The effects of hyaluronan on the adipose-derived stem cell secretome for the treatment of osteoarthritis

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Abstract

Osteoarthritis (OA) can be a debilitating degenerative disease and is the most common form of arthritic disease. There is a general consensus that current non-surgical therapies are insufficient for younger OA sufferers who are not candidates for knee arthroplasties. Adipose-derived mesenchymal stem cell (MSCs) therapy for the treatment of OA can slow disease progression and lead to neocartilage formation. Using Hyaluronan (HA) for delivery of MSC therapy in OA is widespread with no consideration of altered MSC function. The mechanism of action of MSCs is secretion driven. Therefore we sought to elucidate the effects of different concentrations of hyaluronan on MSC growth kinetics and evaluate the effect on the MSC secretome. Using a range of clinically relevant preparations, a titration of hyaluronan concentrations, we assessed MSC adherence and proliferation on both culture plastic surfaces and a novel cartilage-adhesion assay. We employed an adherence time-course and dispersion imaging techniques to assess MSC binding to cartilage. Our data showed HA had profound dose-dependent effects on early growth kinetics and the secretome of MSCs at concentrations up to the hyaluronan entanglement point at 1 mg/mL. At higher concentrations viscosity effects outweighed any benefit of additional HA. The novel cartilage-adhesion assay revealed for the first time that HA-primed MSCs can increase cell attachment to cartilage and that the presence of HA did not.

In conclusion, the investigation showed HA can have profound dose-dependent effects on MSCs. Although early data cytokine data suggested HA would negatively impact MSC mode of action, functional assessments of human osteoarthritic cartilage and synovium demonstrated HA culture modulated negative effects produce by the MSC secretome. Thus we have shown through exploration of the MSC secretome

that MSCs cultured in HA would have a synergistic effect in MSC therapy for the treatment of knee OA.

Declaration

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

Peter Succar – September 2016

List of Publications

[1] **Succar, P.,** Shu, C., Smith, S., Breen, E. J., Walker, P., Smith, M. M., Little, C. B., Herbert, B. R. Hyaluronan-mediated stem cell secretions in osteoarthritis: A pilot study. *Arthritis Research & Therapy* (Submitted, 2016)

[2] **Succar, P**., Medynskyj, M., Breen, E. J., Batterham, T., Molloy, M. P. & Herbert, B. R. Priming Adipose-Derived Mesenchymal Stem Cells with Hyaluronan Alters Growth Kinetics and Increases Attachment to Articular Cartilage. *Stem Cells International* 2016, 13.

[3] **Succar P**, Breen EJ, Kuah D, & Herbert BR (2015). Alterations in the Secretome of Clinically Relevant Preparations of Adipose-Derived Mesenchymal Stem Cells Cocultured with Hyaluronan. *Stem Cells International* 2015: 16.

[4] Meng Q, Jia H, **Succar P**, Zhao L, Zhang R, Duan C*, et al.* (2015). A highly selective and sensitive ON–OFF–ON fluorescence chemosensor for cysteine detection in endoplasmic reticulum. Biosensors and Bioelectronics 74: 461-468.

Communications

Succar P, Medynskyj M, Herbert B. In vitro model using bovine articular cartilage explants for investigation of adipose-derived stem cell kinetics with hyaluronic acid. 22nd Stem Cell Network Workshop, Stem cells and genetic muscle disorders 2015; Sydney, Australia

Succar P, Medynskyj M, Herbert B. In vitro model using bovine articular cartilage explants for investigation of adipose-derived stem cell kinetics with hyaluronic acid. The 12th International Federation for Adipose Therapeutics and Science 2014; Amsterdam, Netherlands.

P. Succar, C. Hill1, S. Blaber, B. Herbert. Adipose-derived stem cells co-cultured with Hyaluronan: changes in secretions and proteomic profile. The 19th Lorne Proteomics Symposium 2014; Victoria, Australia

Table of Abbreviations

Abbreviation	Full Name
ACAN	Aggrecan,
ADAMTS-4	Aggrecanase 1
ADAMTS-5	Aggrecanase-2
BMI	Body mass index
BrdU	5-bromo-2'-deoxyuridine
CCK-8	Cell-Counting Kit-8
CD44	Cluster of Differentiation 44
COL10A1	Collagen type X
COL1A1	Collagen type I
COL2A1	Collagen type II
COX	Cyclooxygenase
CRP	C-reactive protein
CTX-II	C-terminal crosslinking telopeptide of type II collagen
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
Eotaxin	Eosinophil chemotactic protein
FBS	Foetal bovine serum
FDR	False discovery rate
FGF	Fibroblast growth factor
FGF-β	Fibroblast growth factor-basic
GAGs	Glycosaminoglycans
G-CSF	Granulocyte colony-stimulating factor
GI	Gastrointestinal

GM-CSF	Granulocyte macrophage colony-stimulating factor
GROα	Growth-regulated peptide alpha
H&E	Haematoxylin and eosin
HA	Hyaluronic acid; Hyaluronan
HAS	HA synthases
HGF	Hepatocyte growth factor
HRT	Hormone replacement therapy
HYALs	Hyaluronidases
ICAM-1	Intracellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IFN-α2	Interferon alpha-2
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-17	Interleukin-17
IL-1Ra	Interleukin-1 Receptor antagonist
IL-1α	Interleukin-1α
IL-1β	Interleukin 1-beta
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8

IP-10	Interferon gamma-induced protein 10
LIF	Leukemia inhibitory factor
MCP-1	Monocyte chemotactic protein-1
MCP-3	Monocyte chemotactic protein-3
MIF	Macrophage migration inhibitory factor
MIP-1α	Macrophage inflammatory protein-1-alpha
MIP-1β	Macrophage inflammatory protein-1 beta
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NSAIDs	Non-selective non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OH	Hydroxyl radicals
PCR	Polymerase chain reaction
PDGF-$\beta\beta$	Platelet-derived growth factor-$\beta\beta$
PE	Phycoerythrin
PGE2	Prostaglandin E2
PGs	Prostaglandins
PI	Propidium iodide
PMNs	Polymorphonuclear leukocytes
PRP	Platelet rich plasma
RA	Rheumatoid arthritis
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
RFU	Relative fluorescent units
ROS	Reactive oxygen species

