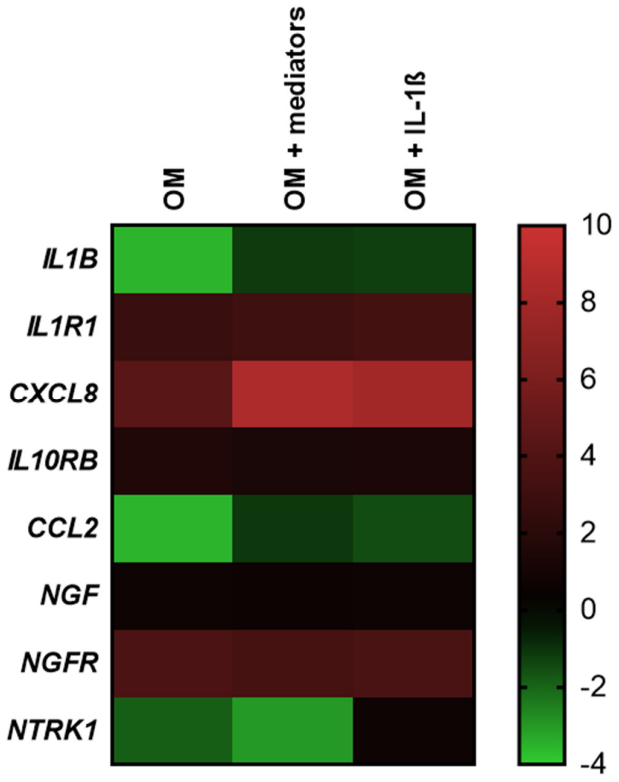


Figure 5.14 Heatmap of inflammatory mediator and receptor gene expression levels

Gene expression levels of inflammatory mediators and their receptors at day 10 of ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *IL1R1* and *CXCL8* were increased in osteogenic conditions containing inflammatory mediators.

Although the expression of *IL1B* did not change following induction of osteogenic differentiation, the expression of this pro-inflammatory cytokines' receptor, *IL1R1*, was increased in OM compared to SM (2.72 $\log_2$ fold change). Furthermore, this increase in expression was exacerbated by the presence of either combined inflammatory mediators or IL-1β alone, as seen by a $\log_2$ fold change of 3.05 or 3.27 from SM, respectively. Another gene affected by the presence of inflammatory mediators was *CXCL8*, encoding for the pro-inflammatory cytokine IL-8. Expression of *CXCL8* was increased in ihBMSCs

cultured in OM by a 4.25 log_2 fold change, but this increase was extended further by the inclusion of inflammatory mediators (8.48 log_2 fold change) or IL-1β (7.89 log_2 fold change) to OM.

Other changes in gene expression were less pronounced than those seen to *IL1R1* and *CXCL8*. Expression of the IL-10 receptor, *IL10RB*, saw a similar log_2 fold change increase from SM in OM conditions regardless of whether inflammatory mediators were present, whilst expression of *CCL2*, encoding for the chemokine MCP-1, saw a similar log_2 fold change decrease in expression. Finally, no significant difference in expression of *NGF* or *NGFR* could be observed from SM, though the NGF receptor *NTRK1* that encodes for TrkA saw a significant decrease in expression in the presence of combined inflammatory mediators (-2.99 log_2 fold change). Expression of *CXCR1*, *IL10*, and *CCR2* could not be detected in this array.

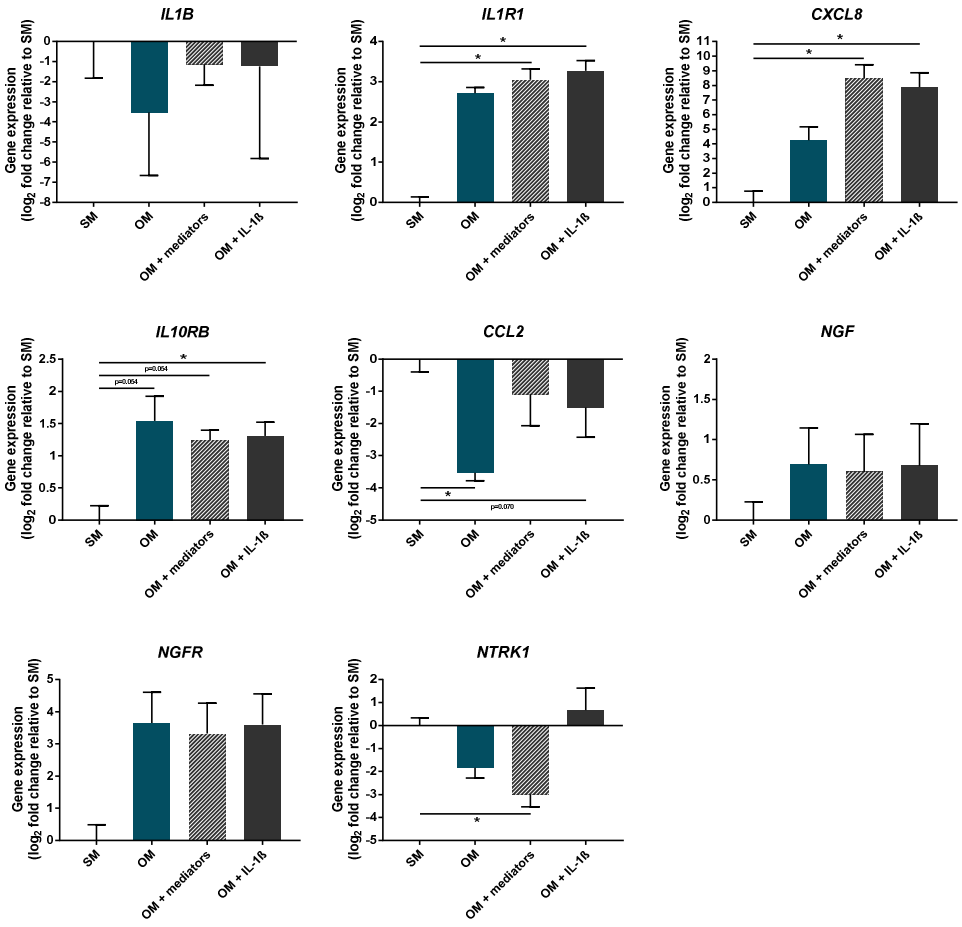


Figure 5.15 Gene expression of inflammatory mediators and their receptors during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for inflammatory mediators and their receptors were analysed at day 10 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. A significant increase in the expression of *IL1R1* and *CXCL8* from SM could be observed due to the presence of inflammatory mediators or IL-1β in OM. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

5.3.5.2 Osteogenic differentiation markers

Expression of genes encoding for classical osteogenic differentiation markers were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **5.16** and Figure **5.17**).

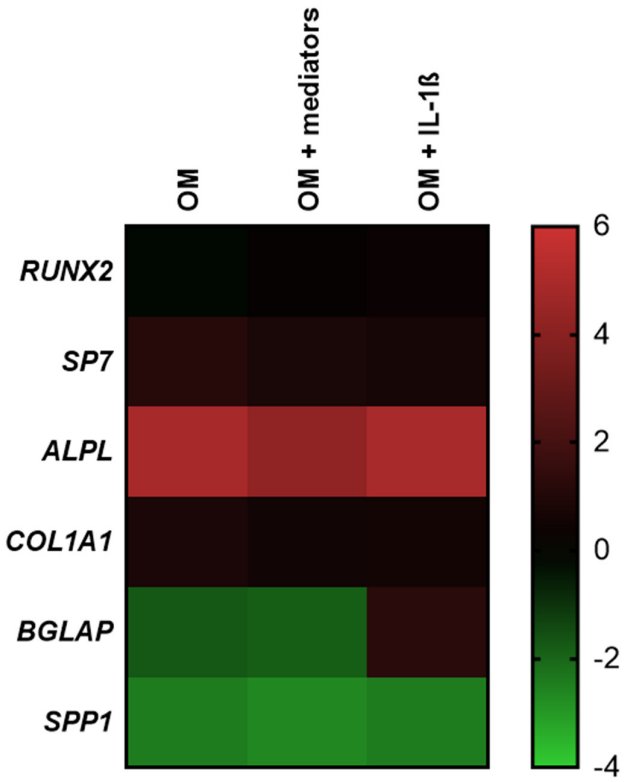


Figure 5.16 Heatmap of osteogenic marker gene expression levels

Gene expression levels of osteogenic markers at day 10 ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *BGLAP* was increased in OM + IL-1β compared to OM.

Expression of *ALPL* was significantly increased in ihBMSCs cultured in OM (4.91 $\log_2$ fold change) compared to SM. In the presence of IL-1β, *ALPL* expression remained significantly increased (4.93 $\log_2$ fold change), but a small reduction was seen in the presence of combined inflammatory mediators (4.19

log_2 fold change) resulting in a loss of significance from SM. Expression of other early differentiation markers (*RUNX2, SP7* and *COL1A1*) showed no significant change in expression from SM in any of the osteogenic treatments, though *SP7* and *COL1A1* showed an increased trend in all osteogenic treatment groups. Interestingly, expression of *BGLAP*, a marker indicative of matrix mineralisation, was increased in the OM + IL-1β treatment group, though this was not significant. The later osteogenic marker, *SPP1*, showed decreased levels of expression which were unaffected by the addition of inflammatory mediators at this timepoint. Expression of *IBSP* could not be detected in this array.

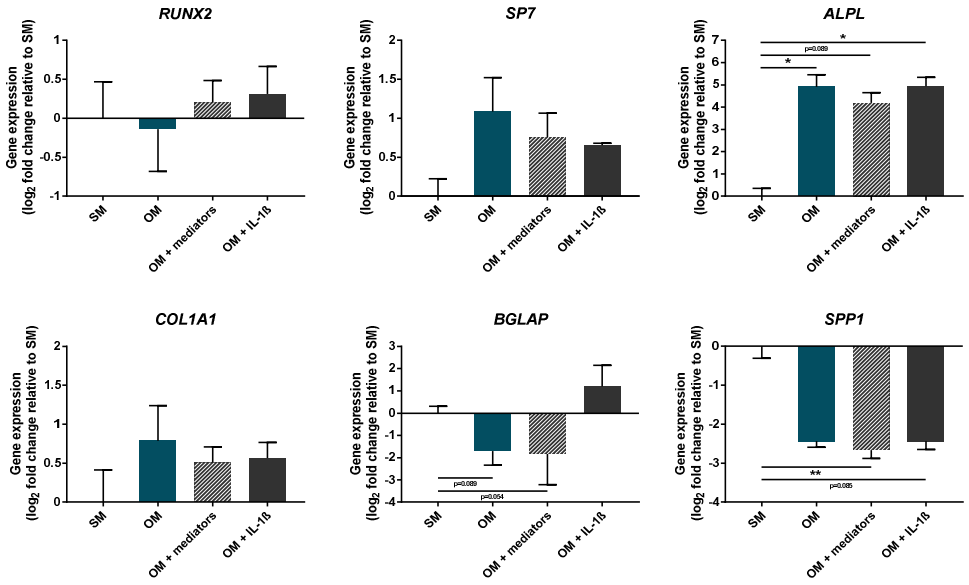


Figure 5.17 Gene expression of osteogenic markers during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for osteogenic differentiation markers were analysed at day 10 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. A significant increase in the expression of the early osteogenic marker *ALPL* could be observed during osteogenesis, whilst expression of *BGLAP* was increased in ihBMSCs cultured in OM + IL-1β, though this was not significant. n=3 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

5.3.5.3 Chondrogenic differentiation markers

Expression of genes encoding for classical chondrogenic differentiation markers were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **5.18** and Figure **5.19**). Although these genes are primarily associated with chondrogenic differentiation, the interplay between a variety of genes is crucial for control and differentiation of MSCs, and thus they were included in the analysis during osteogenic differentiation.

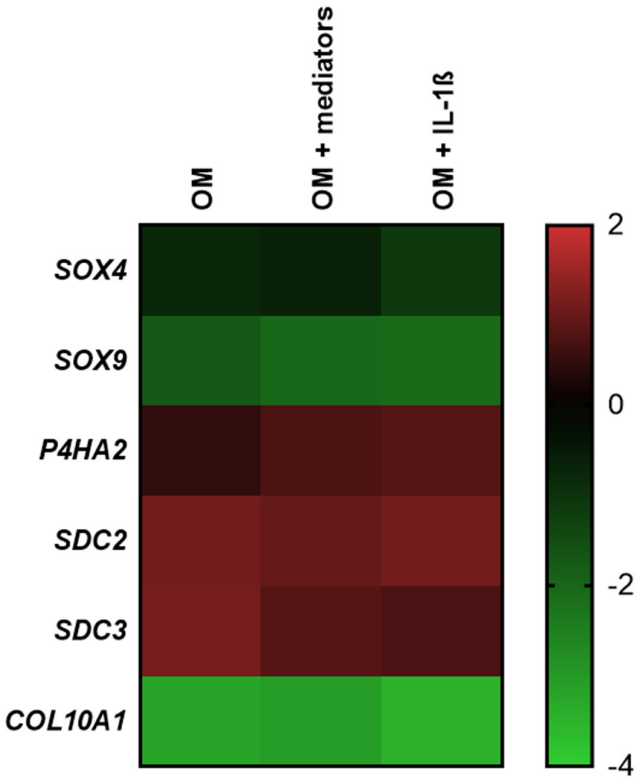


Figure 5.18 Heatmap of chondrogenic marker gene expression levels

Gene expression levels of chondrogenic markers at day 10 of ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. The presence of inflammatory mediators did not significantly affect the gene expression profile of chondrogenic differentiation markers at day 10 of osteogenic differentiation compared to OM.

Expression of *SOX4*, *SOX9* and *COL10A1* was decreased in ihBMSCs undergoing osteogenic differentiation, whereas expression of *P4HA2*, *SDC2* and *SDC3* was increased at day 10. The presence of IL-1β or combined inflammatory mediators did not alter the gene expression profile of differentiating cells. Expression of *COL2A1* could not be detected in this array.

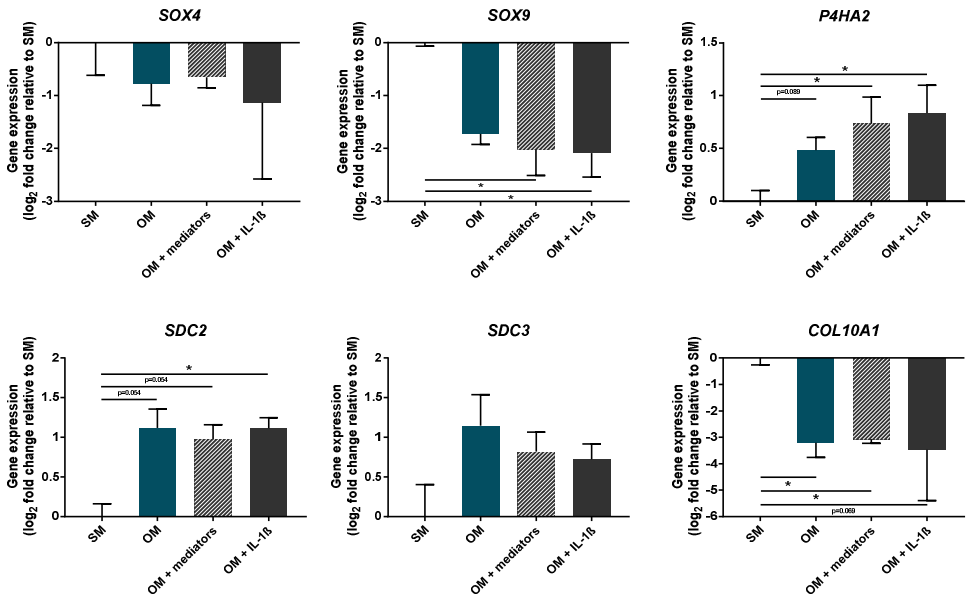


Figure 5.19 Gene expression of chondrogenic markers during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for chondrogenic differentiation markers were analysed at day 10 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The addition of inflammatory mediators did not alter the gene profile of differentiating cells. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

5.3.5.4 MSC immunomodulatory mediators

Expression of genes encoding for MSC immunomodulatory mediators were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **5.20** and Figure **5.21**).

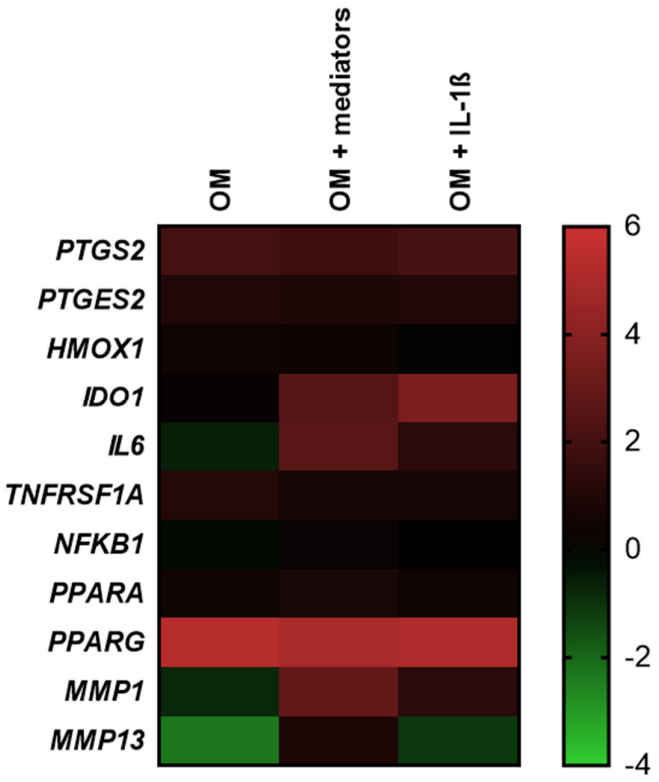


Figure 5.20 Heatmap of immunomodulatory mediator gene expression levels

Gene expression levels of immunomodulatory mediators at day 10 of ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *IDO1*, *IL6* and *MMP1* was increased in the presence of IL-1β or combined inflammatory mediators when compared to SM, whilst *MMP13* expression was only increased in cells exposed to combined inflammatory mediators.

Changes in expression of a number of genes involved in the immunomodulatory function of MSCs was observed. The expression of *PTGS2*, *PTGES2*, *TNFRSF1A*, *PPAR* and *PPARG* were all seen to be increased

in cells undergoing osteogenesis, but the effects of inflammatory mediators did not affect their expression levels compared to OM. However, expression of *IDO1* and *IL-6* were both affected by treatment of inflammatory mediators. In ihBMSCs exposed to IL-1β, *IDO1* expression was seen to be increased (3.55 log_2 fold change), and although the effect was diminished in the presence of combined inflammatory mediators this genes expression was still elevated, as seen by a log_2 fold change of 2.47. With regards to *IL6*, this genes expression was significantly increased by a 2.55 log_2 fold change following exposure to combined inflammatory mediators, whilst IL-1β increased its expression with a 1.24 log_2 fold change, compared to undifferentiated cells in SM. Exposure to inflammatory mediators also impacted on the expression of MMPs, as both *MMP1* and *MMP13* were increased in this treatment group by a 2.87 and 0.82 log_2 fold change respectively. The latter was significantly increased from OM where cell gene expression of *MMP13* had dropped when compared to cells cultured in SM (-2.31 log_2 fold change). Although the presence of inflammatory mediators in OM only increased *NFKB1* expression by a 0.25 log_2 fold change compared to SM, this was nearly significantly increased from expression levels observed in OM conditions (-0.19 log_2 fold change). Osteogenic treatment did not significantly affect *HMOX1* expression, and expression of *NOS1*, *NOS2*, *CSF3*, *IFNG*, *TNF*, *IL1A*, *HLAG* or *MMP3* could not be detected in this array.

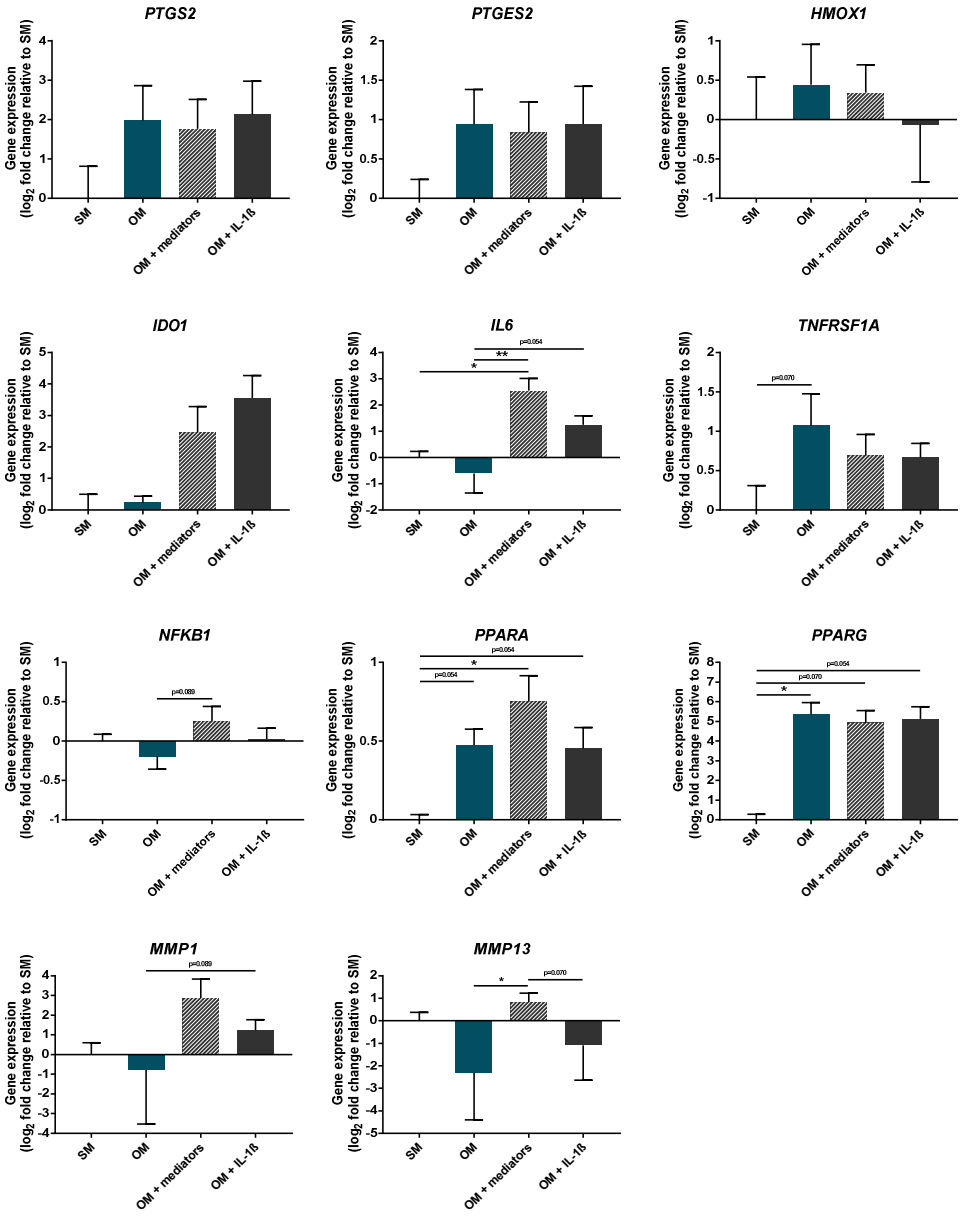


Figure 5.21 Gene expression of immunomodulatory markers during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for immunomodulatory mediators were analysed at day 10 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. Expression of *IL6* was significantly increased in cells cultured in OM + mediators (p=0.007) compared to OM alone. The expression of *MMP1, MMP13* and *IDO1* was

also increased in this treatment group, whilst *IDO1* was further increased by the addition of OM + IL-1β. n=3, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

5.3.5.5 Growth factors and receptors

Expression of genes encoding for growth factors and their receptors were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **5.22** and Figure **5.23**).