SLRPs	Small leucine rich proteoglycans
SOX9	SRY (Sex Determining Region Y)-Box 9
STC-1	Stanniocalcin-1
SVF	Stromal vascular fraction
TGF-β	Transforming growth factor beta
TIMPs	Tissue inhibitor of metalloproteinases
TKR	Total knee arthroplasty
TNF-α	Tumor necrosis factor-α
TNF-β	Tumor necrosis factor-beta
TRAIL	TNF-related apoptosis inducing ligand
TSG-6	TNF alpha stimulated gene 6
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
WISP1	WNT1 Inducible Signaling Pathway Protein 1
WOMAC	Western Ontario and McMaster University Osteoarthritis Index
β-NGF	Nerve growth factor-beta

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for that I thank you.

1

Adipose-derived Mesenchymal stem cells & Hyaluronan for the treatment of Osteoarthritis: Evidence for synergy?

1.1 Overview of Osteoarthritis

Osteoarthritis (OA) is a debilitating degenerative disease and is the most common form of arthritic disease which progressively decreases the function of a joint. Onset of OA is usually in the third and fourth decade of life and has a gradual worsening prognosis over time. In Australia, the prevalence of arthritic conditions in people aged under 25 is less than 1% but increases to 52.1% in the people aged 75 years or older (Figure 1.1). OA accounts for more than half (55.9%) of the arthritic conditions presented in Australia (Australian Bureau of statistics, 2012). In years to come, OA is likely to increase in financial economic burden for governments around the world, in which more and more developed countries are anchoring an aging population. The World Health Organization (WHO) predicts that by the year 2020, OA will become the fourth leading cause of disability [1].
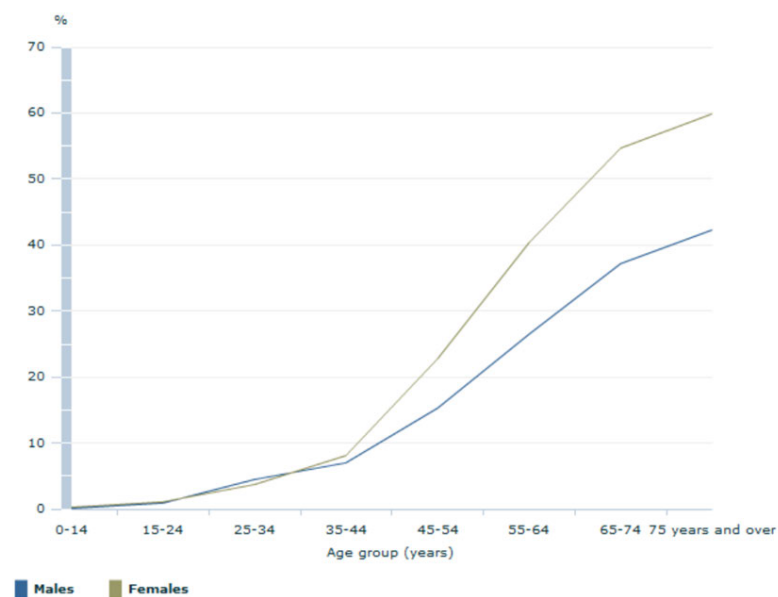
Figure 1.1 Produced by the Australian Bureau of Statistics, 2012

Of persons with arthritis, more than half (55.9%) had osteoarthritis, 13.6% had rheumatoid arthritis, and 37.3% had an unspecified type of arthritis. Note that as it is possible to have more than one type of arthritis, proportions add to more than 100%. The prevalence of arthritis increased with age, from less than 1% of people aged under 25 years to 52.1% of people aged 75 years and over. Women aged 45 years and over were considerably more likely to have arthritis than men. In particular, at ages 75 years and over, 59.9% of women had arthritis compared with 42.3% of men.

1.2 Classification and diagnosis

OA can be classified as either primary or secondary. Primary OA is idiopathic and is thought to be an age related disease and perhaps a case of wear and tear. Secondary OA occurs as a result of injury or other related pathologies. OA most commonly occurs in weight bearing joints such as the hip and knee. The knee joint is the most common site for OA and the incidence is higher in females [2].
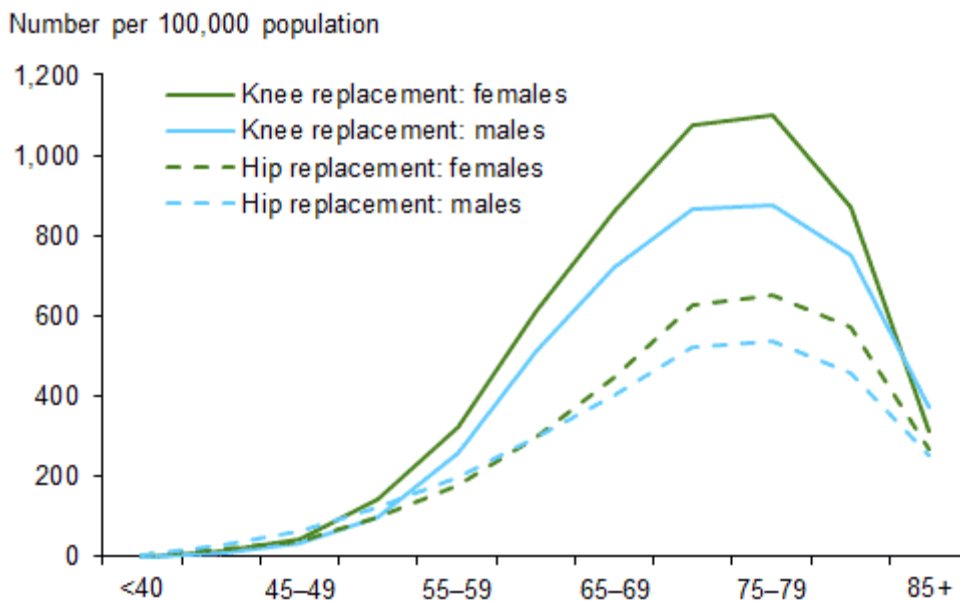
Figure 1.2 Australian institute of Health & Welfare National Hospital Morbidity Database

Rate of total knee and hip replacements for osteoarthritis, by sex and age, 2012–13. OA most commonly occurs in weight bearing joints such as the hip and knee. The knee joint is the most common site for OA and the incidence is higher in females.

The diagnosis of OA can involve patient reported joint symptoms such as pain and stiffness, in which case it is referred to as symptomatic OA. In the case where radiographic changes in joint space, osteophyte formation, subchondral bone sclerosis, loss of articular cartilage, cyst formation, bone remodeling and inflamed or thickened synovium are observed, then it is referred to as radiographic OA. Symptomatic and radiographic OA are not mutually exclusive nor does the existence of one depend on the other. For example, patients may present with symptoms of

pain and reduced joint mobility but have no radiographic evidence of knee OA [3]. Conversely, patients may present with radiographic OA but have no symptoms of knee OA and additionally patients may cumulatively present with both symptomatic and radiographic clinical indications of knee OA. The most commonly used grading system for knee OA takes into account the appearance of osteophytes, joint space narrowing, subchondral bone sclerosis, cyst formation and was first described by Kellgren and Lawrence [4]. The system utilizes these criteria to assign an OA grade (0-4) for various joint sites. Grade 0 indicates there are no features of OA through to grade 4 which indicates severe OA pathology because joint space is greatly impaired accompanied by articular cartilage erosion and sclerosis of the subchondral bone.
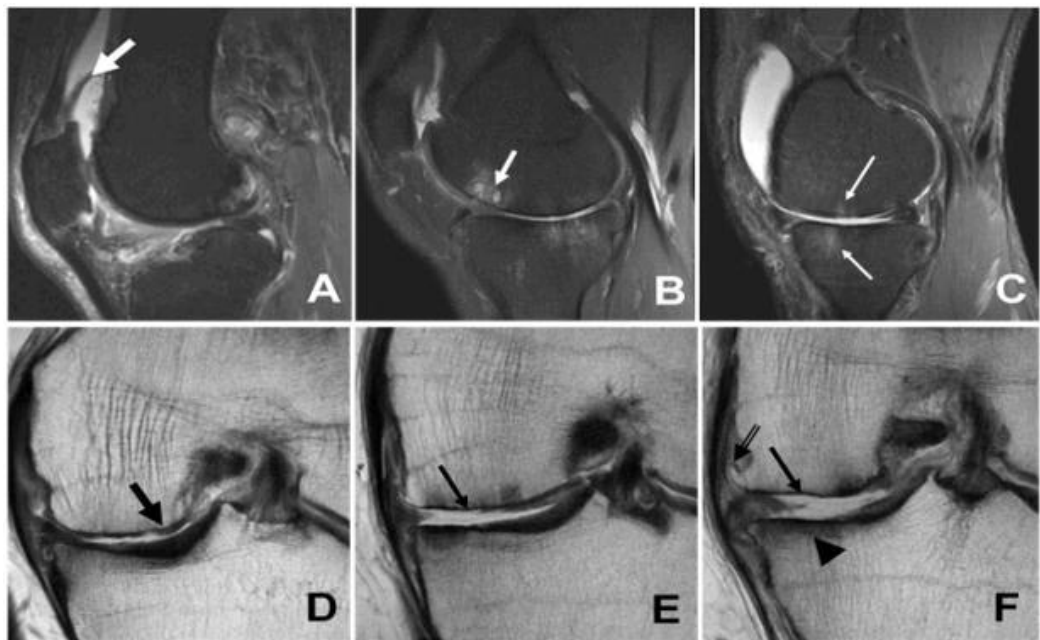
Figure 1.3 Image depicting hallmark changes observed in knee OA

(A) reactive synovitis (thick white arrow), (B) subchondral cyst formation (white arrow), (C) bone marrow edema (thin white arrows), (D) partial thickness cartilage wear (thick black arrow), (E–F) full thickness cartilage wear (thin black arrows), subchondral sclerosis (arrowhead) and marginal osteophyte formation (double arrow). *Image adapted from Loeser et al. [5]

1.2.1 Histopathological changes in OA

Knee OA is a disease which is multifaceted and encompasses all the components of the joint. Manifestations of OA typically begin in the articular cartilage but will also involve the subchondral bone, osteophyte formation, reduced laxity of the surrounding ligaments, degeneration of the meniscus and some variable synovial inflammation. OA represents the failure of the joint as an organ and therefore surrounding tissue in the joint capsule will undergo pathological progression. Such tissue which are also affected include; surrounding fat pads, bursa, nerves and periarticular muscles. For the purpose of this thesis, we will focus on the pathological changes observed in the articular cartilage and synovium.

Articular cartilage of the knee is a smooth and glossy surface which is avascular in its native non-pathological state. Chondrocytes and synoviocytes produce natural lubricants, hyaluronic acid and lubricin within the joint which coupled with the smooth articular surface, provide efficient gliding with a low friction coefficient [6]. The articular cartilage surface carries the greatest amount of shear stress in the knee and therefore it is not surprising that the earliest changes in OA appear in areas under the highest mechanical load [7] i.e. the articular cartilage.

Chondrocytes were initially thought to be dormant and not to have played a major role in cartilage remodeling. Recently however, it was shown at the cellular level in healthy cartilage, chondrocytes are quiescent and contribute to small amounts of cartilage matrix turnover. They secrete hyaluronan and aggrecan which combined with the collagens and other proteoglycans form the rigid but smooth articulating surface (see figure 1.6 and references therein). The activity of chondrocytes is increased in OA, so much so that they become hypertrophic. They begin to proliferate rapidly and increase production of matrix proteins and enzymes which degrade matrix proteins. The conformation of the chondrocytes within the articular cartilage begin to form clusters rather than an evenly dispersed throughout the tissue.