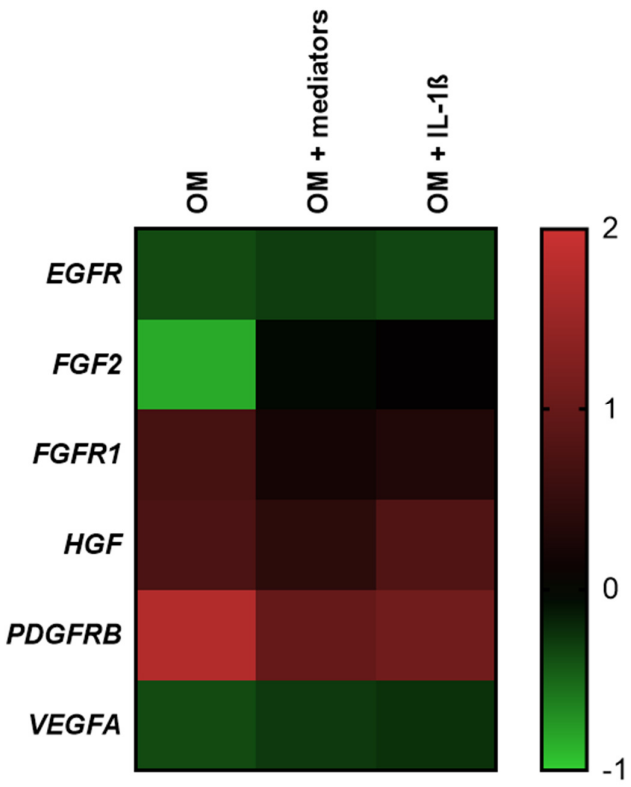


Figure 5.22 Heatmap of growth factor and receptor gene expression levels

Gene expression levels of growth factors and their receptors at day 10 of ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *FGFR1* and *PDGFRB* were decreased in the presence of inflammatory mediators compared to OM, whilst a decrease in *FGF2* expression was prevented by inflammatory mediators.

Expression of both *FGFR1* and *PDGFRB*, receptors for FGF and PDGF respectively, was decreased in cells cultured in OM + mediators or OM + IL-1β when compared to OM alone. For *FGFR1* expression, a $\log_2$ fold change of 0.67 was observed in cells undergoing osteogenic differentiation when compared to those that were not (SM), and this fell when differentiating cells were exposed to combined inflammatory mediators (0.20 $\log_2$ fold change) or IL-1β (0.32 $\log_2$ fold change). In a similar way, *PDGFRB* expression was increased in differentiating cells compared to SM (1.73 $\log_2$ fold change), and this dropped in the presence of combined mediators (0.98 $\log_2$ fold change) or IL-1β (1.10 $\log_2$ fold change). Interestingly, inflammatory mediators prevented a decrease in expression of *FGF2*, but no significant changes in expression could be seen in *EGFR, HGF* or *VEGFA* expression. Expression of *IGF1* could not be detected in this array.

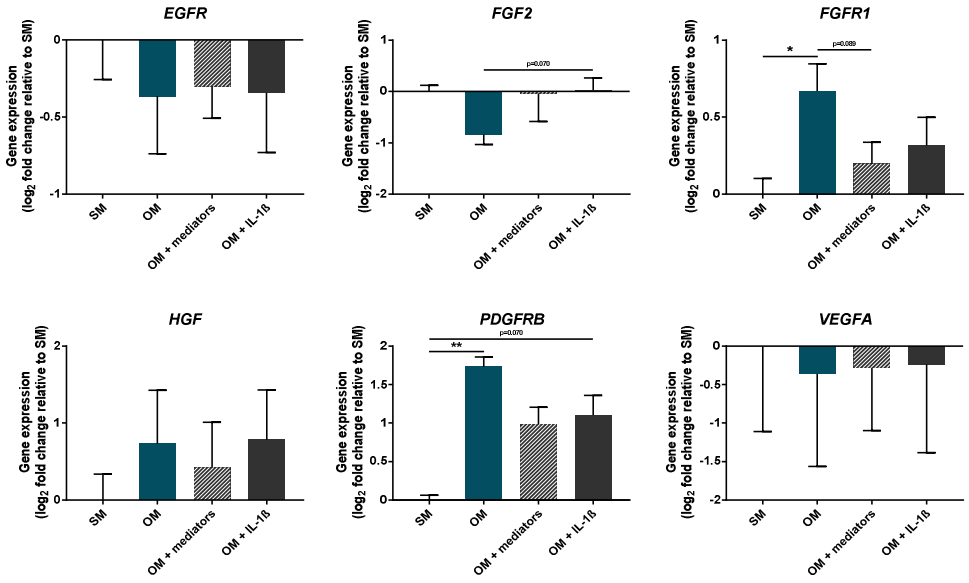


Figure 5.23 Gene expression of growth factors and their receptors during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for growth factors and their receptors were analysed at day 10 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The addition of inflammatory mediators decreased expression of *FGFR1* and *PDGFRB* compared to OM alone. They also prevented a decrease in expression of *FGF2* during osteogenic differentiation. n=3 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

5.3.5.6 TGF-β/BMP signalling

Expression of genes encoding for members of the TGF-β/BMP signalling pathways were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **5.24** and Figure **5.25**).

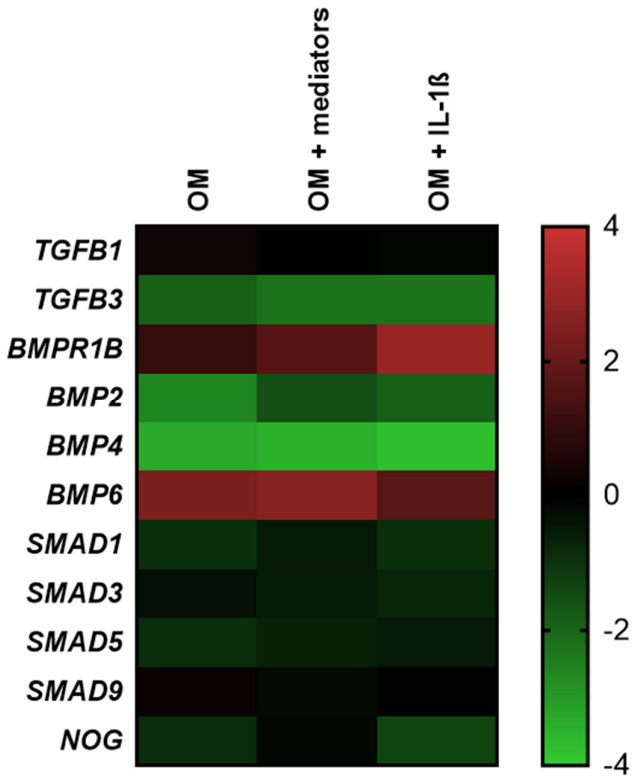


Figure 5.24 Heatmap of TGF-β/BMP signalling gene expression levels

Gene expression levels of TGF-β/BMP signalling molecules at day 10 of ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *BMPR1B* was significantly increased in differentiating cells cultured with IL-1β or combined inflammatory mediators. IL-1β prevented a significant increase in expression of *BMP6* though levels were still elevated.

Expression levels of *BMPR1B* was increased during osteogenic differentiation as seen by a $\log_2$ fold change of 1.03. The addition of inflammatory mediators further elevated this expression, with combined mediators causing a $\log_2$ fold change of 1.67 and IL-1β alone causing a $\log_2$ fold change of 2.95. Expression of *BMP6* was also increased in differentiating cells (2.36 $\log_2$ fold change). Although the presence of combined inflammatory mediators also caused a significant increase (2.64 $\log_2$ fold change), the presence of IL-1β alone reduced this effect (1.73 $\log_2$ fold change). None of the other genes analysed were affected by the presence of inflammatory mediators in OM, with all osteogenic conditions causing decreased expression of *TGFB3*, *BMP2*, *BMP4*, *SMAD1*, *SMAD3* and *SMAD5* compared to SM conditions. No changes could be seen to the expression of *TGFB1*, *SMAD9* or *NOG*. Expression of *GDF2* could not be detected in this array.

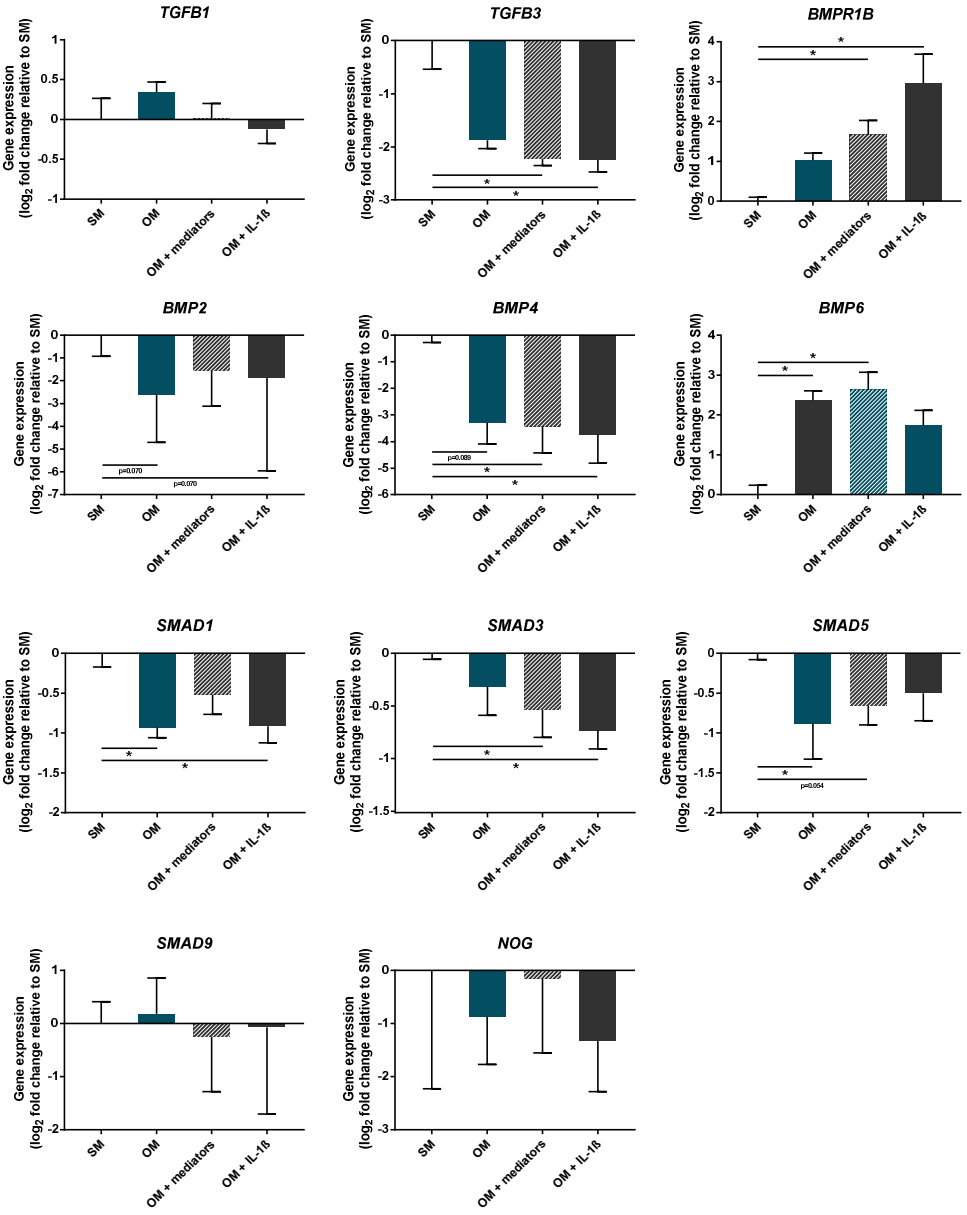


Figure 5.25 Gene expression of TGF-β/BMP signalling molecules during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for TGF-β/BMP signalling molecules were analysed at day 10 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The addition of IL-1β or combined inflammatory mediators to OM caused a significant increase in *BMPR1B* expression. The addition of IL-1β to OM prevented

a significant increase in *BMP6* expression compared to OM. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

5.3.5.7 MAPK signalling

Expression of genes encoding for members of the MAPK signalling pathways were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **5.26** and Figure **5.27**).

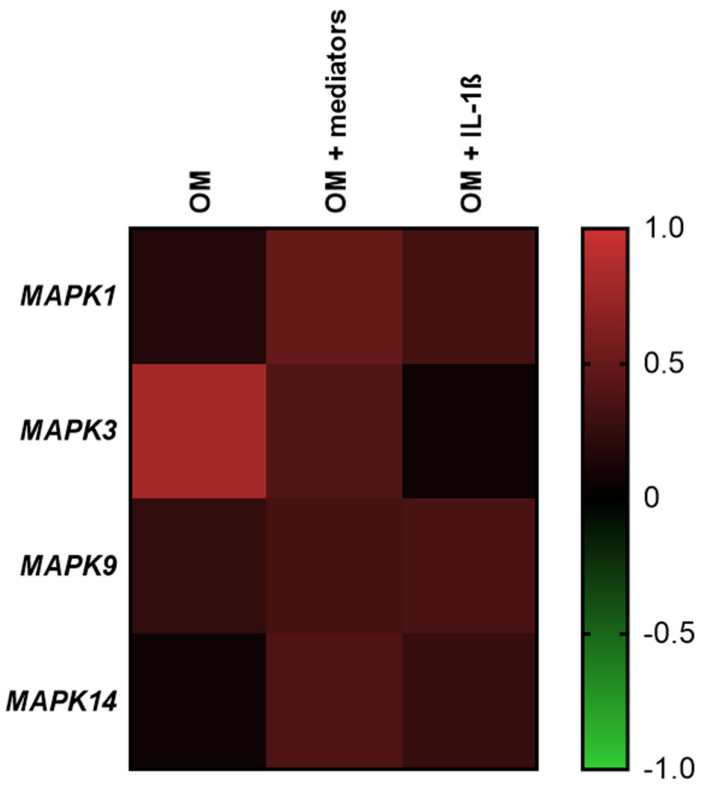


Figure 5.26 Heatmap of MAPK signalling gene expression levels

Gene expression levels of MAPK signalling molecules at day 10 of ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *MAPK3* was significantly increased in differentiating cells, but this effect was lost in the presence of IL-1β. The expression of *MAPK14* was significantly increased in cells cultured with combined inflammatory mediators compared to OM alone.

Expression of *MAPK3*, which encodes for ERK1, was significantly increased in cells undergoing osteogenic differentiation (0.80 $\log_2$ fold change). However, when cells were differentiated in the presence of combined inflammatory mediators this expression was decreased (0.39 $\log_2$ fold change), and the addition of IL-1β alone significantly reduced the effect seen in OM as expression had returned to levels of undifferentiated cells. Another MAPK affected in treatment conditions was *MAPK14*, which encodes for p38*α*. Here the process of differentiation did not affect expression levels of this gene, but the presence of IL-1β alone began to increase expression (0.26 $\log_2$ fold change) and this was elevated further by the addition of other inflammatory markers causing it to be significantly increased (0.37 $\log_2$ fold change). Expression of *MAPK1* and *MAPK9* in osteogenic conditions were not significantly increased from SM.

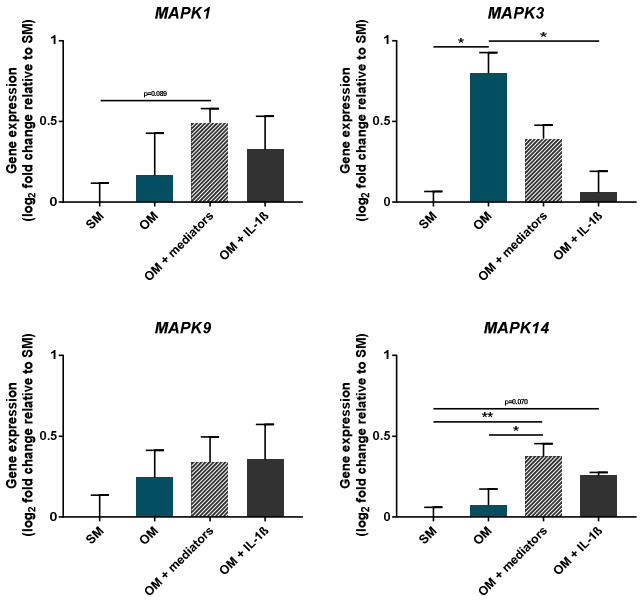


Figure 5.27 Gene expression of MAPK signalling molecules during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for MAPK signalling molecules were analysed at day 10 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The addition of IL-1β to OM prevented the significant increased expression of *MAPK3* seen during osteogenic differentiation. The presence of combined inflammatory mediators significantly increased the expression of *MAPK14*. n=3 biological replicates, * $p<0.05$, ** $p<0.005$. Error bars represent SEM.

5.3.5.8 Wnt signalling

Expression of genes encoding for members of the Wnt signalling pathways were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **5.28** and Figure **5.29**).

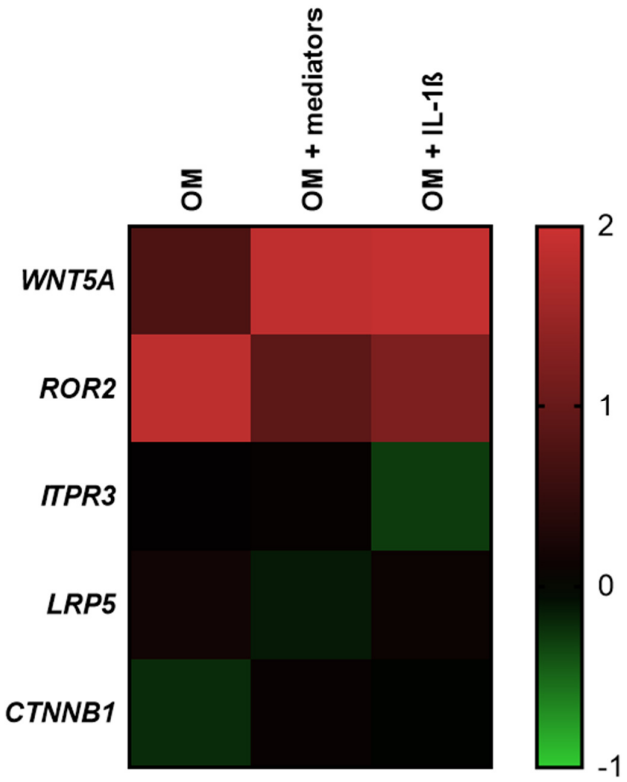


Figure 5.28 Heatmap of Wnt signalling gene expression levels

Gene expression levels of Wnt signalling molecules at day 10 of ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *WNT5A* was significantly increased in the OM + mediators and OM + IL-1β treatment groups. The increased expression of *ROR2* in differentiating cells was diminished in the presence of inflammatory mediators.

The expression of the non-canonical protein *WNT5A* was not significantly increased in cells cultured in OM, but the presence of either IL-1β alone or combined inflammatory mediators significantly increased its expression as

seen by a log_2 fold change of 1.91 and 1.87 respectively. However, the inverse effect could be seen with *ROR2* expression with a significant increase in the OM condition (1.84 log_2 fold change) reduced in both the OM + mediators and OM + IL-1β conditions (0.90 and 1.21 log_2 fold change respectively). Expression of *ITPR3* was unaffected during osteogenic differentiation in a similar way to the genes associated with canonical Wnt signalling, *LRP5* and *CTNNB1*. Expression of *WNT3A* and *WNT7A* could not be detected in this array.

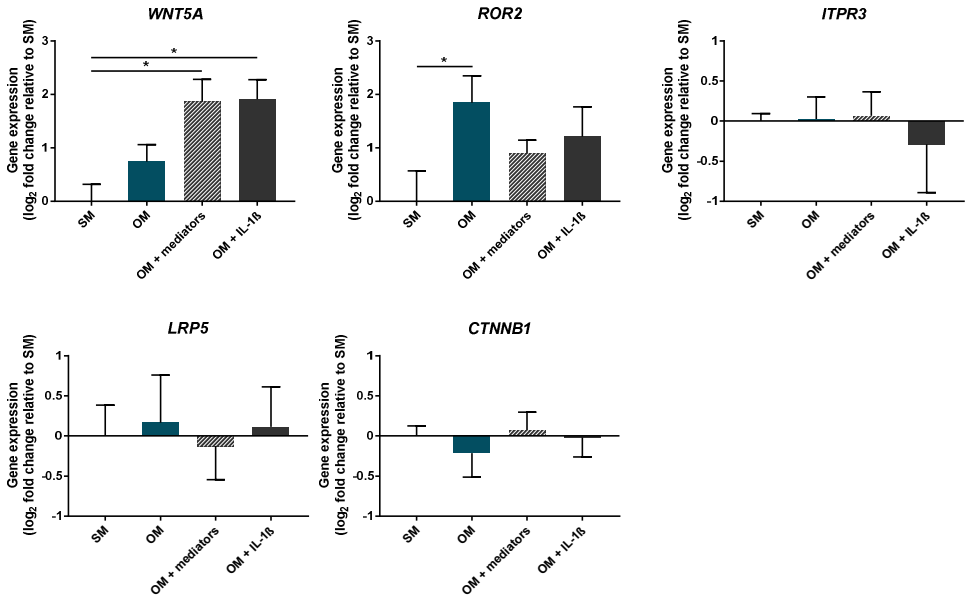


Figure 5.29 Gene expression of Wnt signalling molecules during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for Wnt signalling molecules was analysed at day 10 by TLDAs. Results were reported as log_2 fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, followed by an uncorrected Dunn's post hoc test for multiple comparisons. The addition of combined inflammatory mediators or IL-1β significantly increased the expression of WNT5A in comparison to undifferentiated ihBMSCs. They also prevented a significant increase in *ROR2* signalling that was seen in OM. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

5.3.5.9 Toll like receptor signalling

Expression of genes encoding for TLRs were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **5.30** and Figure **5.31**).