The activation of chondrocytes leads to matrix-remodeling and cartilage calcification [8]. The consensus on exactly how the chondrocytes become activated are still unclear however, injury in the surrounding ligaments or the menisci show a strong correlation to the development and progression of OA. The risk factors for the development of knee OA are discussed in detail in section 1.3.

Synovial inflammation is not typically associated with OA but rather rheumatoid arthritis (RA). Immediately after knee injury however and in the early stages following, inflammation of the synovium (synovitis) is observed [9,10]. Such injury events or synovitis may be the origin of OA development, but the evidence is lacking. Recent developments have shown that synovitis is not exclusively associated with RA, but rather histological observations have exposed synovitis in early stage OA [9], although to a much lesser extent than RA. The prevalence of synovitis gradually increases as OA pathology advances. Krasnokutsy et al. showed that synovitis is both a characteristics feature of advancing knee OA and significantly associated with increased Kellegren Lawrence grade of OA and joint space narrowing [11]. Although the study suffered from a subjective semi-quantitative observations and inter-observer variability. Arthroscopic observations of 422 patients showed OA patients who also displayed synovitis had increased pathology in the cartilage [12]. However these studies are inherently subjective due to the observer determinations. Physical performance assessments can be used to objectively determine changes in knee joint function. A study which followed a cohort of 363 middle aged-women for 11 years, showed that almost a quarter of women would developed radiographic OA. Patients with combined OA and synovitis however, scored lower on walking and stair-climbing times and therefore synovitis was strongly associated with inferior knee function [13].

The synovium lines the knee joint and forms the lining of bursae and fat pads. It is a specialised connective tissue which seals the synovial cavity to maintain synovial

fluid. The subintima is the outer layer of the synovium and is made up of fibrous collagen, areolar tissue and adipose tissue. This outer layer is relatively acellular and is rich in type I collagen, a microvascular blood supply, lymphatic vessels and nerves [14]. The intima is the inner layer of the synovium adjacent to the joint cavity and is lined with a layer of 1-4 synoviocytes deep i.e. 20-40 μm thick compared to the subintima which can be up to 5 mm thick [14].

Synoviocytes which line the intima produce lubricin and hyaluronic acid to control the volume and composition of synovial fluid. Articular cartilage is avascular, therefore the synovial fluid produced by the synoviocytes contribute to chondrocyte nutrition [15]. This lack of intrinsic vasculature or lymphatic supply to articular cartilage means that surrounding tissues, such as the synovium, are required for the removal of products of chondrocytic metabolites and articular matrix turnover [15]. The synovium is a semipermeable membrane encapsulating the joint and therefore acts like a molecular cut-off filter. Small molecules such as growth factors and cytokines are able to diffuse across the membrane, however high molecular weight synovial fluid is retained within the membrane. Equally, high molecular weight plasma proteins are excluded from the joint by the synovial membrane, therefore the viscosity and composition of the synovial fluid is maintained [15]. The disruption of synovial membrane permeability is said to occur during bouts of synovitis. These changes likely contribute to the decreased concentration of hyaluronan and lubricin within the synovial fluid which contribute to the pathogenesis of OA. This can be seen during bouts synovitis or hyperplasia of the synovium, when hyaluronan can be detected in the serum [16]. Changes in hyaluronan concentration are hallmark biomarkers for the progression of OA [17]. Hyaluronan fragmentation which contribute to OA progression are discussed in further detail in chapter 1.5.

The association of synovial pathology contributing to osteoarthritic joint disease was proposed by Oehler et al. In their histological study using synovial specimens

derived from early and late stage osteoarthritic cartilage disease, they clearly

demonstrated OA was significantly associated with synovial pathology.

Histopathological changes observed in the synovium include; Intimal hyperplasia,

Lymphocytic infiltration, subintimal fibrosis and vascularity [18]. These four subtypes

of osteoarthritic synovitis were used in the scoring system as outcome measures for

chapter 5 of this thesis.
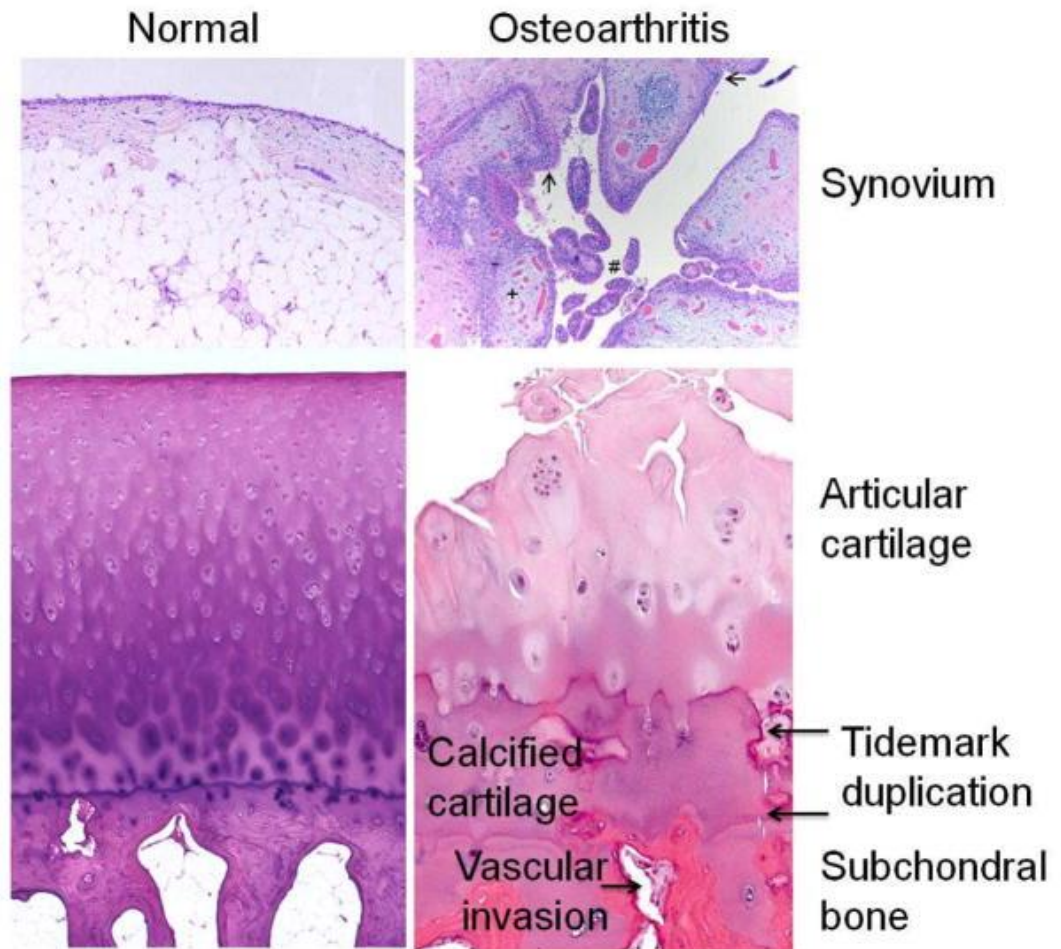
Figure 1.4 Histopathological changes observed in knee OA

The normal synovium has a thin lining layer and a vascularized, loose connective tissue sub-lining

layer. OA synovium demonstrates features of synovial hyperplasia (#), lining hyperplasia (arrows),

increased vascularity (+) and perivascular mononuclear cell (inflammatory) infiltration. In OA

articular cartilage, loss of cells and matrix is accompanied by areas of cell clusters. There is

thickening of the calcified zone and duplication of the tidemark which normally separates articular

cartilage from the underlying calcified cartilage.*Image adapted from Loeser et al. [5]

1.2.2 Molecular changes in OA

Chondrocytes are the only cell type found in articular cartilage but constitute approximately 2% of the cartilage volume [19] with the remainder made up largely of extracellular matrix (ECM). The ECM can be divided into three main zones; the pericellular, territorial and the interterritorial matrix. The pericellular matrix is found adjacent to chondrocytes, rich in perlecan, type VI collagen and various molecules which regulate chondrocyte function. The territorial and interterritorial matrix are conversely composed of type II collagen and aggrecan (see figure 1.5 and references therein).

The tensile strength attributed to articular cartilage is provided by collagen and the anti-compressive properties come about from the proteoglycans which draw water into the matrix. Proteoglycans make up 10-15% of the wet weight in the extracellular matrix of articular cartilage [20], with aggrecan, biglycan, decorin, and fibromodulin the most predominant, and having distinct functions in the tissue. Aggrecan is the principal large proteoglycan of cartilage, containing numerous chondroitin sulphate and keratan sulphate glycosaminoglycan moieties. Its propensity to aggregate with hyaluronan into much larger complexes is essential for the mechanical properties of cartilage [21]. The degradation of both aggrecan and cartilage is central to pathological development of OA.

Aggrecan is long proteoglycan susceptible to proteolysis at numerous sites along the length of the interglobular domain between the N-terminal G1 and G2 globular domains. Cleavage of this site is physiologically significant as it is the glycosaminoglycan-rich moiety of aggrecan and therefore it abolishes the primary function when released from the cartilage matrix. The proteolysis was initially thought to be mediated by metalloproteinases, when matrix metalloproteinase 3 (MMP-3) was shown in vitro at acidic and physiological pH to cleave aggrecan sites [22]. Other MMPs similarly showed they could cleave aggrecan, such as MMP-13

however it was subsequently shown that fragments of aggrecan found in pathological OA synovial fluid were not corroborated to the MMP sensitive sites within the interglobular domain [23]. Cytokine stimulation of chondrocytes and cartilage explants by such cytokines as interleukin 1 (IL-1), activate enzymes that release aggrecan catabolites from cartilage. In vitro studies showed that cytokine-mediated activation of catabolic enzymes could not be inhibited by selective synthetic MMP inhibitors and thus showed that physiological proteolysis of aggrecan and MMP catabolism of aggrecan at the interglobular domain were independent [24].

A Disintegrin and Metalloproteinase with Thrombospondin motifs 4 (aggrecanase 1; ADAMTS-4) was the first identified aggrecanase. It is a member of the ADAMTS protein family that cleaves aggrecan at the glutamic acid-373-alanine-374 bond and was purified from IL-1 stimulated bovine nasal [25]. Aggrecanase 2 (ADAMTS-5), a homologous enzyme was cloned from bovine cartilage soon after [26]. A mutated mice study where aggrecan knockin mice resistant to interglobular domain cleavage by aggrecanase have been used to model aggrecanase selective degradation of cartilage. Little et al., demonstrated that recombinant ADAMTS-5 could not fragment cartilage in situ when surgical methods for inducing OA were applied but rather appeared to stimulate cartilage repair following acute inflammation. Similarly when cartilage explants from the same mouse were stimulated with IL-1 only wildtype mice showed aggrecan fragmentation. Thus showing that blocking aggrecanolysis protects against cartilage erosion [27]. Song et al., then demonstrated similar results in primary human osteoarthritic chondrocytes and cultured human explants by transfecting with small interfering RNA (siRNA) to inhibit the activity of both ADAMTS-4 and ADAMTS-5. He showed cytokine stimulated degradation of aggrecan was attenuated when aggrecanase activity was suppressed [28]. Several synthetic inhibitors are in early stages of development to block the activity of ADAMTS-4 and ADAMTS-5 to delay the cartilage catabolism in OA [29-31].