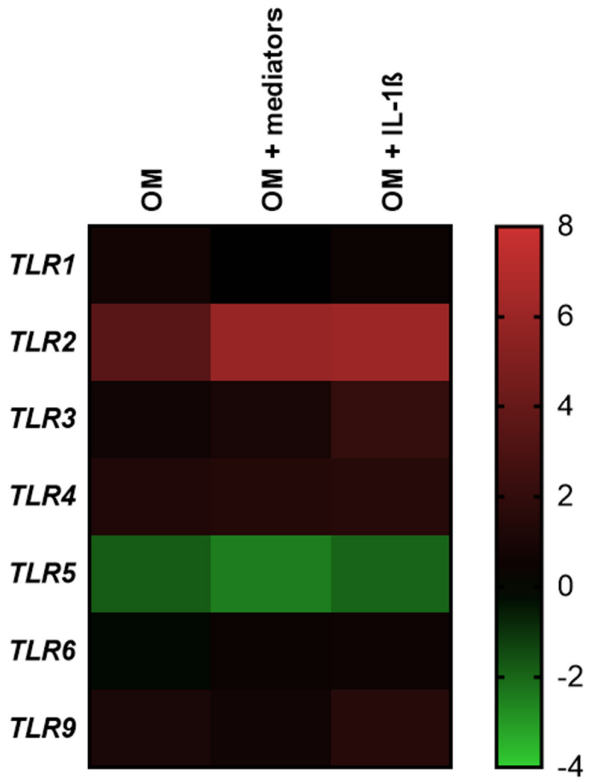


Figure 5.30 Heatmap of toll like receptor gene expression levels

Gene expression levels of TLRs at day 10 of ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. Expression of *TLR2* was increased in differentiating cells, particularly in the presence of inflammatory mediators, whilst IL-1β significantly increased levels of *TLR3* expression.

Expression of *TLR2* was increased in cells undergoing osteogenic differentiation as seen by a $\log_2$ fold change of 3.44, but this expression was exacerbated further by the presence of both combined inflammatory mediators (5.86 $\log_2$ fold change) or IL-1β alone (6.05 $\log_2$ fold change) compared to undifferentiated cells (SM). Another TLR affected in this

experiment was *TLR3.* Although an increase in expression could be observed in the OM (0.65 $\log_2$ fold change) and OM + mediators (0.95 $\log_2$ fold change) treatment groups, IL-1β caused this increase to be significant from SM with a $\log_2$ fold change of 2.00. *TLR4* expression was seen to be decreased during osteogenesis, whilst *TLR5* was increased. Neither *TLR1, TLR6* nor *TLR9* displayed any significant changes from SM in any of the OM treatment groups.

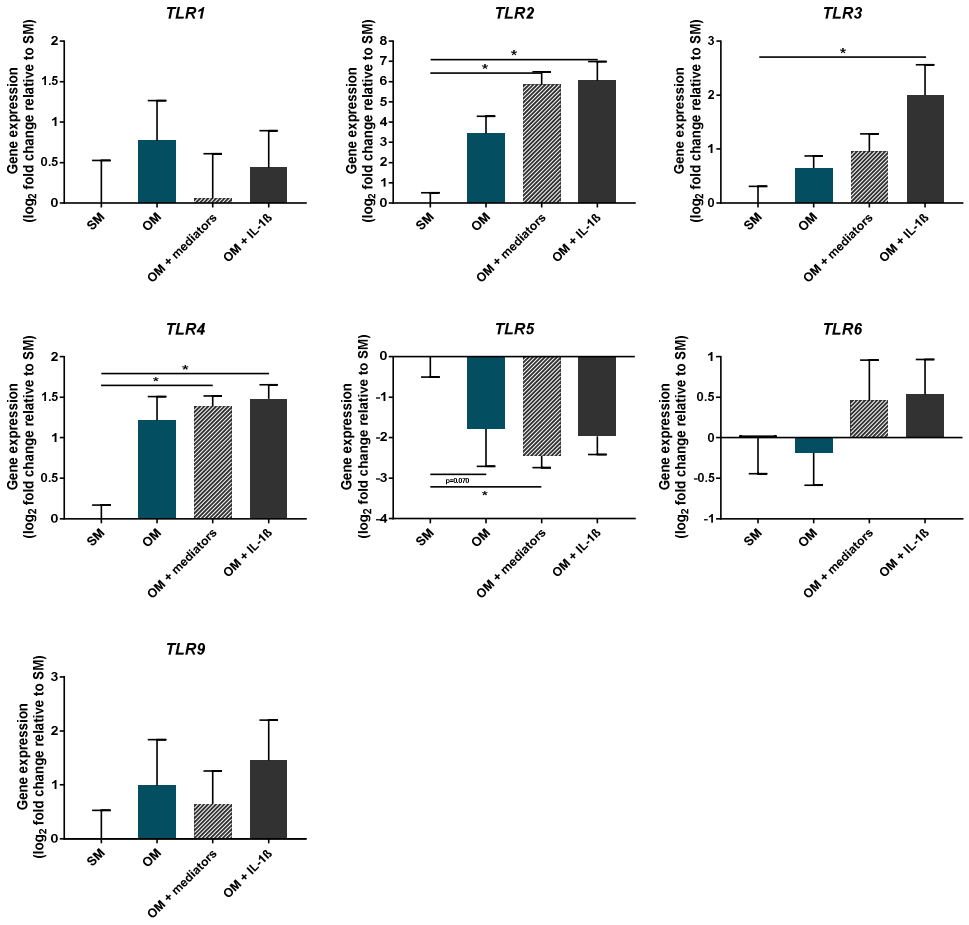


Figure 5.31 Gene expression of TLR signalling molecules during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for TLR signalling molecules was analysed at day 10 by TLDAs. Results were reported as $\log_2$ fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, with an uncorrected Dunn's post hoc test for multiple comparisons. The addition of IL-1β to OM significantly increased expression of *TLR2* and *TLR3*, whilst addition of combined inflammatory mediators significantly increased expression of *TLR3* compared to SM. n=3 biological replicates, * $p<0.05$. Error bars represent SEM.

5.3.5.10 Other signalling pathways

Expression of genes encoding for members of other signalling pathways of interest were analysed to assess the effects of inflammatory mediators on ihBMSCs during differentiation (Figure **5.32** and Figure **5.33**).

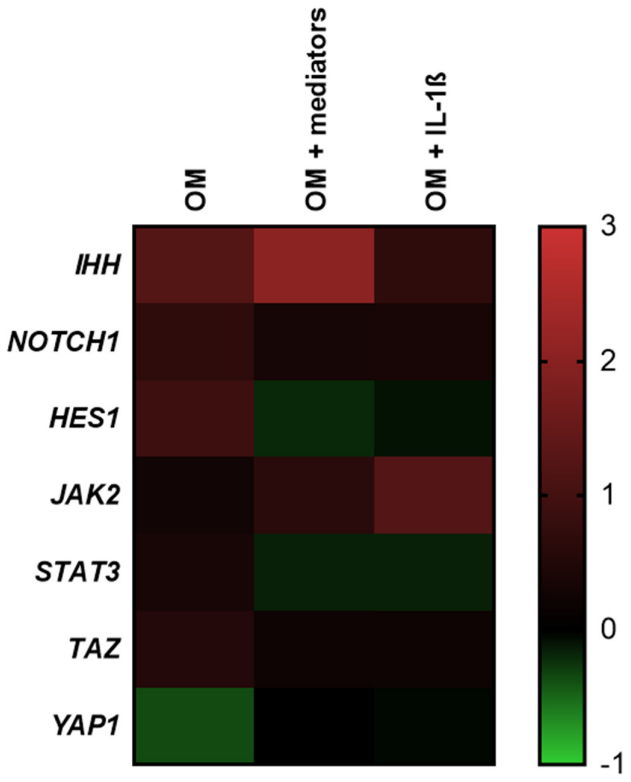


Figure 5.32 Heatmap of other signalling gene expression levels

Gene expression levels of other signalling molecules at day 10 of ihBMSC osteogenic differentiation were calculated and displayed as $\log_2$ fold change relative to SM. No significant changes to gene expression could be detected in ihBMSCs though levels of *IHH* were seen to be increased in comparison to SM.

Expression of genes involved in other signalling pathways not already described revealed no significant changes in expression when compared to OM, or even undifferentiated cells in SM. Though not significant it could be noted that a 2.04 $\log_2$ fold change could be seen in *IHH* expression in cells

cultured with OM + mediators, compared to a log_2 fold change of 1.22 seen in OM alone. Furthermore, OM + IL-1β caused a 1.20 log_2 fold change compared to a 0.25 log_2 fold change seen in OM alone for *JAK2* expression. Expression of *SHH, NOTCH1* or *NELL1* could not be detected in this array.

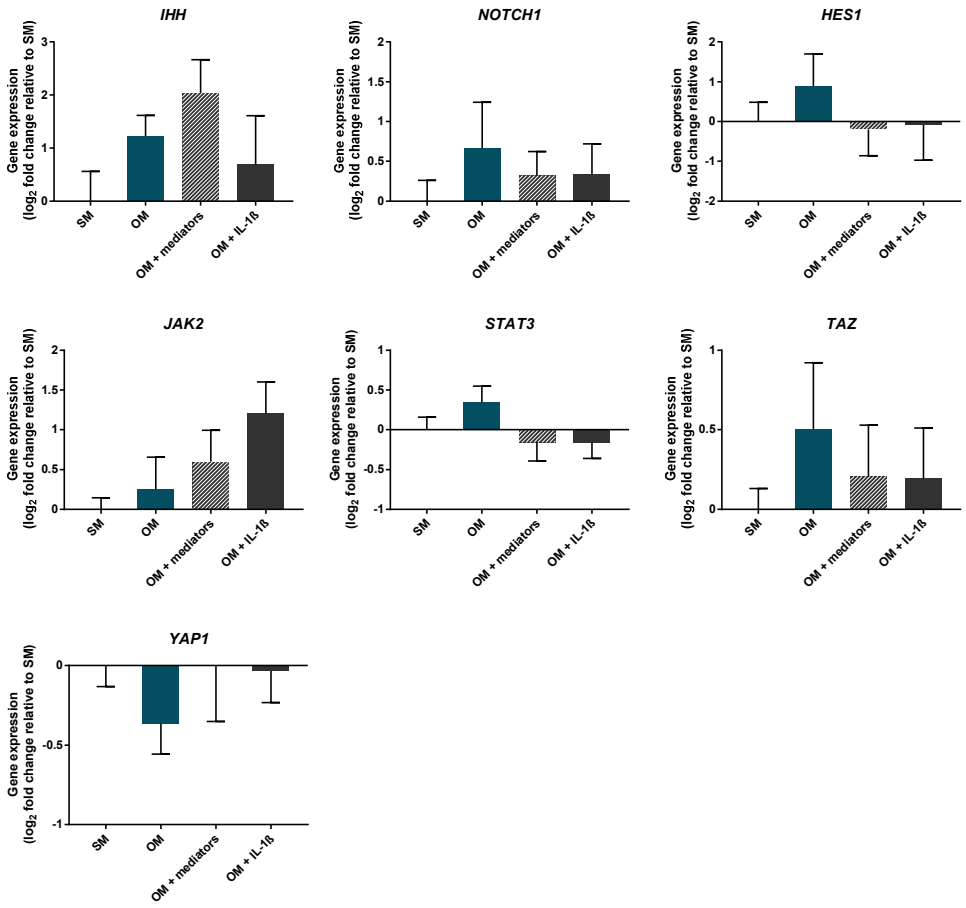


Figure 5.33 Gene expression of other signalling molecules during ihBMSC osteogenic differentiation

To assess the effects of inflammatory mediators on osteogenic differentiation of ihBMSCs, the expression of genes encoding for other signalling molecules was analysed at day 10 by TLDAs. Results were reported as log_2 fold change relative to SM and a Kruskal-Wallis test was performed for statistical analysis, with an uncorrected Dunn's post hoc test for multiple comparisons. No significant change between treatment groups could be observed for any of the genes analysed. n=3 biological replicates. Error bars represent SEM.

5.4 Discussion

Understanding the role of bone remodelling in OA pathogenesis is of paramount importance when searching for novel treatment strategies, as alterations to the subchondral bone are a primary feature of OA. If MSC transplantation is to be a viable treatment option in the future, it is therefore crucial to identify the effects that the OA microenvironment may have on MSCs, as endogenous populations of these cells are a key component of bone remodelling in both the healthy and osteoarthritic joint (Hadjidakis and Androulakis, 2006; Neve, Corrado and Cantatore, 2011). Current research suggests that the inflammatory environment of the diseased joint leads to abnormal bone remodelling, whereby increased bone turnover results in thickening of the subchondral plate and reduced bone quality (Grynpas *et al.*, 1991; Li and Aspden, 1997; Martineau *et al.*, 2017). Moreover, the complex interactions between subchondral bone and articular cartilage means it is also likely to be contributing to the degradation of cartilage seen in OA (Dore *et al.*, 2010; Zhen *et al.*, 2013).

The mechanisms governing the change in osteoarthritic bone remodelling remain unclear due to both an absence of results and contradictory findings. This study aimed to address this issue by determining the effects of inflammatory mediators on the osteogenic differentiation capacity of BMSCs *in vitro*. Each of the mediators presented in this study have been found to be upregulated in the osteoarthritic joint at concentrations similar to those tested, allowing for closer *in vivo* representation. Thus far, various experiments in the literature have cultured BMSCs with individual mediators. Although this helps to discriminate between the actions of mediators, any synergistic relationships that might be present in the osteoarthritic joint are lost. Therefore, to gain a clearer picture of MSC osteogenesis in the osteoarthritic

joint, these findings were extended in this study through the combination of inflammatory mediators. To measure the effects on osteogenic differentiation, both ALP activity and mineral deposition were measured, whilst insight into the mechanisms of observed effects was gained through gene expression analysis.

5.4.1 Effects of inflammatory mediators on BMSCs during osteogenic differentiation

The presence of IL-1β or combined inflammatory mediators in osteogenic medium (OM) significantly altered the temporal regulation of osteogenic differentiation in both murine and ihBMSC cells. Notably, the mediators had inverse effects and either delayed the onset of osteogenic features in murine BMSCs, or stimulated faster differentiation in ihBMSCs. Therefore, the data presented in this chapter brings important new findings for understanding conflicting results in the literature, as it highlights the importance of species differences in both homeostatic functions and disease pathogenesis.

Importantly, this data corroborates findings by Lacey *et al.*, (2009) who showed that the pro-inflammatory cytokines IL-1β and TNFα inhibit osteogenic differentiation of murine MSCs, as characterised by suppression of ALP activity and bone mineralisation. Moreover, these findings are also consistent with those by Sonomoto *et al*. (2012) who demonstrated that IL-1β enhances osteogenic differentiation of human BMSCs. Data presented here extended these findings by showing that the presence of combined inflammatory mediators maintained the same rate of osteogenic differentiation as IL-1β alone, whilst removal of IL-1β from the mediator pool returned differentiation rates to baseline. Combined with additional data that IL-1β exerts its effects in a dose dependent manner, these findings indicated

that IL-1β was responsible for the temporal regulation of MSC differentiation, whilst the presence of other pro-inflammatory cytokines (IL-8, MCP-1) did not appear to exacerbate the observed effects, nor did anti-inflammatory (IL-10) cytokines dampen them. It must be recognised that a limitation of these results was a lack of normalisation to cell number. Although findings were consistent with the literature, and previous experiments in Chapter 3 demonstrated no observable effect of IL-1β on cell proliferation after 10 days 2-D culture in standard medium, future work should include confirmation of this. This could be achieved through the addition of a nuclear stain protocol, such as Hoechst staining. Furthermore, supplementary analysis of the mineralisation of ihBMSCs could be undertaken to establish any phenotypic changes in mineral deposition. This is particularly important with evidence that hypomineralisation is observed in osteoarthritic bone formation (Li and Aspden, 1997), and the finding that the highest dose IL-1β reduced levels of mineral deposition by day 21. Analysis of the medium would be able to identify if this is a false negative effect of *in vitro* culture systems, in which excessive mineralisation is lost into the medium, or a true hypomineralisation effect.

Individually, the other tested inflammatory mediators did not affect osteogenic differentiation of murine BMSCs, ihBMSCs or human primary BMSCs. For the pro-inflammatory chemokine IL-8 this is consistent with the literature, which, though limited, provides evidence that IL-8 does not directly affect osteogenesis (Haleem-Smith *et al.*, 2012). However, previous *in vitro* findings by Van Vlasselaer *et al.*, (1994) indicated that IL-10 inhibits early osteogenesis in mouse BMSCs through the inhibition of TGF-β synthesis. This inhibition occurred before the expression of ALP, and thus experiments presented here could not validate this finding, as ALP was used as the first indicator of osteogenesis. This work also does not support findings by Toben

et al., (2011), who observed *in vivo* that recombination activating gene knockout mice had faster fracture healing times as a result of increased production of anti-inflammatory mediators, notably IL-10. Finally, neither of the effects of MCP-1 nor NGF on MSC differentiation have been widely reported, though it has been noted that MSCs secreting higher levels of MCP-1 have greater osteogenic differentiation potential (Xie *et al.*, 2016), whilst NGF can upregulate osteogenic differentiation of dental pulp stem cells (Yasui *et al.*, 2017). Data presented here does not substantiate these findings with regards to murine BMSC, ihBMSC or human primary BMSC differentiation.

5.4.2 Effects of inflammatory mediators on gene expression of ihBMSCs during osteogenic differentiation

To gain insight into the mechanisms underpinning IL-1β mediated stimulation of ihBMSC osteogenic differentiation, gene expression analysis was undertaken. This allowed for investigation into a variety of signalling pathways involved in normal bone remodelling, and highlighted that members of TLR, TGF-β/BMP and Wnt signalling were affected by the presence of inflammatory mediators. Importantly, the expression of pro-inflammatory genes, such as *IL6*, *CXCL8* and *IDO1*, were also increased. Furthermore, although the gene expression of *IL1B* itself was not seen to be increased in these cells, the expression of the IL-1β receptor, *IL1R1*, was significantly increased in ihBMSCs exposed to IL-1β or combined inflammatory mediators. This means that these cells were more sensitive to the effects of IL-1β in the microenvironment, thereby ensuring IL-1β could continue to successfully exert its effects.

5.4.2.1 Osteogenic differentiation markers

Measurement of osteogenic differentiation markers at day 10 ihBMSC osteogenesis revealed that, although ALP activity was significantly increased in cells exposed to inflammatory mediators, the gene expression of *ALPL* was not significantly increased compared to OM. As the ALP activity assay is dependent on protein expression, the difference could be accounted for by the role of regulatory processes after mRNA production. Moreover, biological variability between cell passages could result in alterations to the temporal pattern of osteogenic differentiation, potentially causing an earlier peak of *ALPL* expression before that seen at day 10. Other earlier osteogenic differentiation markers, *RUNX2* and *SP7*, also showed no significant differences at the gene expression level. Reasons for this include a potential lack of stability and short half-life of RNA, as well as unknown quality of the TaqMan probe used for these genes. In order to expand the gene expression findings, protein expression of relevant markers should be undertaken to allow for any post-transcriptional modifications that may affect production of protein measured in assays such as ALP activity. However, it should be noted that the expression of *BGLAP* was seen to be increased in ihBMSCs exposed to IL-1β compared to cells in OM alone, indicating earlier stimulation of a gene important in regulating mineralisation.

5.4.2.2 Toll like receptors

TLR activity is a key component of the innate immune response, generating signalling cascades that result in the increased secretion of inflammatory mediators, such as IL-6 and IL-8 (Sangiorgi and Panepucci, 2016). These mediators can subsequently act in an autocrine manner and maintain TLR expression, thereby sustaining the immune response. In particular, *TLR2*, *3* and *4* expression has been shown to be increased in human MSCs in response

to inflammatory mediators (Raicevic *et al.*, 2010). Although pro-inflammatory cytokines are often associated with destructive events, they have been shown to play an important role in normal bone remodelling processes, including recruitment and guidance of MSCs into the osteoblast lineage (Mountziaris and Mikos, 2008), thereby implicating TLRs in bone homeostasis. In fact, TLR-2, -3 and -4 have all been shown to increase the osteogenic differentiation capacity of human MSCs (DelaRosa, Dalemans and Lombardo, 2012). The data presented here confirms that TLR expression is altered during osteogenic differentiation, particularly in response to the presence of inflammatory mediators. The two most significant changes could be observed by the increase of *TLR2* and *TLR3*.

Studies investigating the role of TLR-2 in osteogenic differentiation have revealed that it increases osteogenic markers, such as ALP activity and mineralisation (Mountziaris and Mikos, 2008; Muthukuru and Darveau, 2015). The TLDA data provides confirmation of these findings, as increased *TLR2* expression was observed in cells displaying an earlier osteogenic phenotype. Importantly, *TLR2* expression in human articular chondrocytes is increased in response to IL-1β both in the literature and in results presented in Chapter 4 (Su *et al.*, 2005), and these results also demonstrate that the same can be seen in ihBMSCs differentiating towards the osteoblast lineage. Interestingly, TLR-2 in mice has been shown to inhibit tri-lineage differentiation (Pevsner-Fischer *et al.*, 2007). Although no data was collected on murine BMSC gene expression in this study, it is interesting to note that a decrease in both ALP activity and mineral deposition was observed in these cells, and may result from IL-1β upregulation of *TLR2*. The consequences of increased ihBMSC *TLR2* expression in the osteoarthritic joint can be hypothesised using results presented here and previous findings in the literature. Reduction of IL-8 (CXCL8) in human rheumatoid synovial fibroblasts upon neutralisation of

TLR2, directly implicates TLR-2 signalling in secretion of this mediator (Cho *et al.*, 2007). The increase in *TLR2* expression in this dataset is therefore likely to be directly controlling the significant increase in *CXCL8* expression of ihBMSCs exposed to IL-1β or combined inflammatory mediators. IL-8 is important in bone remodelling as it promotes osteoclastogenesis (Bendre *et al.*, 2003), so if secretion of mediators such as this is driven up in MSCs, then these cells could be indirectly contributing to the increased bone resorption observed in OA.