The extracellular matrix found in articular cartilage is primarily composed of type II collagen. Collagen is responsible for the tensile strength of articular cartilage and like aggrecan, collagen degradation is essential to the pathological progression of OA [32]. C-telopeptide fragments of type II collagen (CTX-II) is an indicator of collagen fragmentation and can consequently be detected in urine and synovial fluid. The early molecular events which following knee injury and OA indicate that both aggrecan fragments and CTX-II are detected at significantly higher concentration than in normal synovial fluid [33]. The exact order in which the matrix is catabolised is difficult to ascertain, however aggrecanases are not exclusively involved in the catabolism of cartilage. Chondrocytes produce collagenases which breakdown articular cartilage by cleaving type II collagen which in healthy cartilage may contribute to the normal physiological process for matrix turnover. A study of human osteoarthritic condylar cartilage showed collagenase-3 (MMP-13), may play a significant role in the cleavage and denaturation of type II collagen [34]. However, others have shown by using animal cartilage explant culture that collagen fibrils cannot be degraded until aggrecan is catabolised and fragmented from the matrix [35,36]. This suggests that the preservation of aggrecan in the matrix may offer overall protection to collagen fibrils. Little et al., confirmed that structural cartilage damage in mouse experimental OA is dependent on MMP-13 activity. In MMP-13 knockout mice showed MMP-13 deficiency inhibits cartilage erosion, but not aggrecan depletion [37]. Therefore neither aggrecanase nor collagenase alone is responsible driving OA progression but a combination of both, alongside any yet to be identified mechanisms.

Chondrocytes are responsive to mechanical stimulation via receptors for extracellular matrix (ECM) moieties. Production of matrix-degrading proteinases, inflammatory cytokines and chemokines is thought to be a result of chondrocyte receptor activation [5]. Degradation of type II collagen is efficiently achieved by MMP-13 and once the

collagen network is degraded, unlike aggrecan, the damage is irreversible [5]. MMP-13 expression is increased in OA cartilage and thus it is thought to be the principal collagenase involved in the pathogenesis of OA [34]. It is expressed exclusively in chondrocytes but not synoviocytes and is modulated by two proinflammatory cytokines, IL-1β and TNF-α, in a time- and dose-dependent manner [38]. The expression of MMP-13 is higher in osteoarthritic cartilage compared to healthy cartilage [39]. Clinically tested MMP inhibitors have been associated with a painful, joint-stiffening musculoskeletal side effect that may be due to their lack of selectivity. MMP-13 is unique compared to other collagenases due to its unusually deep S1'subsite. As such inhibitors of MMP-13 have been formulated and are in early stage of therapeutic development. These are able to effectively block cartilage degradation in cytokine stimulated in human osteoarthritic cartilage explants [34] and in bovine cytokine stimulated cartilage [40]. Active MMP13 inhibitors can reduce cartilage damage in vivo without joint fibroplasias in a rat model of musculoskeletal syndrome side effects [41].

1.2.3 Outcome measures in Osteoarthritis

Animal models make a significant contribution to the understanding of the pathogenesis of Osteoarthritis. Imaging modalities in animal models play a pivotal role in understanding the pathogenesis of OA. These have been extensively reviewed in the literature and many advantages and disadvantages arise from each type of model within each species they are undertaken in [42-44]. However the choice of animal model is very dependent on which aspect of the multifaceted osteoarthritis disease needing to be investigated. They can be invasive or non-invasive but none so perfect that they perfectly mirror the idiopathic osteoarthritis seen in humans. The ultimate aim of this thesis was to address the use of experimental MSC therapy in combination with HA via the exploration of the MSC secretome (as defined in the thesis) and MSC growth kinetics. The core experimental work undertaken in this thesis was done using human donors. The thesis concludes with a pilot study using osteoarthritic cartilage and synovium collected from human patients undergoing total knee replacement surgery treated using MSC secretions conditioned with or without HA.

Testing the efficacy of therapeutic interventions in OA relies heavily on patient reported outcomes through self-administered questionnaires. The most widely used measure of symptoms and physical disability is the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). WOMAC was first validated in a double-blind randomized controlled trial testing the efficacy of non-steroidal anti-inflammatory treatment of knee OA [45]. It is a tri-dimensional self-administered questionnaire measuring pain, stiffness and physical function. The score for these three sub-scales is summated for global score and used to determine therapeutic outcomes of interventions in knee OA. Further testing of WOMAC on OA patients have shown that it satisfies test-retest reliability. The sub-scales have also been shown to be internally consistent and associated with radiological OA-severity and

joint range of motion [46]. Another study in 304 patients with symptomatic OA showed that the physical function sub-scale was weakly associated with radiological OA severity. They also found that the sub-scale of pain was inversely correlated with years of formal education [47]. This may suggest a potential limitation of administering the WOMAC in populations where formal education is lacking.

WOMAC only takes into account long-term consequences of knee injury and therefore in the early development it may be lacking. The Knee injury and Osteoarthritis Outcome Score (KOOS) is knee-specific and an extension of WOMAC developed to assess the patients' opinion about their knee and associated problems. Questions from the WOMAC Osteoarthritis Index were included in their full and original form in the KOOS questionnaire and later modified to cater for the wide array of knee injuries that could occur. The KOOS evaluates 42 items in 5 separately scored subscales; Pain, other Symptoms, Function in daily living (ADL), Function in Sport and Recreation (Sport/Rec), and knee-related Quality of Life (QOL) [48]. A study comparing WOMAC and KOOS in patients with definite radiographic knee OA having had meniscectomy 21 years prior using age- and sex-matched controls with no radiographic OA. They confirmed that while both equal subscales had some degree of discrimination in scores, the great discriminant outcomes in all age groups were the two added dimensions of Sport and Recreation Function, and Knee Related QOL [49]. In another study 105 patients underwent total knee replacement were prospectively evaluated using both the WOMAC and KOOS questionnaires. The authors concluded that KOOS improved validity compared to WOMAC but was equivalent on patients responsiveness [50].

1.3 Risk Factors

Risk factors associated with the development of OA include age, gender, obesity, injury, and occupational overuse. The non-modifiable risk factors for OA, such as age and gender, are the strongest predictors of disease. It is well accepted that aging is a significant contributing factor to the development of OA. The prevalence of OA is almost non-existent in people aged under 25 years (Australian Bureau of Statistics, 2015). In China, a cross-sectional community study of 3428 adults, aging was one of the associated risk factors in the development of OA [51]. The way in which aging leads to the development of OA appears multifactorial and may be hinged on age-related systemic and local inflammation [52]. Increased adipose tissue stores in aging can contribute to systemic inflammation. Adipose tissue is a highly active metabolic and endocrine organ, which is capable of secreting cytokines and adipokines [53] and contributes to systemic inflammation by the secretion of pro-inflammatory cytokines such as C-reactive protein (CRP), Interleukin 6 (IL-6) and Tumor necrosis factor-α (TNF-α) [54]. Of these cytokines, IL-6 was the most robust predictor of age-related inflammation [55]. It is unclear whether inflammation is the cause of knee OA or a symptom of the development of knee OA.

Fundamental differences in the physiology of males and females may also contribute to the risk factors in the development of knee OA. In a study of Australian males and females with patient reported knee pain but no signs of radiographic OA, men had significantly larger femoral and patellar volumes of cartilage [56]. Similar differences were found in a much larger cohort with an age range of 21-39 years, in which a statistical differences persisted following adjustments for weight and height [57]. Moreover, female predisposition in the development of knee OA may come about

long before the onset of any clinical knee disease. In an Australian cohort of 271 participants with an age range of 50-79 years and no history of knee pain or OA pathology, a multivariate analyses on baseline magnetic resonance imaging (MRI) and an average of 2.3 year follow-up showed both tibial and patella cartilage volume loss was significantly higher in women compared with men [58]. Therefore even in the absence of disease, female cartilage is more susceptible to erosion than male counterparts. This may help to explain why women aged 45 years and over are considerably more likely to have arthritis than men. At ages 75 years and over, 59.9% of women had arthritis compared with 42.3% of men (Australian Bureau of Statistics, 2015). The prevalence of knee OA in men, is significantly reduced compared to women, in particular post-menopausal women [59]. This suggests changes of either sex steroids in circulation or changes in the response of bodily tissues to sex steroids may influence the pathogenesis of knee OA. Thus hormone replacement therapy may have protective effects on the development of knee OA in post-menopausal women. Indeed the Chingford study tested this hypothesis in 606 post-menopausal women and found a significant protective effect associated with current users of hormone replacement therapy (HRT) for knee OA [60]. However the diagnostic credibility used in the study was inadequate as knee OA was defined by using osteophyte formation and no other radiographic pathology. Nevitt et al., conversely showed in post-menopausal women with cardiac disease, that taking HRT had no significant effect on knee pain [61]. Similarly, Cirillo et al., showed HRT was not associated with reduced total knee arthroplasty (TKR) in community-dwelling women aged between 50-79 years [62].

Genetic factors may also have a role in the pathogenesis of OA. No definitive genetic markers are currently available in the diagnosis of OA, however the evidence suggests there is a strong genetic contribution in the development of OA. Twin

studies can be used to elucidate the genetic components by comparing those with identical genes (monozygotic twins) with non-identical genes (dizygotic twins). In a study of 250 pairs of female twins aged between 48-70 years, the occurrence of radiographic patellofemoral and tibiofemoral pathology was compared between either monozygotic or dizygotic twins. In this study the likelihood of both monozygotic twins having OA pathology was much higher compared with both dizygotic twins suggesting a strong genetic component in the development of knee OA [63]. Moreover, total knee replacements (TKR) are the final treatment option for patients with end stage knee OA. In a study measuring rate of change in knee cartilage volume in 325 adults, half of the recruited cohort were the offspring of subjects who had a TKR for primary knee OA. Using MRI it was shown that the offspring of parents who underwent TKR had greater knee cartilage loss than controls [64]. Another study supporting the inheritability of OA, was a genetic study on the Framingham cohort, which had previously shown age and obesity were strongly associated risk factors in the development of OA. A major advantage of this study is that both generations were studied at adult age with additional risk factors from the initial study. In a total of 337 nuclear families with at least one biological offspring they found using a segregation analysis, there was no correlation between spousal pairs but between parents and offspring or between siblings, the correlation in the development of knee OA was far more pronounced [65]. This further supports the notion of genetic contribution to the development of OA and the Mendelian mode of OA inheritance.

Obesity is an increasing epidemic in both developing [66] and developed nations [67,68]. Obesity is a modifiable risk factor in the development of knee OA. Studies have shown a link between obesity and the development of OA [69]. A decrease in body mass index (BMI) can significantly reduce the risk of developing knee OA, Felson *et*

al., showed that a reduction of 2 BMI points could halve the odds of developing knee OA over a 10 year period [70]. Other epidemiological studies have similarly found a BMI greater than 30 units was significantly associated with knee OA [71], and that the association, was dose-dependent [72]. Interestingly in the same study, obesity was also significantly associated with hand OA [72], which suggests that the mechanism by which obesity contributes to knee OA is more complex than simple weight bearing but rather local and systemic inflammation via cytokine and adipokine release. In a trial of 88 obese adults (BMI > 30), patients were randomized into either a low energy or control diet. Upon examination of body fat composition, the low energy diet showed each body fat percentage lost, correlated to improvements of 10% on the pain index [73]; measured by the Western Ontario and McMaster University Osteoarthritis Index (WOMAC), a disease specific questionnaire used to evaluate research in OA [45].

Injury to the knee joint can lead to the development of/or accelerate knee OA progression compared non-injured cohorts [74]. A longitudinal study of an adolescent patient cohort undergoing a total meniscectomy showed after a 30 year follow-up, using radiographic comparisons, that joint space narrowing and osteophyte formation increased significantly in the operated knee compared to the non-operated knee [75]. Additionally, reconstruction of ruptured knee ligaments, injuries commonly found in athletes, significantly accelerates knee OA disease progression [76], measured by the need for TKR. A meta-analysis of 24 observational studies also showed that injury to the knee was a major risk factor in the development of OA [77].