The expression of *TLR3* in these experiments was significantly increased in the presence of IL-1β. Although expression levels were also increased in the presence of combined inflammatory mediators, the expression pattern was similar to that of OM, suggesting that the addition of other inflammatory mediators may be counteracting the effects of IL-1β on *TLR3* expression. TLR-3 itself is known to promote migration of BMSCs (Tomchuck *et al.*, 2009), as well as their differentiation and mineralisation (Qi, Xiaofeng and Xiaoguang, 2014), but the mechanisms controlling these effects are unclear. TLR-3 has also been linked to acute inflammation, stimulating the production of mediators such as IDO (Qi, Xiaofeng and Xiaoguang, 2014). In a similar way, expression of *IDO1* in these ihBMSCs was primarily increased in those cultured with IL-1β alone, implying a potential link between expression of these two genes. Very little research exists on the role of IDO in bone remodelling, but it may enhance apoptosis of pre-osteoclasts (Bozec *et al.*, 2014).

Taken together, increased expression of both *TLR2* and *TLR3* was seen in conditions that promote faster osteogenic differentiation of ihBMSCs. This not only indicates their importance in osteogenic differentiation, but also signifies the role of an inflammatory environment in their expression. The mechanisms controlling TLR mediated osteogenesis are unknown, but it has been

postulated to occur through different pathways such as Hippo or NF-κB (Cho *et al.*, 2009). In this experiment genes involved in these signalling pathways, such as *TAZ* and *NFKB1* were not affected by the presence of inflammatory mediators in OM, suggesting that the increase in ALP activity and mineral deposition does not occur through either of these pathways. Instead it could be postulated that the stimulation of inflammatory mediator expression by TLRs is driving osteogenic differentiation, for example expression of *IL6*, *CXCL8* and *IDO1*.

5.4.2.3 IL-6

The cytokine IL-6 is constitutively expressed by BMSCs, playing an integral role in MSC proliferation and protection against apoptosis. For this reason, levels of IL-6 are seen to be decreased during bone remodelling in the healthy joint as BMSCs begin to differentiate into cells of the osteoblast lineage (Pricola *et al.*, 2009). Results presented in this chapter showed that that the presence of IL-1β or combined inflammatory mediators prevented this decrease in *IL6* expression during osteogenic differentiation, but this did not negatively affect the rate of osteogenesis as both ALP activity and mineral deposition were increased at this timepoint.

Although expression of *IL6* was increased in the presence of IL-1β alone, it was significantly increased in the presence of combined inflammatory mediators, suggesting that the synergistic action of the mediators exacerbated *IL6* expression. As no observable difference could be seen in ALP activity and mineral deposition of these two treatment groups, expression of *IL6* is unlikely to be a driver of osteogenesis. However, the implications of increased *IL6* expression are highly relevant, as IL-6 has been linked to osteoclast activation (Huh and Lee, 2013) and stimulation of MMPs and aggrecanases that directly

cause cartilage degradation (Akeson and Malemud, 2017). Interestingly, the expression of both *MMP1* and *MMP13* was also increased in ihBMSCs cultured with combined inflammatory mediators, suggesting a potential link between *IL6* and *MMP* expression in these cells. This is consistent with findings in other cells types whereby IL-6 has been shown to increase *MMP1* expression in macrophages and phagocytes through the ERK1/2 and JNK pathways (Sundararaj *et al.*, 2009; Li *et al.*, 2010), and *MMP13* expression in chondrosarcoma cells and fibroblasts (Solís-Herruzo *et al.*, 1999; Tang *et al.*, 2011).

5.4.2.4 TGF-β/BMP signalling

Gene expression data presented in this chapter showed that expression of *BMP2, 4* and *6* in ihBMSCs undergoing osteogenic differentiation was not significantly altered by the presence of inflammatory mediators. However, expression of the BMP receptor, *BMPR1B*, in ihBMSCs was significantly increased by inflammatory mediators at day 10 of osteogenic differentiation. Increased expression of BMP receptors on the surface of these osteoblasts means that they are more sensitive to the action of BMP receptor ligands in the microenvironment, which have been shown to induce osteogenic differentiation (Yamaguchi *et al.*, 2008; Karner, Lee and Long, 2017). These ligands exert their effects through canonical (Smad dependent) signalling or non-canonical signalling via the p38 MAPK pathway. The presented TLDA data did not show any alterations to the expression of chosen Smad dependent genes between osteogenic conditions, however changes to the non-canonical genes *MAPK3* and *MAPK14* could be observed.

The role of MAPK signalling in osteogenesis remains controversial, with studies demonstrating both stimulation or inhibition of osteogenic

differentiation through these pathways (Schindeler and Little, 2006). The conflicting findings are likely to be dependent on variable such as species, MSC source and culture time (Ba *et al.*, 2017). In the results presented here, a significant decrease in expression of *MAPK3*, which encodes for ERK1, could be seen in cells exposed to IL-1β. This could suggest that ERK1 has a negative regulatory role in osteogenesis. Alternatively, early work by Jaiswal *et al.*, (2000) showed that ERK genes displayed sustained expression between days 7 and 11 of *in vitro* differentiation before rapidly returning to basal levels. As the addition of IL-1β induced earlier differentiation, it could be hypothesised that expression of *MAPK3* had already peaked by day 10 and returned to baseline. These findings are validated by more recent work by Li *et al.*, (2016) who showed that ERK signalling peaks at day 9 in human UC-MSCs, and inhibition of this kinase results in the inhibition of osteogenic differentiation, highlighting the integral role of ERK signalling in this process. In order to evaluate this further, the measurement of gene expression at multiple timepoints would need to be undertaken to broaden the understanding of temporal expression of *MAPK3*.

In contrast, ihBMSC expression of *MAPK14*, which encodes for p38α, was increased in the presence of IL-1β or combined inflammatory mediators during osteogenesis, suggesting its role in osteogenic differentiation of ihBMSCs. This data does not correlate with other findings by Li *et al.*, (2016) who postulate that p38 expression is not essential in UC-MSCs. However, this may be a result of differing cell sources, as other studies have shown that p38 boosts the transcriptional potential of *RUNX2* to promote osteogenic differentiation (Artigas *et al.*, 2014). Moreover, p38 activation by IL-1β in both a mouse and human cell line has been shown to induce IL-6 (Rodríguez-Carballo, Gámez and Ventura, 2016), a cytokine whose expression was markedly increased in this experiment. Both IL-1β and IL-6 are known to

activate p38 in human osteoblastic cells (Patil *et al.*, 2004), and in these treatment conditions these cytokines may be promoting self-perpetuation of this signalling pathway for propagation of osteogenic differentiation.

5.4.2.5 Wnt signalling

Canonical Wnt signalling is dependent on the stabilisation of β-catenin for subsequent mediation of Wnt target gene transcription. The role of Wnt/β-catenin signalling in MSCs has linked to both MSC commitment to the osteoblast lineage and repression of osteoclast differentiation (Baron and Kneissel, 2013; Kim *et al.*, 2015; Kobayashi *et al.*, 2016). As the gene expression of known canonical Wnt signalling proteins (*WNT3A* and *WNT7A*) could not be detected in the TLDAs presented in this chapter, it is difficult to speculate the role of this signalling pathway in ihBMSCs from this study. However, gene expression for both LRP5 and β-catenin could be detected at similar levels in cells that were undergoing osteogenesis and those that were not. As cells at day 10 of differentiation have an osteoblast phenotype, this finding indicates that canonical Wnt signalling is not altered in osteoblasts compared to ihBMSCs, though signalling changes during osteoblast commitment (before this time point) and during mineralisation (after this time point) cannot be discounted. It is also important to note that undifferentiated MSCs cultured in SM will not be proliferating at this time point, as confluency will have already been reached. This illustrates a limitation of the protocol, as growth arrest may alter the signalling profile and phenotype of the cells.

Non-canonical Wnt signalling operates independently of β-catenin and exerts its effects through either planar cell polarity signalling, calcium influx or PKC activity (Martineau *et al.*, 2017). One of the glycoproteins known to activate these β-catenin independent signalling pathways is WNT5A, and analysis of

its gene expression using a TLDA protocol showed that the presence of combined inflammatory mediators or IL-1β in OM significantly increased *WNT5A* expression during osteogenic differentiation of ihBMSCs. As there is growing evidence that Wnt signalling pathways interact with each other (Niehrs, 2012), this could account for the lack of altered canonical Wnt gene expression observed in the osteogenic conditions supplemented with inflammatory mediators, as WNT5A preferentially binds to the Frizzled 2 receptor thus inhibiting binding of canonical proteins (Sato *et al.*, 2010). Increased expression of *WNT5A* in the presence of IL-1β during osteogenic differentiation was first demonstrated by Sonomoto et al., (2012). Similar to this experiment, they differentiated human MSCs in OM supplemented with 0.1-10ng/ml IL-1β for 10 days, and reported enhanced osteoblast differentiation and mineralisation through increased expression of *RUNX2*, and both ALP and Alizarin Red S staining. They identified that *WNT5A*, and its major receptor *ROR2*, were induced by IL-1β and confirmed its role in osteogenesis through gene silencing. Data presented in this chapter supports the finding that WNT5A is involved in increased osteogenic differentiation of ihBMSCs. It also extends these findings by demonstrating that supplementation of multiple inflammatory mediators in OM increased non-canonical *WNT5A* signalling, and the presence of an anti-inflammatory mediator, such as IL-10, did not revise this effect. Although this condition only included a representative sample of mediators, it could indicate that a milieu of inflammatory mediators in the osteoarthritic joint could be triggering this signalling cascade *in vivo*, with IL-1β acting as a key mediator of its control. Combined with findings that osteoblasts from OA joints have decreased Wnt/β-catenin and increased expression of the non-canonical WNT5A ligand (Martineau *et al.*, 2017) it can be suggested that the microenvironment in an osteoarthritic joint is promoting the activation of non-canonical Wnt signalling to drive the process of osteogenesis during bone remodelling.

Although Sonomoto *et al.*, (2012) concluded that there may be a potential benefit of IL-1β treated human MSCs for the treatment of damaged bone, caution should be taken with this, as results presented in this chapter provide evidence that the presence of IL-1β in the inflammatory environment of OA joint is also linked to expression of genes involved in osteoclastogenesis, inflammation and cartilage degradation. In fact, WNT5A itself has been linked to enhanced osteoclast differentiation and production of pro-inflammatory cytokines, such as IL-6 and IL-8, through increased production of RANKL (Yamashita, Takahashi and Udagawa, 2012).

5.4.3 Conclusions

Consequences of altered bone remodelling in the osteoarthritic joint include phenotypic changes, such as subchondral bone thickening and osteophyte formation, which contribute to the pain and progression of the disease. When considering MSC transplantation as a viable treatment option for OA, it is therefore important to consider the effects of MSCs in the altered bone remodelling process. This will prevent transplanted cells from contributing to disease progression, and instead allow them to exert therapeutic effects that delay, prevent, or even reverse, joint damage. To achieve a better understanding of the roles of MSCs in bone remodelling, it is crucial to gain a clearer insight into the effects of the osteoarthritic microenvironment on transplanted MSCs.

Data presented here showed that the exposure of BMSCs to IL-1β, a pro-inflammatory cytokine increased in the osteoarthritic joint, significantly altered the initiation and rate of osteogenic differentiation *in vitro*, as measured by ALP activity and mineral deposition. These effects were observed in the presence of IL-1β alone, or in combination with other

inflammatory mediators (IL-8, IL-10, MCP-1 and NGF). In a murine BMSC cell line, these treatment conditions resulted in delayed initiation of osteogenic markers, whilst the opposite effect was seen in an ihBMSC cell line and human primary BMSCs. This illustrates the role of species differences during osteogenic differentiation, and cautions against extrapolating data from separate species when analysing bone remodelling experiments. This is particularly important when considering the variability of results observed in the literature.

To assess how IL-1β was causing altered osteogenic differentiation, analysis of cell signalling pathways revealed the potential role of non-canonical Wnt and TGF-β/BMP signalling, via WNT5A and MAPK14 (p38α) respectively. These pathways are known to be involved in bone formation during homeostatic bone remodelling and were enhanced in this inflammatory environment. Furthermore, this data highlighted the activation of signalling pathways prominent in osteoclastogenesis, such as TLR-2 mediated IL-6 and IL-8 release in osteoblasts, which begins to give a clearer picture of the whole process of bone remodelling *in vivo*.

Although these *in vitro* conditions did not represent the complete complexity of the osteoarthritic joint, it did give insight into the effects of inflammatory mediators on MSCs during OA. Importantly, the effects of IL-1β were neither positively nor negatively affected by the presence of other inflammatory mediators found in the osteoarthritic joint, and continued to promote increased osteogenic differentiation of ihBMSCs. The identification of pathways that are affected by this pro-inflammatory cytokine is important not only to gain a clearer insight into the pathogenesis of OA, but also to determine how MSCs could be used for successful targeted therapy in OA. A

summary of the key changes in ihBMSC gene expression during osteogenic differentiation can be observed in Table **5.1**.

	OM	OM + mediators	OM + IL-1β
Gene	Fold change (log_2)	Fold change (log_2)	Fold change (log_2)
Osteogenic differentiation markers			
BGLAP	-1.71	-1.83	1.21
Inflammatory mediators			
IL1R1	2.71	3.05	3.27
CXCL8	4.25	8.48	7.89
IL6	-0.61	2.55	1.24
IDO1	0.23	2.47	3.55
MMP1	-0.78	2.87	1.24
MMP13	-2.31	0.82	-1.09
Toll like receptor signalling			
TLR2	3.44	5.86	6.05
TLR3	0.65	0.95	2.00
TLR4	1.22	1.39	1.48
TGFβ/BMP signalling			
BMPR1B	1.03	1.67	2.95
MAPK3	0.80	0.39	0.06
MAPK14	0.07	0.37	0.26
Wnt signalling			
WNT5A	0.74	1.87	1.91

Table 5.1 Key changes in ihBMSC gene expression during osteogenic differentiation

Exposure of ihBMSCs to combined inflammatory mediators and IL-1β alone during osteogenic differentiation resulted in significant changes to gene expression. Shown as the log_2 fold change from SM, genes encoding for pro-inflammatory mediators, cartilage degrading enzymes, TLRs, MAPKs and *WNT5A* were significantly increased following exposure to these conditions.

Chapter 6 Final discussion

6.1 Rationale for MSCs used in OA therapy

Despite OA being the most prevalent form of joint disease there is a striking unmet need for treatment strategies (Tonge, Pearson and Jones, 2014; Chen *et al.*, 2017). Current treatments are focused on the relief on pain symptoms whilst the disease is able to progress indefinitely, causing cartilage degradation, subchondral bone remodelling and inflammation (Hunter, 2011).

The inherent properties of MSCs make them an attractive candidate for OA treatment, as they can exert immunomodulatory effects through paracrine signalling, and also differentiate into cell types of the joint, including chondrocytes and osteoblasts (Wyles *et al.*, 2015; Kong *et al.*, 2017). Moreover, they can be easily sourced and expanded (Man and Mologhianu, 2014; Kim and Park, 2017). These features give them a critical advantage over chondrocytes that are used in established treatments such as autologous chondrocyte implantation, which is only recommended for treatment of small articular lesions, thereby ruling out most people with OA (NICE, 2017).

Recent advances in our understanding on the paracrine activity of MSCs has highlighted the trophic and immunomodulatory abilities of these cells (Baraniak and McDevitt, 2010; Kong *et al.*, 2017). Although exploitation of MSC paracrine effects could be instrumental in prevention of disease progression and provide relief of pain symptoms, their effects on endogenous cartilage regeneration is limited by a lack of progenitor source in cartilage (Grogan *et al.*, 2009; Koelling *et al.*, 2009), and thus the ability of MSCs to differentiate is important with a view to full disease treatment and joint regeneration. Therefore, development of treatment strategies that harness

both the immunomodulatory and differentiation potential of MSCs is paramount to success.

However, current clinical trials assessing the effectiveness of MSCs for OA treatment do not provide unequivocal evidence for cartilage regeneration, and although significant improvements in pain and physical function of the treated joint have been reported (Jo *et al.*, 2014; Lamo-Espinosa *et al.*, 2018), a systematic review of these trials suggests that poor study design, large heterogeneity of results and high-risk of bias impedes the quality of the results meaning these clinical trials are insufficient at providing strong evidence for the efficacy of MSC transplantation (Iijima *et al.*, 2018).

One potential cause for the lack of observed success in clinical trials of MSC transplantation is the effect of the local inflammatory environment on the osteoarthritic joint. Recent studies suggest that endogenous populations of MSCs have been shown to display an altered phenotype and differentiation potential (Coleman *et al.*, 2010; Wyles *et al.*, 2015), and properties of a recently described, novel population of MSCs suggest that they may even be contributing towards the OA phenotype (Jayasuriya *et al.*, 2018). Therefore, identification of factors and cell signalling pathways that influence MSC function is critical for ensuring MSCs promote reparative effects and do not contribute to disease pathology. These findings will subsequently help to develop targeted MSC-based treatments for OA that can regenerate joint tissue, modulate the immune response and relieve pain. To explore this, this study aimed to identify the effects that inflammatory mediators (IL-1β, IL-8, IL-10, MCP-1 and NGF) known to be involved in OA pathology have on BMSCs.

6.2 Effects of inflammatory mediators on BMSCs

To assess the effects of an inflammatory environment on ihBMSCs, these cells were cultured in the presence of inflammatory mediators that are known to contribute towards OA pathology. Although these mediators did not affect the proliferative capacity or viability of these cells over 10 days in culture, their gene expression profile was altered in the presence of IL-1β and combined mediators. These treatment conditions promoted increased expression of genes encoding for pro-inflammatory cytokines (IL-1β, IL-6, IL-8, MCP-1) and cartilage degrading enzymes (MMP-13), as well as genes involved in immunosuppression (COX-2, IDO, HGF), illustrating opposing actions of MSC paracrine activity in response to IL-1β. Moreover, both IL-6 and COX-2 are involved in propagation of OA pain (Laine *et al.*, 2008; Lin *et al.*, 2017). Importantly, the presence of combined inflammatory mediators counteracted some effects seen by individual IL-1β exposure, suggesting that other mediators may have an antagonistic effect on the actions of IL-1β. This was primarily observed in expression of TLRs, whereby combined inflammatory mediators prevented the upregulated expression seen in individual IL-1β treatment. This information could be useful when considering MSCs in OA treatment strategies, as TLRs have been linked to generation of signalling cascades that promote secretion of pro-inflammatory cytokines (Sangiorgi and Panepucci, 2016). Further investigation of the synergistic and antagonistic effects of the inflammatory mediators would yield useful results with regards to identifying the cause of this differential gene expression, and could be achieved by culturing the cells in different combinations of the mediators with IL-1β.

As the differentiation capacity of MSCs is also a critical parameter for success of future OA therapies, the effects of an inflammatory environment upon

ihBMSC chondrogenic differentiation was assessed. The data presented here corroborates findings in the literature that IL-1β impedes the chondrogenic differentiation capacity of MSCs (Wehling *et al.*, 2009; Liu *et al.*, 2017), as measured by the decreased concentration of GAGs in the IL-1β treated chondrogenic pellets. Importantly, this work extends these findings by showing that a combination of mediators also reduced the concentration of GAGs; although this decrease was less pronounced than the individual treatment of IL-1β. In a similar way to the results following exposure during standard cell culture, this may be a result of antagonistic actions of inflammatory mediators on IL-1β regulated signalling pathways, and provides an indication that the reported effects of IL-1β *in vitro* may not be fully representative of effects in the OA inflammatory environment. Moreover, it may help to identify specific mediators that could abrogate the negative effects of IL-1β during chondrogenic differentiation of BMSCs, and so future work could include exploration of this for validation.

Gene expression analysis of chondrogenic pellets revealed that the decreased quantification of GAGs was associated with decreased expression of the gene encoding for aggrecan, but increased expression of genes encoding for the cartilage degrading enzymes MMPs. This suggests that IL-1β may be able to exert its effects through both prevention of proteoglycan synthesis and degradation of ECM components. A limitation of this study is the assessment of chondrogenic differentiation at one time-point, which does not allow for discrimination between the potential outcomes of altered *ACAN* and *MMP* expression. Future work assessing GAG concentration throughout the differentiation process could help to identify if this altered gene expression works synergistically to decrease GAG levels or if IL-1β stimulated effects favour one pathway. Furthermore, this analysis would allow for investigation

into the observed decreased cell number in these conditions and identify if IL-1β conditions reduce proliferation or promote cell death upon differentiation.

In parallel to these gene expression findings, IL-1β treatment of chondrogenic pellets was also linked to increased expression of genes that may act as markers of chondrocyte hypertrophy (*RUNX2, ALPL, IBSP*). These findings suggest that IL-1β might drive MSCs towards terminal differentiation, which would be undesirable for regeneration of cartilage, as this might lead to endochondral ossification and formation of painful osteophytes in the OA joint (Van der Kraan and Van den Berg, 2012). The gene expression analysis also began to unravel the signalling pathways that are activated by IL-1β, and could therefore act as potential therapeutic targets for prevention of aberrant MSC chondrogenic differentiation. This includes the MAPK signalling pathway, corroborating findings by Wang et al., 2011, and *TLR2*, which are both known to augment expression of MMPs. Moreover, it was observed that IL-1β limited the expression of anabolic growth factors, such as *TGF-β3* and *IGF-1* that contribute to synthesis of ECM proteins (Tang *et al.*, 2009).

In contrast to the observations during chondrogenic differentiation, the osteogenic differentiation of ihBMSCs was enhanced by IL-1β. Here the temporal regulation of osteogenesis was altered so that ihBMSCs began to differentiate more quickly down the osteoblast lineage, as shown by earlier induction of both ALP activity and mineral deposition, corroborating findings by Sonomoto *et al.*, (2012). Importantly, the present work extends these findings by demonstrating that combined inflammatory mediators maintained this change in differentiation, but was still mediated specifically by IL-1β. Gene expression analysis of ihBMSCs undergoing osteogenic differentiation was able to provide some insight into the signalling pathways that might be involved in this enhanced differentiation, highlighting changes

to both MAPK signalling and non-canonical Wnt signalling. The former suggests that increased expression of the gene encoding for p38α may be linked to promotion of osteogenic differentiation, whilst the latter is in keeping with findings by Sonomoto *et al.,* (2012) who showed that IL-1β enhanced osteoblast differentiation and mineralisation through *WNT5A* signalling. Although, this group concluded that there may be potential benefits to treatment of human MSCs with IL-1β, results from the present gene expression analysis cautions against this. This is due to the finding that IL-1β was also seen to increase expression of pro-inflammatory cytokines, such as IL-6 and IL-8, which have been linked to catabolic events like osteoclastogenesis and stimulation of MMPs. This may occur through the upregulation of *TLR2* and *TLR3* in these cells. These findings are relevant with regards to the use of MSCs as a therapeutic option, as exposure to an IL-1β mediated inflammatory environment appears to both enhance osteogenic differentiation of human BMSCs whilst simultaneously increasing gene expression of factors involved in bone resorption. This could suggest that MSCs may act to increase the rate of bone remodelling, a key feature of OA pathology that is also linked to increased pain. As this altered bone turnover is also marked by hypomineralisation (Li and Aspden, 1997) it would be interesting to assess the quality of the mineralisation observed in these cells to explore this hypothesis. A limitation of this study was a lack of normalisation to cell number. Although results on cell proliferation do not suggest any observable effect of IL-1β in standard culture, it would be important to confirm this in osteogenic conditions.

6.3 Future considerations

The data presented in this thesis provides an initial dataset supporting future investigation into the factors and signalling pathways altering the biological

functions of ihBMSCs in an inflammatory environment. For example, one interesting follow up could be looking into the effects of increased *TLR2* expression. Expression of this gene was significantly increased by ihBMSCs in response to IL-1β during both chondrogenic and osteogenic differentiation. As TLR-2 has been linked to increased production of pro-inflammatory mediators it could be hypothesised that this receptor is driving the increased gene expression of *IL6* and *CXCL8* in these conditions, which may have detrimental downstream effects via promotion of osteoclastogenesis and cartilage breakdown. Adding to this hypothesis, this receptor is particularly interesting as it has been reported to inhibit tri-lineage differentiation in mice (Pevsner-Fischer *et al.*, 2007), and data in this study observed a decreased capacity of murine BMSCs to undergo osteogenic differentiation in the presence of IL-1β. Therefore, further investigation into this receptor's role may reveal a potential therapeutic target.

This data could generally be extended and improved in the ways already discussed, including identification and validation of any antagonistic effects of the tested mediators, measurement of chondrogenic differentiation at multiple time points and normalisation of osteogenic differentiation to cell number. Further limitations of the *in vitro* protocol should also be noted, as the customary medium change every 2-3 days alters the MSC microenvironment and thereby limits the autocrine effects of secreted factors by these cells.

Although gene expression analysis is a useful tool for the measure of mediator driven changes, it can be limited by the potential lack of stability and short half-life of RNA that results in discrepancies between gene expression levels and proteins levels. Therefore, it would be important to validate these findings and allow for the presence of post-transcriptional modifications through

analysis of protein expression of relevant markers. This could be achieved through immunocytochemistry and coupled flow cytometry and microscopy observation for quantitative and qualitative evaluation. Furthermore, although the TLDA method was useful for assessing treatment effects on a large number of genes, it could not allow for optimisation and thus key genes such as *COL2A1* could not detected. Separate analysis of any other genes of interest that could not be detected in the array, or have since become interesting upon analysis of results, could be assessed using real time-PCR and may be beneficial to expansion of findings.

6.4 Conclusions

Although *in vitro* conditions do not represent the complete complexity of the osteoarthritic joint, the combination of inflammatory mediators does begin to give deeper insight into the effects of the inflammatory environment on BMSCs. Taken together, these findings suggest that MSCs might have a detrimental effect on OA pathology in an IL-1β mediated inflammatory environment, as they can contribute to increased levels of pro-inflammatory cytokines and cartilage degrading enzymes. Furthermore, cells exposed to IL-1β show both limited capacity to undergo chondrogenic differentiation and increased ability to differentiate towards the osteoblast lineage, suggesting that these cells may be more likely to contribute to subchondral bone remodelling than cartilage regeneration. A graphical summary of the key findings from this thesis can be seen in Figure **6.1.**

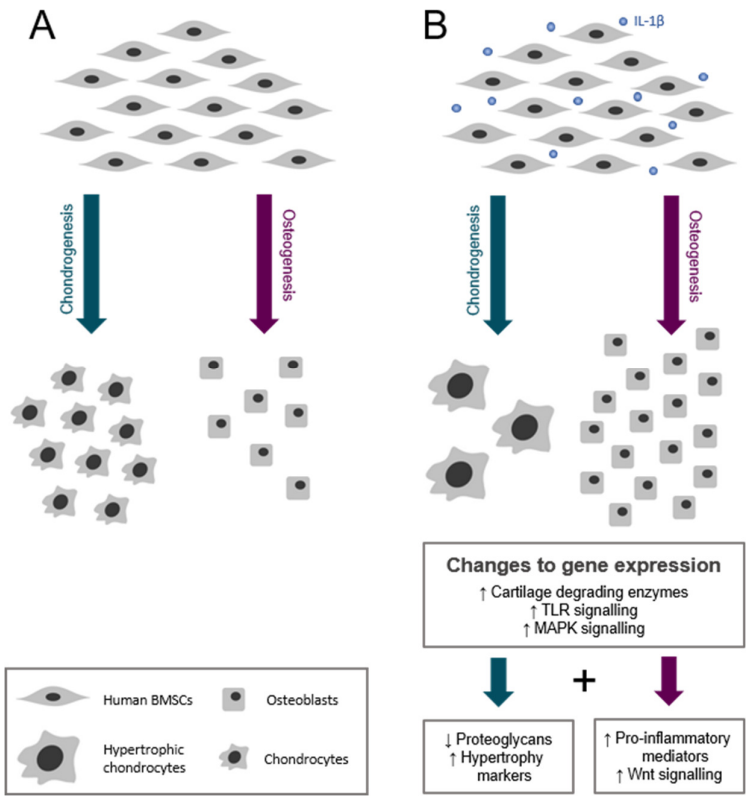


Figure 6.1. A graphical abstract of the key findings from this thesis

In the right conditions human BMSCs have the ability to differentiate into chondrocytes and osteoblasts, making them an attractive candidate for novel OA treatment strategies (**A**). However, it is hypothesised that the OA inflammatory environment may affect the functional capacity of these cells, and thus this thesis aimed to explore this hypothesis. In these experiments, exposure of human BMSCs to the pro-inflammatory mediator IL-1β, whether alone or in combination with other inflammatory mediators, was seen to affect the differentiation response of these cells under chondrogenic and osteogenic conditions (**B**). The observed changes are defined by reduced levels of chondrogenic differentiation and increased levels of osteogenic differentiation *in vitro*. Gene expression analysis revealed increased expression of genes associated with cartilage degrading enzymes (e.g. MMPs), TLR signalling and MAPK signalling during both chondrogenic and osteogenic differentiation. Furthermore, a reduction in the expression of genes encoding for proteoglycans and an increase in the expression of chondrocyte hypertrophy markers was seen during chondrogenic differentiation, whilst increased expression of genes encoding for pro-inflammatory mediators and non-canonical Wnt signalling molecules was observed during osteogenic differentiation. Together, these findings suggest that an IL-1β mediated inflammatory

environment, such as that seen in OA, may limit the therapeutic potential of BMSCs, as it indicates that these cells may be more likely to contribute to subchondral bone remodelling than cartilage regeneration.

References

Abdelgawad, M. E. *et al.* (2016) 'Early reversal cells in adult human bone remodeling: osteoblastic nature, catabolic functions and interactions with osteoclasts', *Histochemistry and Cell Biology*. Springer Berlin Heidelberg, 145(6), pp. 603–615. doi: 10.1007/s00418-016-1414-y.

Ahmed, A. S. I. *et al.* (2017) 'Effect of aging on stem cells', *World Journal of Experimental Medicine*, 7(1), p. 1. doi: 10.5493/wjem.v7.i1.1.

Akeson, G. and Malemud, C. J. (2017) 'A role for soluble IL-6 receptor in osteoarthritis', *Journal of Functional Morphology and Kinesiology*, 2(3), p. 27. doi: 10.1016/j.chemosphere.2012.12.037.Reactivity.

Akiyama, H. *et al.* (2002) 'The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6', *Genes and Development*, 16(21), pp. 2813–28. doi: 10.1101/gad.1017802.overt.

Alvarez, C. V. *et al.* (2012) 'Defining stem cell types: understanding the therapeutic potential of ESCs, ASCs, and iPS cells', *Journal of Molecular Endocrinology*, 49(2), pp. R89-111. doi: 10.1530/JME-12-0072.

Arikawa, T., Omura, K. and Morita, I. (2004) 'Regulation of bone morphogenetic protein-2 expression by endogenous prostaglandin E2 in human mesenchymal stem cells', *Journal of Cellular Physiology*, 200(3), pp. 400–6. doi: 10.1002/jcp.20031.

Arthritis Research UK (2013) *Osteoarthritis in general practice: data and perspectives*.

Artigas, N. *et al.* (2014) 'Mitogen-activated protein kinase (MAPK)-regulated interactions between osterix and Runx2 are critical for the transcriptional

osteogenic program', *Journal of Biological Chemistry*, 289(39), pp. 27105–27117. doi: 10.1074/jbc.M114.576793.

Aubin, J. (2001) 'Regulation of osteoclast formation and function', in *Rev. Endocr. Metab. Disord.*, pp. 95–104.

Ayral, X. *et al.* (2005) 'Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis - Results of a 1 year longitudinal arthroscopic study in 422 patients', *Osteoarthritis and Cartilage*, 13(5), pp. 361–7. doi: 10.1016/j.joca.2005.01.005.

Ba, P. *et al.* (2017) 'Differential effects of p38 and Erk1/2 on the chondrogenic and osteogenic differentiation of dental pulp stem cells', *Molecular Medicine Reports*, 16(1), pp. 63–8. doi: 10.3892/mmr.2017.6563.

Badlani, N. *et al.* (2009) 'Use of bone morphogenic protein-7 as a treatment for osteoarthritis', *Clinical Orthopaedics and Related Research*, 467(12), pp. 3221–9. doi: 10.1007/s11999-008-0569-9.

Bajada, S. *et al.* (2008) 'Updates on stem cells and their applications in regenerative medicine', *Journal of Tissue Engineering and Regenerative Medicine*, 2(4), pp. 169–83. doi: 10.1002/term.

Baraniak, P. R. and McDevitt, T. C. (2010) 'Stem cell paracrine actions and tissue regeneration', *Regenerative Medicine*, 5(1), pp. 121–43. doi: 10.2217/rme.09.74.Stem.

Baron, R. and Kneissel, M. (2013) 'WNT signaling in bone homeostasis and disease: from human mutations to treatments', *Nature Medicine*. Nature Publishing Group, 19(2), pp. 179–192. doi: 10.1038/nm.3074.

Batra, H. and Charnley, J. (1969) 'Existence and incidence of osteoid in osteoarthritis femoral heads. A preliminary report.', *The Journal of Bone and Joint Surgery British Volume*, 51(2), pp. 366–71.

Bellayr, I. H. and Li, Y. (2009) 'Stem Cells: It's Good To Have Choices', *Journal of the American College of Certified Wound Specialists*. Mosby, Inc, 1(3), pp. 92–4. doi: 10.1016/j.jcws.2009.05.004.

Bendre, M. S. *et al.* (2003) 'Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease', *Bone*, 33(1), pp. 28–37. doi: 10.1016/S8756-3282(03)00086-3.

Bhosale, A. M. and Richardson, J. B. (2008) 'Articular cartilage: Structure, injuries and review of management', *British Medical Bulletin*, 87(1), pp. 77–95. doi: 10.1093/bmb/ldn025.

Bigg, H. F. *et al.* (2007) 'Activity of matrix metalloproteinase-9 against native collagen types I and III', *FEBS Journal*, 274(5), pp. 1246–55. doi: 10.1111/j.1742-4658.2007.05669.x.

Birmingham, E. *et al.* (2012) 'Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche', *European Cells and Materials*, 23(353), pp. 13–27. doi: 10.22203/eCM.v023a02.

Blaney Davidson, E. N. *et al.* (2009) 'Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice', *The Journal of Immunology*, 182(12), pp. 7937–45. doi: 10.4049/jimmunol.0803991.

Bohme, K. *et al.* (1992) 'Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: The role of insulin-like growth factor-I, insulin, or thyroxine', *Journal of Cell Biology*, 116(4), pp. 1035–42. doi: 10.1083/jcb.116.4.1035.

Borzi, R. M. *et al.* (1999) 'Flow cytometric analysis of intracellular chemokines in chondrocytes in vivo: constitutive expression and enhancement in osteoarthritis and rheumatoid arthritis', *FEBS Letters*, 455(3), pp. 238–42. doi: 10.1016/S0014-5793(99)00886-8.

Borzi, R. M. *et al.* (2002) 'Growth-related oncogene α induction of apoptosis in

osteoarthritis chondrocytes', *Arthritis and Rheumatism*, 46(12), pp. 3201–11. doi: 10.1002/art.10650.

Borzi, R. M. *et al.* (2014) 'Chemokines in cartilage degradation', *Clinical Orthopaedics and Related Research2*, 427, pp. S53–S61.

Bosetti, M. *et al.* (2007) 'Effect of different growth factors on human osteoblasts activities: A possible application in bone regeneration for tissue engineering', *Biomolecular Engineering*, 24(6), pp. 613–618. doi: 10.1016/j.bioeng.2007.08.019.

Boyden, L. *et al.* (2002) 'High bone density due to a mutation in LDL-receptor-related protein 5', *The New England Journal of Medicine*, 342(20), pp. 1513–21.

Bozec, A. *et al.* (2014) 'BONE DISEASE T Cell Costimulation Molecules CD80 / 86 Inhibit Osteoclast Differentiation by Inducing the IDO / Tryptophan Pathway', *Science Translational Medicine*, 6(May), pp. 1–11. doi: 10.1126/scitranslmed.3007764.T.

Brandt, K. D. (1999) 'Osteophytes in osteoarthritis. Clinical aspects', *Osteoarthritis and Cartilage*, 7(3), pp. 334–5. doi: 10.1053/joca.1998.0187.

Bryder, D., Rossi, D. J. and Weissman, I. L. (2006) 'Hematopoietic stem cells: The paradigmatic tissue-specific stem cell', *American Journal of Pathology*, 169(2), pp. 338–46. doi: 10.2353/ajpath.2006.060312.

Buckland-Wright, C. (2004) 'Subchondral bone changes in hand and knee osteoarthritis detected by radiography', *Osteoarthritis and Cartilage*, 12, pp. S10-19. doi: 10.1016/j.joca.2003.09.007.

Buckwalter, J. A. and Mankin, H. J. (1998) 'Articular cartilage: tissue design and chondrocyte-matrix interactions', *Instructional Course Lectures*, 47(1), pp. 477–86.

Burr, D. B. and Gallant, M. A. (2012) 'Bone remodelling in osteoarthritis', *Nature Reviews Rheumatology*. Nature Publishing Group, 8(11), pp. 665–73. doi:

10.1038/nrrheum.2012.130.

Burrage, P. S., Mix, K. S. and Brinckerhoff, C. E. (2006) 'Matrix metalloproteinases: role in arthritis', *Frontiers in Bioscience: A Journal and Virtual Library*, 1(11), pp. 529–43.

Caplan, A. (1991) 'Mesenchymal stem cells.', *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*, 9(5), pp. 641–50. doi: 10.1002/jor.1100090504.

Caplan, A. I. and Correa, D. (2011a) 'PDGF in bone formation and regeneration: New insights into a novel mechanism involving MSCs', *Journal of Orthopaedic Research*, 29(12), pp. 1795–1803. doi: 10.1002/jor.21462.

Caplan, A. I. and Correa, D. (2011b) 'The MSC: an injury drugstore', *Cell Stem Cell*, 9(1), pp. 11–15. doi: 10.1016/j.stem.2011.06.008.THE.

Caplan, A. I. and Dennis, J. E. (2006) 'Mesenchymal stem cells as trophic mediators', *Journal of Cellular Biochemistry*, 98(5), pp. 1076–84. doi: 10.1002/jcb.20886.

Carlson, M. E. and Conboy, I. M. (2007) 'Loss of stem cell regenerative capacity within aged niches', *Aging Cell*, 6(3), pp. 371–82. doi: 10.1111/j.1474-9726.2007.00286.x.

Casarosa, S., Bozzi, Y. and Conti, L. (2014) 'Neural stem cells: ready for therapeutic applications?', *Molecular and Cellular Therapies*, 2(1), p. 31. doi: 10.1186/2052-8426-2-31.

Chauffier, K. *et al.* (2012) 'Induction of the chemokine IL-8/Kc by the articular cartilage: possible influence on osteoarthritis', *Joint Bone Spine*, 79(6), pp. 604–9. doi: 10.1016/j.jbspin.2011.12.013.

Chen, D. *et al.* (2017) 'Osteoarthritis: toward a comprehensive understanding of pathological mechanism', *Bone Research*. Nature Publishing Group,

5(August 2016). doi: 10.1038/boneres.2016.44.

Chen, F. S., Frenkel, S. R. and Di Cesare, P. E. (1999) 'Repair of articular cartilage defects: part I. Basic science of cartilage healing', *American Journal of Orthopedics*, 28(1), pp. 31–3.

Chen, H. *et al.* (2014) 'Runx2 regulates endochondral ossification through control of chondrocyte proliferation and differentiation', *Journal of Bone and Mineral Research*, 29(12), pp. 2653–65. doi: 10.1002/jbmr.2287.Runx2.

Chen, S. *et al.* (2015) 'Strategies to minimize hypertrophy in cartilage engineering and regeneration', *Genes and Diseases*. Elsevier Ltd, 2(1), pp. 76–95. doi: 10.1016/j.gendis.2014.12.003.

Chen, W. H. *et al.* (2009) 'In vitro stage-specific chondrogenesis of mesenchymal stem cells committed to chondrocytes', *Arthritis and Rheumatism*, 60(2), pp. 450–9. doi: 10.1002/art.24265.

Cho, H. H. *et al.* (2009) 'NF-ÎºB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression', *Journal of Cellular Physiology*, (November 2009), p. n/a-n/a. doi: 10.1002/jcp.22024.

Cho, M. La *et al.* (2007) 'Toll-like receptor 2 ligand mediates the upregulation of angiogenic factor, vascular endothelial growth factor and interleukin-8/CXCL8 in human rheumatoid synovial fibroblasts', *Immunology Letters*, 108(2), pp. 121–128. doi: 10.1016/j.imlet.2006.11.005.

Choi, J. J. *et al.* (2008) 'Mesenchymal stem cells overexpressing interleukin-10 attenuate collagen-induced arthritis in mice', *Clinical and Experimental Immunology*, 153(2), pp. 269–76. doi: 10.1111/j.1365-2249.2008.03683.x.

Clar, C. *et al.* (2005) *Clinical and cost-effectiveness of autologous chondrocyte implantation for cartilage defects in knee joints: systematic review and economic evaluation*, *https://www.ncbi.nlm.nih.gov/books/NBK62302/*.

Coleman, C. M. *et al.* (2010) 'Mesenchymal stem cells and osteoarthritis: remedy or accomplice?', *Human Gene Therapy*, 21(10), pp. 1239–50. doi: 10.1089/hum.2010.138.

Collison, J. (2018) 'Low-level inflammation promotes bone growth', *Nature Reviews Rheumatology*, 14(5), p. 249.

Conboy, I. M. *et al.* (2003) 'Notch-mediated restoration of regenerative potential to aged muscle', *Science*, 302(5650), pp. 1575–7.

Couchourel, D. *et al.* (2009) 'Altered mineralization of human osteoarthritic osteoblasts is attributable to abnormal type I collagen production', *Arthritis and Rheumatism*, 60(5), pp. 1438–1450. doi: 10.1002/art.24489.

Crane, J. L. and Cao, X. (2014) 'Bone marrow mesenchymal stem cells andTGF-ß signaling in bone remodeling', *Journal of Clinical Investigation*, 124(2), pp. 466–472. doi: 10.1172/JCI70050.466.

Cutler, C. *et al.* (2013) 'Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation', *Blood*, 122(17), pp. 3074–81. doi: 10.1182/blood-2013-05-503177.

Daley, G. Q. (2012) 'The promise and perils of stem cell therapeutics', *Cell Stem Cell*, 10(6), pp. 740–9. doi: 10.1016/j.stem.2012.05.010.The.

Dean, D. D. and Woessner, J. F. (1984) 'Extracts of human articular cartilage contain an inhibitor of tissue metalloproteinases', *The Biochemical Journal*, 218(1), pp. 277–80.

DelaRosa, O., Dalemans, W. and Lombardo, E. (2012) 'Toll-Like Receptors as Modulators of Mesenchymal Stem Cells', *Frontiers in Immunology*, 3(July), pp. 1–8. doi: 10.3389/fimmu.2012.00182.

Deshmane, S. L. *et al.* (2009) 'Monocyte chemoattractant protein-1 (MCP-1): an Overview', *Journal of Interferon & Cytokine Research*, 29(6), pp. 313–26. doi:

10.1089/jir.2008.0027.

Dhingra, S. *et al.* (2015) 'Modulation of alloimmune responses by interleukin-10 prevents rejection of implanted allogeneic smooth muscle cells and restores postinfarction ventricular function', *Cell Transplantation*, 24(6), pp. 1013–29. doi: 10.3727/096368914X681036.

Dimmeler, S. *et al.* (2014) 'Translational strategies and challenges in regenerative medicine', *Nature Medicine*, 20, pp. 814–21.

Dimmeler, S. and Leri, A. (2008) 'Aging and disease as modifiers of efficacy of cell therapy Stefanie', *Circulation Research*, 102(11), pp. 1319–30. doi: 10.1161/CIRCRESAHA.108.175943.Aging.

Dinarello, C. A. (2011) 'Interleukin-1 in the pathogenesis and treatment of inflammatory diseases', *Blood*, 117(14), pp. 3720–32.

Dodds, R. A. *et al.* (1994) 'Expression of mRNA for IL1 beta, IL6 and TGF beta 1 in developing human bone and cartilage', *Journal of Histochemistry and Cytochemistry*, 42(6), pp. 733–44.

Dore, D. *et al.* (2010) 'Bone marrow lesions predict site-specific cartilage defect development and volume loss: a prospective study in older adults', *Arthritis Research & Therapy*. BioMed Central Ltd, 12(6), p. R222. doi: 10.1186/ar3209.

Dumond, H. *et al.* (2003) 'Evidence for a key role of leptin in osteoarthritis', *Arthritis & Rheumatism*, 48(11), pp. 3118–29. doi: 10.1002/art.11303.

Ekenstedt, K. J. *et al.* (2006) 'Effects of chronic growth hormone and insulin-like growth factor 1 deficiency on osteoarthritis severity in rat knee joints', *Arthritis and Rheumatism*, 54(12), pp. 3850–8. doi: 10.1002/art.22254.

Eriksen, E. F. (2010) 'Cellular mechanisms of bone remodeling', *Reviews in Endocrine and Metabolic Disorders*, 11(4), pp. 219–227. doi: 10.1007/s11154-010-9153-1.

Fan, H. *et al.* (2012) 'Pre-treatment with IL-1β enhances the efficacy of MSC transplantation in DSS-induced colitis', *Cellular and Molecular Immunology*, 9(6), pp. 473–81. doi: 10.1038/cmi.2012.40.

Fan, Z. *et al.* (2007) 'Activation of interleukin-1 signaling cascades in normal and osteoarthritis articular cartilage', *American Journal of Pathology*, 171(3), pp. 938–46. doi: 10.2353/ajpath.2007.061083.

Farahat, M. N. *et al.* (1993) 'Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis', *Annals of the Rheumatic Diseases*, 52(12), pp. 870–5. doi: 10.1136/ard.52.12.870.

Felson, D. T. *et al.* (2001) 'The association of bone marrow lesions with pain in knee osteoarthritis', *Annals of Internal Medicine*, 134(7), pp. 541–9.

Fernandes, J. C., Martel-Pelletier, J. and Pelletier, J. P. (2002) 'The role of cytokines in osteoarthritis pathophysiology', *Biorheology*, 39(1–2), pp. 237–46.

Ferreira, E. *et al.* (2013) 'Inflammatory cytokines induce a unique mineralizing phenotype in mesenchymal stem cells derived from human bone marrow', *Journal of Biological Chemistry*, 288(41), pp. 29494–505. doi: 10.1074/jbc.M113.471268.

Findlay, D. M. and Atkins, G. J. (2014) 'Osteoblast-chondrocyte interactions in osteoarthritis', *Current Osteoporosis Reports*, 12(1), pp. 127–134. doi: 10.1007/s11914-014-0192-5.

Florencio-Silva, R. *et al.* (2015) 'Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells', *BioMed Research International*, 2015, pp. 1–17. doi: 10.1155/2015/421746.

Forbes, S. J. and Rosenthal, N. (2014) 'Preparing the ground for tissue regeneration: from mechanism to therapy', *Nature Medicine*. Nature Publishing Group, 20(8), pp. 857–69. doi: 10.1038/nm.3653.

Fox, A. J. S., Bedi, A. and Rodeo, S. A. (2009) 'The basic science of articular cartilage: structure, composition, and function', *Sports Health*, 1(6), pp. 461–8. doi: 10.1055/s-0032-1313741.

François, M. *et al.* (2012) 'Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation', *Molecular Therapy*. Nature Publishing Group, 20(1), pp. 187–95. doi: 10.1038/mt.2011.189.

Fransen, M. and Neal, B. (2004) 'Non-steroidal anti-inflammatory drugs for preventing heterotopic bone formation after hip arthroplasty', *The Cochrane Database of Systematic Reviews*, 3, p. CD001160. doi: 10.1002/14651858.CD001160.pub2.www.cochranelibrary.com.

Freemont, A. J. *et al.* (1997) 'Gene expression of matrix metalloproteinases 1, 3, and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific', *Annals of the Rheumatic Diseases*, 56(9), pp. 542–8. doi: 10.1136/ard.56.9.542.

Friedenstein, A. J., Piatetzky-Shapiro, I. I. and Petrakova, K. V (1966) 'Osteogenesis in transplants of bone marrow cells', *Journal of Embryology and Experimental Morphology*, 16(3), pp. 381–90.

Fukumoto, T. *et al.* (2003) 'Combined effects of insulin-like growth factor-1 and transforming growth factor-β1 on periosteal mesenchymal cells during chondrogenesis in vitro', *Osteoarthritis and Cartilage*, 11(1), pp. 55–64. doi: 10.1053/joca.2002.0869.

Goldring, M. B. (2012) 'Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis', *Therapeutic Advances in Musculoskeletal Disease*, 4(4), pp. 269–85. doi: 10.1177/1759720X12448454.

Goldring, M. B. and Goldring, S. R. (2010) 'Articular cartilage and subchondral

bone in the pathogenesis of osteoarthritis', *Annals of the New York Academy of Sciences*, 1192, pp. 230–7.

Goldring, M. B. and Marcu, K. B. (2009) 'Cartilage homeostasis in health and rheumatic diseases', *Arthritis Research and Therapy*, 11(3), pp. 224–40. doi: 10.1186/ar2592.

Goldring, S. R. and Goldring, M. B. (2016) 'Changes in the osteochondral unit', *Nature Publishing Group*. Nature Publishing Group, 12(11), pp. 632–44. doi: 10.1038/nrrheum.2016.148.

Gómez-Gaviro, M. V. *et al.* (2012) 'The vascular stem cell niche', *Journal of Cardiovascular Translational Research*, 5(5), pp. 618–30. doi: 10.1007/s12265-012-9371-x.

Gómez, R. *et al.* (2014) 'TLR4 signalling in osteoarthritis — finding targets for candidate DMOADs', *Nature Publishing Group*. Nature Publishing Group, 11(3), pp. 159–170. doi: 10.1038/nrrheum.2014.209.

Gong, Y. *et al.* (2001) 'LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development', *Cell*, 107(4), pp. 513–523. doi: 10.1016/S0092-8674(01)00571-2.

Gregory, C. A. *et al.* (2004) 'An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction', *Analytical Biochemistry*, 329(1), pp. 77–84.

Grogan, S. P. *et al.* (2009) 'Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis', *Arthritis Research and Therapy*, 11(3), p. R85. doi: 10.1186/ar2719.

Grynpas, M. *et al.* (1991) 'Subchondral bone in osteoarthritis', *Calcified Tissue International*, 49(1), pp. 20–6.

Gupta, P. K. *et al.* (2012) 'Mesenchymal stem cells for cartilage repair in

osteoarthritis', *Stem Cell Research & Therapy*, 3(4), p. 25.

Gwilym, S. E. *et al.* (2009) 'Psychophysical and functional imaging evidence supporting the presence of central sensitization in a cohort of osteoarthritis patients', *Arthritis Care and Research*, 61(9), pp. 1226–34. doi: 10.1002/art.24837.

Hadjidakis, D. J. and Androulakis, I. I. (2006) 'Bone remodeling', *Annals of the New York Academy of Sciences*, 1092, pp. 385–396. doi: 10.1196/annals.1365.035.

Haleem-Smith, H. *et al.* (2012) 'Biological responses of human mesenchymal stem cells to titanium wear debris particles', *Journal of Orthopaedic Research*, 30(6), pp. 853–63. doi: 10.1002/jor.22002.Biological.

Harigai, M. *et al.* (1993) 'Monocyte chemoattractant protein-1 (MCP-1) in inflammatory joint diseases and its involvement in the cytokine network of rheumatoid synovium', *Clinical Immunology and Immunopathology*, 69(1), pp. 83–91.

Harris, Q. *et al.* (2013) 'Monocyte chemotactic protein-1 inhibits chondrogenesis of synovial mesenchymal progenitor cells: an in vitro study', *Stem Cells*, 31(10), pp. 2253–65. doi: 10.1002/stem.1477.

Hayrapetyan, A., Jansen, J. A. and van den Beucken, J. J. (2015) 'Page 1 of 42 1', *Tissue Engineering Part B Reviews*, 21(1), pp. 75–87. doi: 10.1165/rcmb.2008-0455OC.

Haywood, L. *et al.* (2003) 'Inflammation and angiogenesis in osteoarthritis', *Arthritis and Rheumatism*, 48(8), pp. 2173–7. doi: 10.1002/art.11094.

Hellingman, C. A. *et al.* (2011) 'Smad signaling determines chondrogenic differentiation of bone-marrow-derived mesenchymal stem cells: inhibition of Smad1/5/8P prevents terminal differentiation and calcification', *Tissue Engineering Part A*, 17(7–8), pp. 1157–67. doi: 10.1089/ten.tea.2010.0043.

Heng, B. C., Cao, T. and Lee, E. H. (2004) 'Directing stem cell differentiation

into the chondrogenic lineage in vitro', *Stem Cells*, 22(7), pp. 1152–67. doi: 10.1634/stemcells.2004-0062.

Henrotin, Y. E. *et al.* (2000) 'Modulation of human chondrocyte metabolism by recombinant human interferon', *Osteoarthritis and Cartilage*, 8(6), pp. 474–82. doi: 10.1053/joca.2000.0323.

Hiraide, A. *et al.* (2005) 'Repair of articular cartilage defect by intraarticular administration of basic fibroblast growth factor gene, using adeno-associated virus vector', *Human Gene Therapy*, 16(12), pp. 1413–21. doi: 10.1089/hum.2005.16.1413.

Hofer, H. R. and Tuan, R. S. (2016) 'Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies', *Stem Cell Research & Therapy*. Stem Cell Research & Therapy, 7(131), pp. 1–14. doi: 10.1186/s13287-016-0394-0.

Homandberg, G. A., Hui, F. and Wen, C. (1996) 'Fibronectin fragment mediated cartilage chondrolysis. I. Suppression by anti-oxidants', *Biochimica et Biophysica Acta*, 1317(2), pp. 134–142. doi: 10.1016/S0925-4439(96)00046-4.

Hou, Y. *et al.* (2014) 'IL-8 enhances the angiogenic potential of human bone marrow mesenchymal stem cells by increasing vascular endothelial growth factor', *Cell Biology International*, 38(9), pp. 1050–9. doi: 10.1002/cbin.10294.

Houard, X., Goldring, M. B. and Berenbaum, F. (2013) 'Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis', *Current Rheumatology Reports*, 15(11), p. 375. doi: 10.1007/s11926-013-0375-6.

Huang, F., Wang, J. and Chen, A. (2006) 'Effects of co-grafts mesenchymal stem cells and nerve growth factor suspension in the repair of spinal cord injury', *Journal of Huazhong University of Science and Technology*, 26(2), pp. 206–10.

Huang, H. *et al.* (2013) 'Nerve growth factor induced after temporomandibular

joint inflammation decelerates chondrocyte differentiation', *Oral Diseases*, 19(6), pp. 604–10. doi: 10.1111/odi.12045.

Huang, W. and Olsen, B. R. (2015) 'Skeletal defects in Osterix-Cre transgenic mice', *Transgenic Research*, 24(1), pp. 167–172. doi: 10.1007/s11248-014-9828-6.

Huang, Z. *et al.* (2007) 'The Sequential Expression Profiles of Growth Factors from Osteroprogenitors to Osteoblasts *In Vitro*', *Tissue Engineering*, 13(9), pp. 2311–2320. doi: 10.1089/ten.2006.0423.

Huh, J. E. and Lee, S. Y. (2013) 'IL-6 is produced by adipose-derived stromal cells and promotes osteogenesis', *Biochimica et Biophysica Acta - Molecular Cell Research*. Elsevier B.V., 1833(12), pp. 2608–2616. doi: 10.1016/j.bbamcr.2013.06.025.

Hui, A. Y. *et al.* (2012) 'A systems biology approach to synovial joint lubrication in health, injury and disease', *Wiley Interdisciplinary Reviews Systems Biology and Medicine*, 4(1), pp. 15–37. doi: 10.1002/wsbm.157.A.

Hunter, D. J. (2011) 'Pharmacologic therapy for osteoarthritis—the era of disease modification', *Nature Reviews Rheumatology*, 7, pp. 13–22.

Iannone, F. *et al.* (2001) 'Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes', *Clinical and Experimental Rheumatology*, 19(2), pp. 139–45.

Iannone, F. and Lapadula, G. (2003) 'The pathophysiology of osteoarthritis', *Aging Clinical and Experimental Research*, 15(5), pp. 364–72. doi: 10.1007/BF03327357.

Iijima, H. *et al.* (2018) 'Effectiveness of mesenchymal stem cells for treating patients with knee osteoarthritis: a meta-analysis toward the establishment of effective regenerative rehabilitation', *Regenerative Medicine*. Springer US, 3(1), p. 15. doi: 10.1038/s41536-018-0041-8.

Im, H. *et al.* (2007) 'Basic fibroblast growth factor accelerates matrix degradation via a neuro-endocrine pathway in human adult articular chondrocytes', *Journal of Biological Chemistry*, 282(15), pp. 11110–21. doi: 10.1074/jbc.M609040200.Basic.

Jaiswal, R. K. *et al.* (2000) 'Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase', *Journal of Biological Chemistry*, 275(13), pp. 9645–52. doi: 10.1074/jbc.275.13.9645.

James, A. W. (2013) 'Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation', *Scientifica*, 2013, pp. 1–17. doi: 10.1155/2013/684736.

Jayasuriya, C. T. *et al.* (2018) 'Molecular characterization of mesenchymal stem cells in human osteoarthritis cartilage reveals contribution to the OA phenotype', *Scientific Reports*. Springer US, 8(1), pp. 1–14. doi: 10.1038/s41598-018-25395-8.

Jo, C. H. *et al.* (2014) 'Intra-articular injection of mesenchymal stem cells for the treatment of osteoarthritis of the knee: A proof-of-concept clinical trial', *Stem Cells*, 32(5), pp. 1254–66. doi: 10.1002/stem.1634.

Johnstone, B. *et al.* (1998) 'In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells', *Experimental Cell Research*, 238(1), pp. 265–72. doi: 10.1006/excr.1997.3858.

Jones, D. L. and Wagers, A. J. (2008) 'No place like home: anatomy and function of the stem cell niche', *Nature Reviews Molecular Cell Biology*, 9, pp. 11–21.

Kaneko, S. *et al.* (2000) 'Interleukin–6 and interleukin–8 levels in serum and synovial fluid of patients with osteoarthritis', *Cytokines, Cellular & Molecular Therapy*, 6(2), pp. 71–9. doi: 10.1080/13684730050515796.

Karner, C. M., Lee, S.-Y. and Long, F. (2017) 'Bmp Induces Osteoblast Differentiation through both Smad4 and mTORC1 Signaling', *Molecular and Cellular Biology*, 37(4), pp. e00253-16. doi: 10.1128/MCB.00253-16.

Kaul, G. *et al.* (2006) 'Local stimulation of articular cartilage repair by transplantation of encapsulated chondrocytes overexpressing human fibroblast growth factor 2 (FGF-2) in vivo', *Journal of Gene Medicine*, 8(1), pp. 100–11. doi: 10.1002/jgm.819.

Kennedy, A. *et al.* (2011) 'Macrophages in synovial inflammation', *Frontiers in Immunology*, 2, p. 52.

Khosla, S. (2001) 'Minireview: The OPG/RANKL/RANK System', *Endocrinology*, 142(12), pp. 5050–5055. doi: 10.1210/endo.142.12.8536.

Kidd, B. (2012) 'Mechanisms of Pain in Osteoarthritis', *HSS Journal*, 8, pp. 26–28. doi: 10.1007/s11420-011-9263-7.

Kim, H. J. and Park, J.-S. (2017) 'Usage of Human Mesenchymal Stem Cells in Cell-based Therapy: Advantages and Disadvantages', *Development & Reproduction*, 21(1), pp. 1–10. doi: 10.12717/DR.2017.21.1.001.

Kim, J. A. *et al.* (2015) 'Regulation of mesenchymal stromal cells through fine tuning of canonical Wnt signaling', *Stem Cell Research*. Elsevier B.V., 14(3), pp. 356–368. doi: 10.1016/j.scr.2015.02.007.

Kim, J. H. *et al.* (2009) 'The Mechanism of Osteoclast Differentiation Induced by IL-1', *The Journal of Immunology*, 183(3), pp. 1862–1870. doi: 10.4049/jimmunol.0803007.

Kobayashi, Y. *et al.* (2016) 'Regulation of bone metabolism by Wnt signals', *Journal of Biochemistry*, 159(4), pp. 387–392. doi: 10.1093/jb/mvv124.

Koelling, S. *et al.* (2009) 'Migratory chondrogenic progenitor cells from repair tissue during the later stages of human osteoarthritis', *Cell Stem Cell*. Elsevier

Ltd, 4(4), pp. 324–35. doi: 10.1016/j.stem.2009.01.015.

Kolf, C. M., Cho, E. and Tuan, R. S. (2007) 'Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation', *Arthritis Research and Therapy*, 9(1), pp. 1–10. doi: 10.1186/ar2116.

Komori, T. *et al.* (1997) 'Targeted Disruption of Cbfa1 Results in a Complete Lack of Bone Formation owing to Maturational Arrest of Osteoblasts', *Cell*, 89, pp. 755–764. Available at: https://www.cell.com/action/showPdf?pii=S0092-8674%2800%2980258-5.

Kong, L. *et al.* (2017) 'Role of mesenchymal stem cells in osteoarthritis treatment', *Journal of Orthopaedic Translation*. Elsevier Ltd, 9(April), pp. 89–103. doi: 10.1016/j.jot.2017.03.006.

Koshy, P. J. T. *et al.* (2002) 'The modulation of matrix metalloproteinase and ADAM gene expression in human chondrocytes by interleukin-1 and oncostatin M: A time-course study using real-time quantitative reverse transcription-polymerase chain reaction', *Arthritis and Rheumatism*, 46(4), pp. 961–7. doi: 10.1002/art.10212.

Koskinen, A. *et al.* (2011) 'Adiponectin associates with markers of cartilage degradation in osteoarthritis and induces production of proinflammatory and catabolic factors through mitogen-activated protein kinase pathways', *Arthritis Research & Therapy*. BioMed Central Ltd, 13(6), p. R184. doi: 10.1186/ar3512.

Kostoulas, G. *et al.* (1999) 'Stimulation of angiogenesis through cathepsin B inactivation of the tissue inhibitors of matrix metalloproteinases\r1\rDA - 19990816\rIS - 0014-5793 (Print)\rIS - 0014-5793 (Linking)\rLA - eng\rPT - Journal Article\rPT - Research Support, Non-U.S. Gov't\r', *FEBS Lett.*, 455(3), pp. 286–90. doi: S0014-5793(99)00897-2 [pii].

van der Kraan, P. M. and van den Berg, W. B. (2007) 'Osteophytes: relevance and biology', *Osteoarthritis and Cartilage*, 15(3), pp. 237–244. doi: 10.1016/j.joca.2006.11.006.

Van der Kraan, P. M. and Van den Berg, W. B. (2012) 'Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration?', *Osteoarthritis and Cartilage*. Elsevier Ltd, 20(3), pp. 223–32. doi: 10.1016/j.joca.2011.12.003.

van der Kraan, P. M., Blaney Davidson, E. N. and van den Berg, W. B. (2010) 'Bone Morphogenetic Proteins and articular cartilage. To serve and protect or a wolf in sheep clothing's?', *Osteoarthritis and Cartilage*. Elsevier Ltd, 18(6), pp. 735–741. doi: 10.1016/j.joca.2010.03.001.

Krasnokutsky, S. *et al.* (2011) 'Quantitative MRI evidence of synovial proliferation is associated with radiographic severity of knee osteoarthritis', *Arthritis & Rheumatology*, 63(10), pp. 2983–91. doi: 10.1002/art.30471.Quantitative.

Kreuz, P. C. *et al.* (2006) 'Results after microfracture of full-thickness chondral defects in different compartments in the knee', *Osteoarthritis and Cartilage*, 14(11), pp. 1119–25. doi: 10.1016/j.joca.2006.05.003.

Kubota, E. *et al.* (1997) 'Interleukin-1 in the pathogenesis and treatment of inflammatory diseases', *Journal of Oral and Maxillofacial Surgery*, 55(1), pp. 20–7.

Kumarasinghe, D. D. *et al.* (2012) 'Evidence for the dysregulated expression of TWIST1, TGFβ1 and SMAD3 in differentiating osteoblasts from primary hip osteoarthritis patients', *Osteoarthritis and Cartilage*. Elsevier Ltd, 20(11), pp. 1357–1366. doi: 10.1016/j.joca.2012.07.005.

Kusuma, G. D. *et al.* (2017) 'Effect of the microenvironment on mesenchymal stem cell paracrine signaling: opportunities to engineer the therapeutic effect',

Stem Cells and Development, 26(9), pp. 617–31. doi: 10.1089/scd.2016.0349.

Kuyinu, E. L. *et al.* (2016) 'Animal models of osteoarthritis: classification, update, and measurement of outcomes', *Journal of Orthopaedic Surgery and Research*. Journal of Orthopaedic Surgery and Research, 11, p. 19. doi: 10.1186/s13018-016-0346-5.

Kyrtatos, P. G. *et al.* (2009) 'Magnetic tagging increases delivery of circulating progenitors in vascular injury', *JACC: Cardiovascular Interventions*. American College of Cardiology Foundation, 2(8), pp. 794–802. doi: 10.1016/j.jcin.2009.05.014.

Kyurkchiev, D. (2014) 'Secretion of immunoregulatory cytokines by mesenchymal stem cells', *World Journal of Stem Cells*, 6(5), p. 552. doi: 10.4252/wjsc.v6.i5.552.

Lacey, D. C. *et al.* (2009) 'Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation', *Osteoarthritis and Cartilage*. Elsevier Ltd, 17(6), pp. 735–42. doi: 10.1016/j.joca.2008.11.011.

Laine, L. *et al.* (2008) 'COX-2 selective inhibitors in the treatment of asteoarthritis', *Seminars in Arthritis and Rheumatism*. Elsevier Inc., 38(3), pp. 165–87. doi: 10.1016/j.semarthrit.2007.10.004.

Lamo-Espinosa, J. M. *et al.* (2018) 'Intra-articular injection of two different doses of autologous bone marrow mesenchymal stem cells versus hyaluronic acid in the treatment of knee osteoarthritis: Long-term follow up of a multicenter randomized controlled clinical trial (phase I/II)', *Journal of Translational Medicine*. BioMed Central, 16(1), pp. 1–5. doi: 10.1186/s12967-018-1591-7.

Lane, N. E. *et al.* (2010) 'Tanezumab for the treatment of pain from osteoarthritis of the knee', *New England Journal of Medicine*, 363(16), pp. 1521–

31. doi: 10.1056/NEJMoa0901510.

de Lange-Brokaar, B. J. E. De *et al.* (2012) 'Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review', *Osteoarthritis and Cartilage*. Elsevier Ltd, 20(12), pp. 1484–99. doi: 10.1016/j.joca.2012.08.027.

Lanyon, P. *et al.* (1998) 'Radiographic assessment of simptomatic knee osteoarthritis in the community: definition and normal joint space', *Annals of the Rheumatic Diseases*, 57, pp. 595–601. Available at: papers2://publication/uuid/50BC56F8-A283-425E-B923-7CACC3086A57.

Li, B. and Aspden, R. M. (1997) 'Mechanical and material properties of the subchondral bone plate from the femoral head of patients with osteoarthritis or osteoporosis', *Annals of the Rheumatic Diseases*, 56(4), pp. 247–254. doi: 10.1136/ard.56.4.247.

Li, C. *et al.* (2016) 'Activation of the extracellular signal-regulated kinase signaling is critical for human umbilical cord mesenchymal stem cell osteogenic differentiation', *BioMed Research International*, 2016, p. 3764372.

Li, X. *et al.* (2012) 'Species-specific biological effects of FGF-2 in articular cartilage: implication for distinct roles within the FGF receptor family', *Journal of Cellular Biochemistry*, 113(7), pp. 2532–42. doi: 10.1002/jcb.24129.

Li, Y. *et al.* (2010) 'IL-6 and high glucose synergistically upregulate MMP-1 expression by U937 mononuclear phagocytes via ERK1/2 and JNK pathways and c-Jun', *Journal of Cellular Biochemistry*, 110(1), pp. 248–259. doi: 10.1002/jcb.22532.

Liang, X. *et al.* (2014) 'Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives', *Cell Transplantation*, 23(9), pp. 1045–59. doi: 10.3727/096368913X667709.

Lin, A. C. *et al.* (2009) 'Modulating hedgehog signaling can attenuate the severity of osteoarthritis', *Nature Medicine*, 15(12), pp. 1421–1425. doi:

10.1038/nm.2055.

Lin, Y. *et al.* (2017) 'Inhibition of interleukin-6 function attenuates the central sensitization and pain behavior induced by osteoarthritis', *European Journal of Pharmacology*. Elsevier B.V., 811(20), pp. 260–7. doi: 10.1016/j.ejphar.2017.06.032.

Little, C. B. and Smith, M. M. (2008) 'Animal models of osteoarthritis', *Current Rheumatology Reviews*, 4(3), pp. 178–82.

Little, R. D. *et al.* (2002) 'A Mutation in the LDL Receptor–Related Protein 5 Gene Results in the Autosomal Dominant High–Bone-Mass Trait', *The American Journal of Human Genetics*, 70(1), pp. 11–19. doi: 10.1086/338450.

Liu-Bryan, R. and Terkeltaub, R. (2010) 'Chondrocyte innate immune MyD88-dependent signaling drives pro-catabolic effects of the endogenous TLR2/TLR4 ligands LMW-HA and HMGB1', *Arthritis & Rheumatism*, 62(7), pp. 2004–12. doi: 10.1002/art.27475.Chondrocyte.

Liu, W. *et al.* (2017) 'IL-1β impedes the chondrogenic differentiation of synovial fluid mesenchymal stem cells in the human temporomandibular joint', *International Journal of Molecular Medicine*, 39(2), pp. 317–26. doi: 10.3892/ijmm.2016.2832.

Livshits, G. *et al.* (2009) 'Interleukin-6 is a significant predictor of radiographic knee osteoarthritis: The Chingford Study', *Arthritis and Rheumatism*, 60(7), pp. 2037–45. doi: 10.1002/art.24598.

Loeser, R. F. *et al.* (2002) 'Detection of nitrotyrosine in aging and osteoarthritic cartilage: correlation of oxidative damage with the presence of interleukin-1β and with chondrocyte resistance to insulin-like growth factor 1', *Arthritis and Rheumatism*, 46(9), pp. 2349–57. doi: 10.1002/art.10496.

Lu, Z. *et al.* (2017) 'Nerve growth factor from Chinese cobra venom stimulates chondrogenic differentiation of mesenchymal stem cells', *Nature Publishing*

Group. Nature Publishing Group, 8(5), pp. e2801-14. doi: 10.1038/cddis.2017.208.

Mackie, E. J. *et al.* (2008) 'Endochondral ossification: How cartilage is converted into bone in the developing skeleton', *International Journal of Biochemistry and Cell Biology*, 40(1), pp. 46–62. doi: 10.1016/j.biocel.2007.06.009.

Maeda, K. *et al.* (2012) 'Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis', *Nature Medicine*. Nature Publishing Group, 18(3), pp. 405–412. doi: 10.1038/nm.2653.

Man, G. and Mologhianu, G. (2014) 'Osteoarthritis pathogenesis – a complex process that involves the entire joint', *Journal of Medicine and Life*, 7(1), pp. 37–41. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3956093/%5Cnhttp://www.ncbi.nlm.nih.gov/pmc/articles/PMC3956093/pdf/JMedLife-07-37.pdf.

Mao, C. *et al.* (2016) 'Double-edged-sword effect of IL-1β on the osteogenesis of periodontal ligament stem cells via crosstalk between the NF-κB, MAPK and BMP/Smad signaling pathways', *Cell Death and Disease*, 7, p. e2296.

Mariani, E., Pulsatelli, L. and Facchini, A. (2014) 'Signaling pathways in cartilage repair', *International Journal of Molecular Sciences*, 15(5), pp. 8667–98. doi: 10.3390/ijms15058667.

Martin, G. R. (1981) 'Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells', *Proceedings of the National Academy of Sciences of the United States of America*, 78(12), pp. 7634–8. doi: 10.1073/pnas.78.12.7634.

Martineau, X. *et al.* (2017) 'Alteration of Wnt5a expression and of the non-canonical Wnt/PCP and Wnt/PKC-Ca2+pathways in human osteoarthritis osteoblasts', *PLoS ONE*, 12(8), pp. 1–21. doi: 10.1371/journal.pone.0180711.

Maruotti, N., Corrado, A. and Cantatore, F. P. (2017) 'Osteoblast role in

osteoarthritis pathogenesis', *Journal of Cellular Physiology*, 232(11), pp. 2957–2963. doi: 10.1002/jcp.25969.

Massicotte, F. *et al.* (2002) 'Can altered production of interleukin-1β, interleukin-6, transforming growth factor-β and prostaglandin E2 by isolated human subchondral osteoblasts identity two subgroups of osteoarthritic patients', *Osteoarthritis and Cartilage*, 10(6), pp. 491–500. doi: 10.1053/joca.2002.0528.

Mathiessen, A. and Conaghan, P. G. (2017) 'Synovitis in osteoarthritis : current understanding with therapeutic implications', *Arthritis Research & Therapy*. Arthritis Research & Therapy, 19(18), pp. 1–9. doi: 10.1186/s13075-017-1229-9.

Matsukawa, A. *et al.* (1995) 'Neutrophil accumulation and activation by homologous IL-8 in rabbits. IL-8 induces destruction of cartilage and production of IL-1 and IL-1 receptor antagonist in vivo', *The Journal of Immunology*, 154(10), pp. 5418–25.

McDougall, J. J. (2006) 'Arthritis and pain: neurogenic origin of joint pain', *Arthritis Research and Therapy*, 8(6), p. 220. doi: 10.1186/ar2069.

Melchiorri, C. *et al.* (1998) 'Enhanced and coordinated in vivo expression of inflammatory cytokines and nitric oxide synthase by chondrocytes from patients with osteoarthritis', *Arthritis and Rheumatism*, 41(12), pp. 2165–74. doi: 10.1002/1529-0131(199812)41:12<2165::AID-ART11>3.0.CO;2-O.

Melgarejo, E. *et al.* (2009) 'Monocyte chemoattractant protein-1: A key mediator in inflammatory processes', *International Journal of Biochemistry and Cell Biology*, 41(5), pp. 998–1001. doi: 10.1016/j.biocel.2008.07.018.

Merz, D. *et al.* (2003) 'IL-8/CXCL8 and growth-related oncogene α/CXCL1 induce chondrocyte hypertrophic differentiation', *The Journal of Immunology*, 171(8), pp. 4406–15. doi: 10.4049/jimmunol.171.8.4406.

Miller, R. E. *et al.* (2012) 'CCR2 chemokine receptor signaling mediates pain in

experimental osteoarthritis', *Proceedings of the National Academy of Sciences of the United States of America*, 109(50), pp. 20602–7. doi: 10.1111/j.1471-0528.1991.tb13414.x.

Miller, R. E. *et al.* (2015) 'Damage-associated molecular patterns generated in osteoarthritis directly excite murine nociceptive neurons through Toll-like receptor 4', *Arthritis & Rheumatology*, 67(11), pp. 2933–43. doi: 10.1016/j.trsl.2014.08.005.The.

Miller, R. E., Miller, R. J. and Malfait, A.-M. (2014) 'Osteoarthritis joint pain: the cytokine connection', *Cytokine*, 70(2), pp. 185–93. doi: 10.1038/jid.2014.371.

Mimeault, M., Hauke, R. and Batra, S. (2007) 'Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies', *Clinical Pharmacology and Therapeutics*, 82(3), pp. 252–64.

Mohan, S. and Baylink, D. (1991) 'Bone growth factors', *Clinical Orthopaedics and Related Research*, 263, pp. 30–48.

Moser, B. and Willimann, K. (2004) 'Chemokines: role in inflammation and immune surveillance', *Annals of the Rheumatic Diseases*, 63(SUPPL. 2), pp. 84–89. doi: 10.1136/ard.2004.028316.

Mountziaris, P. M. and Mikos, A. G. (2008) 'Modulation of the Inflammatory Response for Enhanced Bone Tissue Regeneration', *Tissue Engineering Part B: Reviews*, 14(2), pp. 179–186. doi: 10.1089/ten.teb.2008.0038.

Muddasani, P. *et al.* (2007) 'Basic fibroblast growth factor activates the MAPK and NFκB pathways that converge on Elk-1 to control production of matrix metalloproteinase-13 by human adult articular chondrocytes', *Journal of Biological Chemistry*, 282(43), pp. 31409–21. doi: 10.1074/jbc.M706508200.

Müller, F., Snyder, E. Y. and Loring, J. F. (2006) 'Gene therapy: can neural stem cells deliver?', *Nature Reviews Neuroscience*, 7(1), pp. 75–84.

Murawski, C. D., Foo, L. F. and Kennedy, J. G. (2010) 'A review of arthroscopic bone marrow stimulation techniques of the talus: the good, the bad, and the causes for concern', *Cartilage*, 1(2), pp. 137–44. doi: 10.1177/1947603510364403.

Murphy, J. M. *et al.* (2002) 'Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis', *Arthritis and Rheumatism*, 46(3), pp. 704–713. doi: 10.1002/art.10118.

Muthukuru, M. and Darveau, R. P. (2015) 'TLR signaling that induces weak inflammatory response and SHIP1 enhances osteogenic functions', *Bone Research*, 2(August), pp. 1–13. doi: 10.1038/boneres.2014.31.

Najar, M. *et al.* (2016) 'Mesenchymal stromal cells and immunomodulation: A gathering of regulatory immune cells', *Cytotherapy*. Elsevier Inc., 18(2), pp. 160–71. doi: 10.1016/j.jcyt.2015.10.011.

Nakasaki, M. *et al.* (2008) 'IGF-I secreted by osteoblasts acts as a potent chemotactic factor for osteoblasts', *Bone*. Elsevier Inc., 43(5), pp. 869–879. doi: 10.1016/j.bone.2008.07.241.

National Joint Registry (2014) *11th Annual Report 2014*.

Nauta, A. J. and Fibbe, W. E. (2007) 'Review in translational hematology Immunomodulatory properties of mesenchymal stromal cells', *Blood*, 110(10), pp. 3499–506. doi: 10.1182/blood-2007-02-069716.

Neogi, T. *et al.* (2016) 'Association of joint inflammation with pain sensitization in knee osteoarthritis: the multicentre osteoarthritis study', *Arthritis & Rheumatology*, 68(3), pp. 654–61. doi: 10.1016/j.trsl.2014.08.005.The.

Nettelbladt, E. G. and Sundblad, L. K. M. (1959) 'Protein patterns in synovial fluid and serum in rheumatoid arthritis and osteoarthritis', *Arthritis and Rheumatism*, 2(2), pp. 144–51.

Neve, A., Corrado, A. and Cantatore, F. P. (2011) 'Osteoblast physiology in

normal and pathological conditions', *Cell and Tissue Research*, 343(2), pp. 289–302. doi: 10.1007/s00441-010-1086-1.

NICE (2017) *Autologous chondrocyte implantation for treating symptomatic articular cartilage defects of the knee*, *https://www.nice.org.uk/guidance/ta477/chapter/1-Recommendations*.

Niehrs, C. (2012) 'The complex world of WNT receptor signalling', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 13(12), pp. 767–779. doi: 10.1038/nrm3470.

Oh, C. *et al.* (2000) 'Opposing role of mitogen-activated protein kinase subtypes, Erk-1/2 and p38, in the regulation of chondrogenesis of mesenchymes', *The Journal of Biological Chemistry*, 275(8), pp. 5613–9. doi: 10.1074/jbc.275.8.5613.

Okada, Y. *et al.* (1992) 'Matrix metalloproteinase 9 (92 kDa gelatinase/type IV gelatinase) from HT 1080 human fibrosarcoma cells', *The Journal of Biological Chemistry*, 267(30), pp. 21712–9.

Okamoto, T. *et al.* (2002) 'Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells', *Biochemical and Biophysical Research Communications*, 295, pp. 354–361.

Opitz, C. A. *et al.* (2009) 'oll-like receptor engagement enhances the immunosuppressive properties of human bone marrow-derived mesenchymal stem cells by inducing indoleamine-2,3-dioxygenase-1 via interferon-beta and protein kinase R', *Stem Cells*, 27(4), pp. 909–19. doi: 10.1002/stem.7.

Orita, S. *et al.* (2011) 'Associations between proinflammatory cytokines in the synovial fluid and radiographic grading and pain-related scores in 47 consecutive patients with osteoarthritis of the knee.', *BMC musculoskeletal disorders*. BioMed Central Ltd, 12(1), p. 144. doi: 10.1186/1471-2474-12-144.

Van Osch, G. J. V. M. *et al.* (1996) 'The relation between cartilage damage and osteophyte size in a murine model for osteoarthritis in the knee', *Rheumatology International*, 16(3), pp. 115–119. doi: 10.1007/BF01409983.

Van Osch, G. J. V. M. *et al.* (2009) 'Cartilage repair: past and future - lessons for regenerative medicine', *Journal of Cellular and Molecular Medicine*, 13(5), pp. 792–810. doi: 10.1111/j.1582-4934.2009.00789.x.

Palfrey, A. J. and Davies, D. V (1966) 'The fine structure of chondrocytes', *Journal of Anatomy*, 100(Pt 2), pp. 213–26.

Pankhurst, Q. A. *et al.* (2009) 'Progress in applications of magnetic nanoparticles in biomedicine', *Journal of Physics D: Applied Physics*, 42(22), p. Article 224001. doi: 10.1088/0022-3727/42/22/224001.

Park, C. W. *et al.* (2009) 'Cytokine secretion profiling of human mesenchymal stem cells by antibody array', *International Journal of Stem Cells*, 2(1), pp. 59–68. doi: 10.15283/ijsc.2009.2.1.59.

Patil, C. *et al.* (2004) 'p38 MAPK regulates IL-1beta induced IL-6 expression through mRNA stability in osteoblasts.', *Immunological investigations*, 33(2), pp. 213–233. doi: 10.1081/imm-120034231.

Pesesse, L. *et al.* (2014) 'Bone sialoprotein as a potential key factor implicated in the pathophysiology of osteoarthritis', *Osteoarthritis and Cartilage*. Elsevier Ltd, 22(4), pp. 547–556. doi: 10.1016/j.joca.2014.01.010.

Pevsner-Fischer, M. *et al.* (2007) 'Toll-like receptors and their ligands control mesenchymal stem cell functions', *Differentiation*, 109(4), pp. 1422–1432. doi: 10.1182/blood-2006-06-028704.The.

Pfander, D. *et al.* (2004) 'Tenascin and aggrecan expression by articular chondrocytes is influenced by interleukin 1β: A possible explanation for the changes in matrix synthesis during osteoarthritis', *Annals of the Rheumatic Diseases*, 63(3), pp. 240–4. doi: 10.1136/ard.2002.003749.

Pierzchala, A. W., Kusz, D. J. and Hajduk, G. (2011) 'CXCL8 and CCL5 expression in synovial fluid and blood serum in patients with osteoarthritis of the knee', *Archivum Immunologiae et Therapiae Experimentalis*, 59(2), pp. 151–5. doi: 10.1007/s00005-011-0115-4.

Pittenger, M. F. *et al.* (1999) 'Multilineage potential of adult human mesenchymal stem cells', *Science*, 284(5411), pp. 143–7.

Pittenger, M. F. (1999) 'Multilineage Potential of Adult Human Mesenchymal Stem Cells', *Science*, 284(5411), pp. 143–147. doi: 10.1126/science.284.5411.143.

Porée, B. *et al.* (2008) 'Interleukin-6 (IL-6) and/or soluble IL-6 receptor down-regulation of human type II collagen gene expression in articular chondrocytes requires a decrease of Sp1·Sp3 ratio and of the binding activity of both factors to the COL2A1 promoter', *Journal of Biological Chemistry*, 283(8), pp. 4850–65. doi: 10.1074/jbc.M706387200.

Prasadam, I. *et al.* (2010) 'ERK-1/2 and p38 in the regulation of hypertrophic changes of normal articular cartilage chondrocytes induced by osteoarthritic subchondral osteoblasts', *Arthritis and Rheumatism*, 62(5), pp. 1349–60. doi: 10.1002/art.27397.

Pricola, K. *et al.* (2009) 'Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism', *Journal of Cellular Biochemistry*, 108(3), pp. 577–588. doi: 10.1002/jcb.22289.Interleukin-6.

Qi, C., Xiaofeng, X. and Xiaoguang, W. (2014) 'Effects of toll-like receptors 3 and 4 in the osteogenesis of stem cells', *Stem Cells International*, 2014. doi: 10.1155/2014/917168.

Quarles, L. D. *et al.* (1992) 'Distinct proliferation and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development', *J Bone Miner Res*, 7(6), pp. 683–692.

Raggatt, L. J. and Partridge, N. C. (2010) 'Cellular and molecular mechanisms of bone remodeling', *Journal of Biological Chemistry*, 285(33), pp. 25103–25108. doi: 10.1074/jbc.R109.041087.

Raicevic, G. *et al.* (2010) 'Inflammation modifies the pattern and the function of Toll-like receptors expressed by human mesenchymal stromal cells', *Human Immunology*. Elsevier Inc., 71(3), pp. 235–244. doi: 10.1016/j.humimm.2009.12.005.

Redondo-Castro, E. *et al.* (2017) 'Interleukin-1 primes human mesenchymal stem cells towards an anti-inflammatory and pro-trophic phenotype in vitro', *Stem Cell Research & Therapy*. Stem Cell Research & Therapy, 8(1), p. 79. doi: 10.1186/s13287-017-0531-4.

Regan, J. and Long, F. (2014) 'Notch signaling and bone remodeling', 11(2), pp. 126–129. doi: 10.1007/s11914-013-0145-4.Notch.

Reimann, I., Mankin, H. J. and Trahan, C. (1977) 'Quantitative histologic analyses of articular cartilage and subchondral bone from osteoarthritic and normal human hips', *Acta Orthopaedica*, 48(1), pp. 63–73. doi: 10.3109/17453677708985113.

Rezza, A., Sennett, R. and Rendl, M. (2014) 'Adult stem cell niches: cellular and molecular components', *Current Topics in Developmental Biology*, 107, pp. 333–72. doi: 10.1016/B978-0-12-416022-4.00012-3.

Richter, F. *et al.* (2010) 'Tumor necrosis factor causes persistent sensitization of joint nociceptors to mechanical stimuli in rats', *Arthritis and Rheumatism*, 62(12), pp. 3806–14. doi: 10.1002/art.27715.

Rodríguez-Carballo, E., Gámez, B. and Ventura, F. (2016) 'p38 MAPK Signaling in Osteoblast Differentiation', *Frontiers in Cell and Developmental Biology*, 4(May), pp. 1–20. doi: 10.3389/fcell.2016.00040.

Rodriguez-Raecke, R. *et al.* (2009) 'Brain gray matter decrease in chronic pain

is the consequence and not the cause of pain', *Journal of Neuroscience*, 29(44), pp. 13746–50. doi: 10.1523/JNEUROSCI.3687-09.2009.

Roemer, F. W. *et al.* (2011) 'Advances in imaging of osteoarthritis and cartilage', *Radiology*, 260(2), pp. 332–54. doi: 10.1148/radiol.11101359.

van Roon, J. *et al.* (1996) 'Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4', *Arthritis and Rheumatism*, 39(5), pp. 829–35.

Rousselle, A. V. and Heymann, D. (2002) 'Osteoclastic acidification pathways during bone resorption', *Bone*, 30(4), pp. 533–540. doi: 10.1016/S8756-3282(02)00672-5.

Rowan, A. D. *et al.* (2001) 'Synergistic effects of glycoprotein 130 binding cytokines in combination with interleukin-1 on cartilage collagen breakdown', *Arthritis and Rheumatism*, 44(7), pp. 1620–32. doi: 10.1002/1529-0131(200107)44:7<1620::AID-ART285>3.0.CO;2-B.

Sabokar, A. *et al.* (1994) 'A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro', *Bone and Mineral*, 27(1), pp. 57–67.

Sangiorgi, B. and Panepucci, R. A. (2016) 'Modulation of Immunoregulatory Properties of Mesenchymal Stromal Cells by Toll-Like Receptors: Potential Applications on GVHD', *Stem Cells International*. Hindawi Publishing Corporation, 2016. doi: 10.1155/2016/9434250.

Sato, A. *et al.* (2010) 'Wnt5a regulates distinct signalling pathways by binding to Frizzled2', *EMBO Journal*. Nature Publishing Group, 29(1), pp. 41–54. doi: 10.1038/emboj.2009.322.

Scanzello, C. *et al.* (2009) 'Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease', *Osteoarthritis and Cartilage*. Elsevier Ltd, 17(8), pp. 1040–8. doi:

10.1016/j.joca.2009.02.011.

Scanzello, C. R. (2012) 'Pathologic and pathogenic Processes in Osteoarthritis: The Effects of Synovitis', *Hospital for Special Surgery Journal*, 8(1), p. 2022. doi: 10.1007/s11420-011-9228-x.

Scanzello, C. R. and Goldring, S. R. (2012) 'The role of synovitis in osteoarthritis pathogenesis', *Bone*, 51(2), pp. 249–57. doi: 10.1016/j.bone.2012.02.012.The.

Schaffler, M. B. and Kennedy, O. D. (2012) 'NIH Public Access', 10(2), pp. 118–125. doi: 10.1007/s11914-012-0105-4.Osteocyte.

Schaible, H. G. *et al.* (2009) 'Joint pain', *Experimental Brain Research*, 196(1), pp. 153–62.

Schaible, H. G. (2012) 'Mechanisms of chronic pain in osteoarthritis', *Current Rheumatology Reports*, 14(6), pp. 549–56. doi: 10.1007/s11926-012-0279-x.

Schaible, H. G., Ebersberger, A. and Natura, G. (2011) 'Update on peripheral mechanisms of pain: Beyond prostaglandins and cytokines', *Arthritis Research and Therapy*. BioMed Central Ltd, 13(2), p. 210. doi: 10.1186/ar3305.

Schaible, H. G., Schmelz, M. and Tegeder, I. (2006) 'Pathophysiology and treatment of pain in joint disease', *Advanced Drug Delivery Reviews*, 58(2), pp. 323–42. doi: 10.1016/j.addr.2006.01.011.

Scharstuhl, A. *et al.* (2002) 'Inhibition of Endogenous TGF- During Experimental Osteoarthritis Prevents Osteophyte Formation and Impairs Cartilage Repair', *The Journal of Immunology*, 169(1), pp. 507–514. doi: 10.4049/jimmunol.169.1.507.

Scharstuhl, A. *et al.* (2003) 'Reduction of Osteophyte Formation and Synovial Thickening by Adenoviral Overexpression of Transforming Growth Factor ??/Bone Morphogenetic Protein Inhibitors during Experimental

Osteoarthritis', *Arthritis and Rheumatism*, 48(12), pp. 3442–3451. doi: 10.1002/art.11328.

Schindeler, A. and Little, D. G. (2006) 'Ras-MAPK signaling in osteogenic differentiation: Friend or foe?', *Journal of Bone and Mineral Research*, 21(9), pp. 1331–1338. doi: 10.1359/jbmr.060603.

Schmitz, N. *et al.* (2010) 'Basic methods in histopathology of joint tissues', *Osteoarthritis and Cartilage*. Elsevier Ltd, 18(SUPPL. 3), pp. S113–S116. doi: 10.1016/j.joca.2010.05.026.

Schofield, R. (1978) 'The relationship between the spleen colony-forming cell and the haemopoietic stem cell', *Blood Cells*, 4(1–2), pp. 7–25.

Shakibaei, M. *et al.* (2005) 'Curcumin protects human chondrocytes from IL-1β-induced inhibition of collagen type II and β1-integrin expression and activition of caspase-3: An immunomorphological study', *Annals of Anatomy*, 187(5–6), pp. 487–97. doi: 10.1016/j.aanat.2005.06.007.

Shirjang, S. *et al.* (2017) 'Toll-like receptors as a key regulator of mesenchymal stem cell function: An up-to-date review', *Cellular Immunology*, 315, pp. 1–10. doi: 10.1016/j.cellimm.2016.12.005.

Shlopov, B. V., Gumanovskaya, M. L. and Hasty, K. A. (2000) 'Autocrine regulation of collagenase 3 (matrix metalloproteinase 13) during osteoarthritis', *Arthritis and Rheumatism*, 43(1), pp. 195–205. doi: 10.1002/1529-0131(200001)43:1<195::AID-ANR24>3.0.CO;2-G.

Simonet, W. . *et al.* (1997) 'Osteoprotegerin: A Novel Secreted Protein Involved in the Regulation of Bone Density', *Cell*, 89(2), pp. 309–319. doi: 10.1016/S0092-8674(00)80209-3.

Singh, P. *et al.* (2018) 'Phenotypic instability of chondrocytes in osteoarthritis: On a path to hypertrophy', *Annals of the New York Academy of Sciences*, pp. 1–18. doi: 10.1111/nyas.13930.

Smith, M. D. *et al.* (1997) 'Synovial membrane inflammation and cytokine production in patients with early osteoarthritis', *The Journal of Rheumatology*, 24(2), pp. 265–71.

Smith, M. D. (2011) 'The Normal Synovium', 5(Suppl 1:M2), pp. 100–6.

Sobue, T. *et al.* (2002) 'Regulation of Fibroblast Growth Factor 2 and Fibroblast Growth Factor Receptors by Transforming Growth Factor β in Human Osteoblastic MG-63 Cells', *Journal of Bone and Mineral Research*, 17(3), pp. 502–512. doi: 10.1359/jbmr.2002.17.3.502.

Sofat, N., Ejindu, V. and Kiely, P. (2011) 'What makes osteoarthritis painful? The evidence for local and central pain processing', *Rheumatology*, 50(12), pp. 2157–65. doi: 10.1093/rheumatology/ker283.

Sohn, D. H. *et al.* (2012) 'Plasma proteins present in osteoarthritic synovial fluid can stimulate cytokine production via Toll-like receptor 4', *Arthritis Research and Therapy*, 14(1), pp. 1–13. doi: 10.1186/ar3555.

Sokolove, J. and Lepus, C. M. (2013) 'Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations', *Therapeutic Advances in Musculoskeletal Disease*, 5(2), pp. 77–94. doi: 10.1177/1759720X12467868.

Solchaga, L. A., Penick, K. J. and Welter, J. F. (2011) 'Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells: tips and tricks', *Methods in Molecular Biology*, 698, pp. 253–78. doi: 10.1007/978-1-60761-999-4.

Solís-Herruzo, J. A. *et al.* (1999) 'Interleukin-6 increases rat metalloproteinase-13 gene expression through stimulation of activator protein 1 transcription factor in cultured fibroblasts', *Journal of Biological Chemistry*, 274(43), pp. 30919–30926. doi: 10.1074/jbc.274.43.30919.

Somoza, R. A. *et al.* (2014) 'Chondrogenic differentiation of mesenchymal stem cells: challenges and unfulfilled expectations', *Tissue Engineering Part B:*

Reviews, 20(6), pp. 596–608. doi: 10.1089/ten.teb.2013.0771.

Sonomoto, K. *et al.* (2012) 'Interleukin-1β induces differentiation of human mesenchymal stem cells into osteoblasts via the wnt-5a/receptor tyrosine kinase-like orphan receptor 2 pathway', *Arthritis and Rheumatism*, 64(10), pp. 3355–3363. doi: 10.1002/art.34555.

Stannus, O. *et al.* (2010) 'Circulating levels of IL-6 and TNF-α are associated with knee radiographic osteoarthritis and knee cartilage loss in older adults', *Osteoarthritis and Cartilage*. Elsevier Ltd, 18(11), pp. 1441–7. doi: 10.1016/j.joca.2010.08.016.

Stanokovic, A. *et al.* (2009) 'Serum and synovial fluid concentrations of CCL2 (MCP-1) chemokine in patients suffering rheumatoid arthritis and osteoarthritis reflect disease activity', *Bratislava Medical Journal*, 110(10), pp. 641–6.

Stone, J. E. *et al.* (1994) 'Interaction of 1,9-dimethylmethylene blue with glycosaminoglycans', *Annals of Clinical Biochemistry*, 31(2), pp. 147–152. doi: 10.1177/000456329403100206.

Stöve, J. *et al.* (2000) 'Interleukin-1β induces different gene expression of stromelysin, aggrecan and tumor-necrosis-factor-stimulated gene 6 in human osteoarthritic chondrocytes in vitro', *Pathobiology*, 68(3), pp. 144–9. doi: 10.1159/000055915.

Su, S. L. *et al.* (2005) 'Expression and regulation of Toll-like receptor 2 by IL-1β and fibronectin fragments in human articular chondrocytes', *Osteoarthritis and Cartilage*, 13(10), pp. 879–886. doi: 10.1016/j.joca.2005.04.017.

Sui, Y. *et al.* (2009) 'Mechanical injury potentiates proteoglycan catabolism induced by interleukin-6 with soluble interleukin-6 receptor and tumor necrosis factor α in immature bovine and adult human articular cartilage', *Arthritis and Rheumatism*, 60(10), pp. 2985–96. doi: 10.1002/art.24857.

Sundararaj, K. P. *et al.* (2009) 'Interleukin-6 released from fibroblasts is essential for up-regulation of matrix metalloproteinase-1 expression by U937 macrophages in coculture: Cross-talking between fibroblasts and U937 macrophages exposed to high glucose', *Journal of Biological Chemistry*, 284(20), pp. 13714–13724. doi: 10.1074/jbc.M806573200.

Sura, H. S. *et al.* (2008) 'Gene expression changes in stem cells following targeted localisation in a flow system using magnetic particle technology', *European Cells and Materials*, 16(S3), p. 18.

Takeshita, M. *et al.* (2012) 'Sensory innervation and inflammatory cytokines in hypertrophic synovia associated with pain transmission in osteoarthritis of the hip: A case-control study', *Rheumatology*, 51(10), pp. 1790–5. doi: 10.1093/rheumatology/kes173.

Tang, C. H. *et al.* (2011) 'IL-6 increases MMP-13 expression and motility in human chondrosarcoma cells', *Journal of Biological Chemistry*, 286(13), pp. 11056–11066. doi: 10.1074/jbc.M110.204081.

Tang, J. *et al.* (2015) 'Effects of mesenchymal stem cells on interleukin-1β-treated chondrocytes and cartilage in a rat osteoarthritic model', *Molecular Medicine Reports*, 12(2), pp. 1753–60. doi: 10.3892/mmr.2015.3645.

Tang, Q. O. *et al.* (2009) 'TGF-β3: a potential biological therapy for enhancing chondrogenesis', *Expert Opinion on Biological Therapy*, 9(6), pp. 689–701. doi: 10.1517/14712590902936823.

Thomson, J. A. *et al.* (1998) 'Embryonic stem cell lines derived from human blastocysts', *Science*, 282(5391), pp. 1145–7. doi: 10.1126/science.282.5391.1145.

Toben, D. *et al.* (2011) 'Fracture healing is accelerated in the absence of the adaptive immune system', *Journal of Bone and Mineral Research*, 26(1), pp. 113–24. doi: 10.1002/jbmr.185.

Tomchuck, S. L. *et al.* (2009) 'Toll-Like Receptors on Human Mesenchymal

Stem Cells Drive their Migration and Immunomodulating Responses', *Stem Cells*, 26(1), pp. 99–107. doi: 10.1634/stemcells.2007-0563.Toll-Like.

Tomchuck, S. L. *et al.* (2012) 'Toll-like receptor 3 and suppressor of cytokine signalling proteins regulate CXCR4 and CXCR7 expression in bone marrow-derived human multipotent stromal cells', *Public Library of Science One*, 7(6), p. e39592.

Tonge, D. P., Pearson, M. J. and Jones, S. W. (2014) 'The hallmarks of osteoarthritis and the potential to develop personalised disease-modifying pharmacological therapeutics', *Osteoarthritis and Cartilage*. Elsevier Ltd, 22(5), pp. 609–21. doi: 10.1016/j.joca.2014.03.004.

Valdes, A. M. *et al.* (2010) 'Genetic variation in the SMAD3 gene is associated with hip and knee osteoarthritis', *Arthritis and Rheumatism*, 62(8), pp. 2347–52. doi: 10.1002/art.27530.

Van Vlasselaer, P. *et al.* (1994) 'Interleukin 10 inhibits transforming growth factor-β (TGFβ) synthesis required for osteogenic commitment of mouse bone marrow cells', *Journal of Cell Biology*, 124(4), pp. 569–77.

Wagner, E. R. *et al.* (2010) 'Therapeutic implications of PPARγ in human osteosarcoma', *PPAR Research*, 2010, p. 956427. doi: 10.1155/2010/956427.

Wang, X. *et al.* (2011) 'Effects and relationship of ERK1 and ERK2 in interleukin-1β-induced alterations in MMP3, MMP13, type II collagen and aggrecan expression in human chondrocytes', *International Journal of Molecular Medicine*, 27(4), pp. 583–9. doi: 10.3892/ijmm.2011.611.

Waterman, R. S. *et al.* (2010) 'A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype', *PLoS ONE*, 5(4), p. e10088. doi: 10.1371/journal.pone.0010088.

Watt, F. M. and Huck, W. T. S. (2013) 'Role of the extracellular matrix in regulating stem cell fate', *Nature Medicine*, 14(467–73).

Wehling, N. *et al.* (2009) 'Interleukin-1 and tumor necrosis factor-α inhibit chondrogenesis by human mesenchymal stem cells through NF-κB dependent pathways', 60(3), pp. 801–12. doi: 10.1002/art.24352.Interleukin-1.

Weilbaecher, K. N., Guise, T. A. and McCauley, L. K. (2011) 'Cancer To Bone: a Fatal Attraction', *Nature Reviews Cancer*, 11(6), pp. 411–425. doi: 10.1038/nrc3055.Cancer.

Wenham, C. Y. J. and Conaghan, P. G. (2010) 'The role of synovitis in osteoarthritis', *Therapeutic Advances in Musculoskeletal Disease*, 2(6), pp. 349–59. doi: 10.1177/1759720X10378373.

Wieland, H. A. *et al.* (2005) 'Osteoarthritis — an untreatable disease?', *Nature Reviews Drug Discovery*, 4, pp. 331–44.

Wojdasiewicz, P., Poniatowski, L. A. and Szukiewicz, D. (2014) 'The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis', *Mediators of Inflammation*, 2014, p. 561459. doi: 10.1016/j.cyto.2013.12.016.

Wyles, C. C. *et al.* (2015) 'Mesenchymal stem cell therapy for osteoarthritis: Current perspectives', *Stem Cells and Cloning: Advances and Applications*, 8, pp. 117–24. doi: 10.2147/SCCAA.S68073.

Xian, L. *et al.* (2012) 'NIH Public Access', 18(7), pp. 1095–1101. doi: 10.1038/nm.2793.Matrix.

Xie, K. (2001) 'Interleukin-8 and human cancer biology', *Cytokine and Growth Factor Reviews*, 12(4), pp. 375–91. doi: 10.1016/S1359-6101(01)00016-8.

Xie, Z. *et al.* (2016) 'MCP1 triggers monocyte dysfunctions during abnormal osteogenic differentiation of mesenchymal stem cells in ankylosing spondylitis', *Journal of Molecular Medicine*. Journal of Molecular Medicine, 95(2), pp. 143–54. doi: 10.1007/s00109-016-1489-x.

Xu, F. *et al.* (2010) 'Chemokines mediate mesenchymal stem cell migration toward gliomas in vitro', *Oncology Reports*, 23, pp. 1561–7. doi: 10.3892/or.

Xu, M., McCanna, D. J. and Sivak, J. G. (2015) 'Use of the viability reagent PrestoBlue in comparison with alamarBlue and MTT to assess the viability of human corneal epithelial cells', *Journal of Pharmacological and Toxicological Methods*. Elsevier Inc., 71, pp. 1–7. doi: 10.1016/j.vascn.2014.11.003.

Xu, Y. K. *et al.* (2015) 'The role of MCP-1-CCR2 ligand-receptor axis in chondrocyte degradation and disease progress in knee osteoarthritis', *Biological Research*. BioMed Central, 48, p. 64. doi: 10.1186/s40659-015-0057-0.

Yamaguchi, A. *et al.* (2008) 'Regulation of osteoblast differentiation mediated by BMP, Notch, and CCN3/NOV', *Japanese Dental Science Review*. Japanese Association for Dental Science, 44(1), pp. 48–56. doi: 10.1016/j.jdsr.2007.11.003.

Yamaguchi, A., Komori, T. and Suda, T. (2009) 'Regulation of Osteoblast Differentiation By Micrornas', *Endocrine Reviews*, 21(November), pp. 393–411.

Yamashita, T., Takahashi, N. and Udagawa, N. (2012) 'New roles of osteoblasts involved in osteoclast differentiation', *World Journal of Orthopedics*, 3(11), p. 175. doi: 10.5312/wjo.v3.i11.175.

Yang, J. *et al.* (2015) 'The Hedgehog signalling pathway in bone formation', *International Journal of Oral Science*, 7(2), pp. 73–79. doi: 10.1038/ijos.2015.14.

Yang, X. *et al.* (2001) 'TGF-β/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage', *The Journal of Cell Biology*, 153(1), pp. 35–46.

Yasui, T. *et al.* (2017) 'Isolation of dental pulp stem cells with high osteogenic potential', *Inflammation and Regeneration*. Inflammation and Regeneration, 37(1), p. 8. doi: 10.1186/s41232-017-0039-4.

Yoo, H. J. *et al.* (2011) 'Gene expression profile during chondrogenesis in

human bone marrow derived mesenchymal stem cells using a cDNA microarray', *Journal of Korean Medical Science*, 26(7), pp. 851–8.

Yoo, J. *et al.* (1998) 'The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells', *The Journal of Bone and Joint Surgery. American Volume*, 80(12), pp. 1745–57.

Zhang, L. *et al.* (2010) 'Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems', *Biotechnology Letters*, 32(9), pp. 1339–46. doi: 10.1007/s10529-010-0293-x.

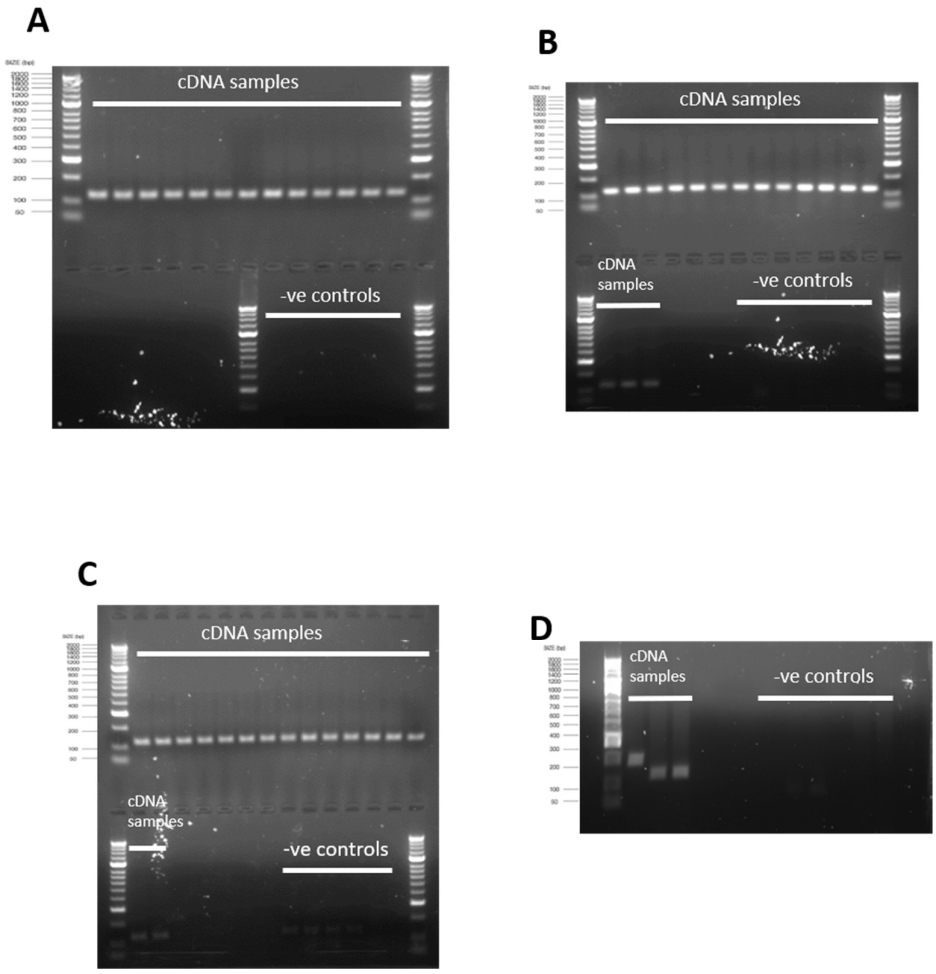
Zhang, X. *et al.* (2002) 'Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair', *The Journal of Clinical Investigation*, 109(11), pp. 1405–15. doi: 10.1172/JCI200215681.Introduction.

Zhao, Q., Ren, H. and Han, Z. (2016) 'Mesenchymal stem cells: immunomodulatory capability and clinical potential in immune diseases', *Journal of Cellular Immunotherapy*. Elsevier Ltd, 2(1), pp. 3–20. doi: 10.1016/j.jocit.2014.12.001.

Zhen, G. *et al.* (2013) 'Inhibition of TGF–β signaling in subchondral bone mesenchymal stem cells attenuates osteoarthritis', *Nature Medicine*, 19(6), pp. 704–712. doi: 10.1038/nm.3143.Inhibition.

Zhu, F. *et al.* (2012) 'The trancription factor Osterix (SP7) regulates BMP6-induces human osteoblast differentiation', *Journal of Cellular Physiology*, 227(6), pp. 2677–85. doi: 10.1021/nl061786n.Core-Shell.

Appendix I – cDNA validation



Appendix Figure. CDNA validation was carried out before use with TLDAs through RT-PCR and agarose gel electrophoresis.

Successful validation of chondrogenic (**A**) and osteogenic (**B**) samples was obtained. Exposure samples showed bands in negative controls (**C**) so the negative controls and representative cDNA samples were run again to confirm RT-PCR contamination and not contamination of the cDNA (**D**). Negative controls included: RNA negative, RT negative, cDNA negative, PCR negative, No primers, No Taq polymerase

Appendix II – PIP Reflective Statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student. The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIPS Reflective Statement

As part of my PhD I undertook my 3 month internship in the Research Liaison and Evaluation team at Arthritis Research UK. During this placement I was lucky enough to work on a number of different projects within the research team, though my primary role was based in Research Liaison. Here I worked in a small team that acts as the communication centre between the funded research, the organisation and the public, whose aim is to ensure that the impacts of the charity's research investments are available and visible to all. I particularly enjoyed this work because I was able to translate and celebrate the exciting knowledge coming out of academic research in a more accessible way to people outside of the research environment. I mainly achieved this by writing lay summaries of newly funded research projects, which are available on their public website, but I also had more direct interaction by responding to research enquiries from the public as they came in, which often required a very quick turnaround, and thus good time management. Another way of communicating research outcomes was through update reports to trusts who fund the research which required me to utilise another different style of communication.

During this placement I was able to utilise my already existing knowledge on osteoarthritis and stem cell research, but this placement gave me a much wider view of the field and a clearer picture of how research impacts and translates outside of the academic world. It was also invaluable for helping me develop my communication skills particularly in reference to tailoring to the target audience.

Whilst I undertook this placement I also had the opportunity to gain insight into the charity as a whole, as I engaged in other projects, such as evaluation of the investments of the charity and assisting with the peer review process for new funding calls. As well as providing me with a wider view on how the charity works it also allowed me to develop my teamwork and time management skills as I worked simultaneously across the teams and projects.

A separate project I worked on during the placement was cataloguing and creating a review of all the research that the charity funding with regards to stem cell research; proposed due to the enquiries that the charity receives about this field of work. This allowed me to work independently and develop the skills I had already established from academia, such as the ability to quickly and accurately review literature.

After returning from my placement I was better able to use the skills I gained on this placement throughout the rest of my PhD and overall it was a very valuable experience, both in terms of skill development and also for my future career, as it is a career path I would like to pursue.