The demands of physical work can lead to occupational overuse which contributes to the development of knee OA. At present there is limited evidence for direct causation, however observational studies showed for occupations involving prolonged kneeling or squatting, have a significantly increased risk of developing

knee OA [78,79,80]. Another study showed frequent stairs climbing was an overuse associated with increased risk of developing knee OA [81].

1.4 Conventional OA therapy

Osteoarthritis of the knee involves all the tissues of the knee joint, however the most striking pathological features are the degenerated articular cartilage and remodelling in the adjacent bone. Therapies for knee OA can be classified as non-pharmacological, pharmacological and surgical. Primary clinical intervention typically involves alleviating pain with systemic pharmacotherapy such as analgesics and anti-inflammatories which are targeted at relieving pain and reducing inflammation. Non-pharmacological treatments are commonly used in conjunction with systemic pharmacotherapies to maintain range of motion/functionality in the joint and relieving pain through braces, muscle strengthening and controlling mechanical overload through weight loss.

Modern day bracing can provide pain relief to sufferers of knee OA by achieving condylar separation i.e. knee joint distraction. Such devices are anchored using straps at both the tibia and femur, which rotate along parallel hinges at either lateral ligaments providing flexible support of the knee. In a study which showed patients improved an average of 33% in their WOMAC scores, no changes were observed in the biomechanics or radiographic joint space narrowing [82], this may have been because the amount of time the brace was worn was not controlled or measured. Another randomized trial of 126 patients suffering from patellofemoral OA, patients wore the brace for an average of 7.4 hours a day. MRI comparisons after 6 weeks showed patients had significantly reduced volumes of patellofemoral bone marrow lesions and decreased knee pain [83].

Quadriceps are essential muscles surrounding the knee and contribute to the proper mechanical function of the joint. The effect of knee bracing on surrounding muscles, especially the quadriceps muscles has been explored. A study testing maximum

voluntary contraction of quadriceps in 108 knee OA patients, showed after six weeks of knee bracing there was no difference to control [84]. The eccentric contraction of the quadriceps is especially important as it protects the knee from high loads in the weight-acceptance phase of gait. This cushioning of load has a cartilage protective role and thus quadriceps muscle strength may be of clinical benefit to patients suffering from knee OA. In a five year longitudinal study which included 2404 elderly knee OA patients, women were 28% more likely to have worsening knee pain from decreased quadriceps strength [85] but men showed no such association. Muraki *et al*., showed in a cohort of 2152 knee OA patients differing results, in that both women and men had significantly associated knee pain with decreased quadriceps muscle strength and, that the association was independent of radiographic knee OA [86]. Additionally in a controlled study of 60 men, changes in quadriceps muscles and muscle morphology were compared between Kellgren and Lawrence grade 0 and grades 1 & 2 (i.e. early onset of knee OA). The control group (grade 0; i.e. no knee OA) was found to have significantly higher eccentric knee extensor torque and eccentric activation in the vastus lateralis muscle of the quadriceps compared to those with early onset of knee OA [87]. Nonetheless, strengthening of quadriceps muscles using non-weight bearing exercises, neuromuscular electrical stimulation of quadriceps, weighted knee extensions and flexions all produce improvements in physical function and pain in patients suffering from radiographic knee OA [88, 89, 90]. This evidence suggest quadriceps muscle strength is of fundamental importance for the treatment of knee OA.

Adipose tissue can contribute to systemic inflammation via the secretion of cytokines and adipokines. Evidence from a meta-analysis of 12 studies showed, the risk of developing knee OA increases with body mass index (BMI) dose-dependently and the risk increases exponentially for a BMI greater than 30 [91]. In a trial of obese adults

with a BMI greater than 30, knee OA patients were randomized into either a low energy or control diet. The study showed each body fat percentage lost correlated to improvements of 10% on the WOMAC pain index [73]. Although it was not determined whether the pain relief came about from reduced systemic inflammation or mechanical factors. However, it is known that knee joint loading can have significant effects on joint space narrowing which suggests unloading the joint could be beneficial mechanically. In a study of 157 obese patients with knee OA, a 16 week dietary intervention resulted in a 13.5% loss of weight and a reduction of 30% in knee pain. Importantly, a comparison of biomechanical measurements at baseline revealed for every one kilogram of weight loss, patients obtained more than a two kilogram reduction in peak knee loading on the knee joint [92].

Local inflammation of the knee joint may be a symptom of knee OA, or it may contribute to disease pathogenesis. The diffuse neuroendocrine system is a ubiquitous network of specialized neuroendocrine cells which secrete hormones in response to stimuli. Substance P is secreted by nerves and inflammatory cells of the diffuse neuroendocrine system and has proinflammatory effects on the musculoskeletal system and contributes to pain signaling [93]. Topical creams such as capsaicin containing creams can be used to reduce pain in knee OA but may not affect disease progression. These are available as over the counter analgesics and are considered a non-pharmacological treatment. Capsaicin is a naturally occurring neurotoxin which can deplete the contents of Substance P in neuroendocrine cells [94]. In a double-blind placebo controlled studies of OA associated pain, 0.025% capsaicin significantly reduced knee OA pain compared to placebo or vehicle control [95,96]. A single blinded study compared the 0.025% formulation applied four time daily and a 0.25% formulation applied twice daily over 28 days. The high strength formulation provided greater relief from OA associated pain compared to the lower strength [97].

Moreover, a recent Cochrane review found that the use of topical capsaicin for the relief of osteoarthritic pain was only moderate and not significant. It concluded capsaicin extract would not improve pain, however, neither of the three studies above were included and the assessment was based on a single study [98].

Oral analgesics are considered first line treatment for the relief of pain in a range of diseases. Some common analgesics include paracetamol, Non-steroidal anti-inflammatories, cyclo-oxygenase 2 inhibitors and opioids. However, these do not treat the underlying disease progression and merely provide temporary symptomatic relief from associated pain.

In the case of knee OA, paracetamol has traditionally been recommended as a first line analgesic for pain relief as it is considered a safe analgesic because of the minimal side-effect profile. A recent systematic review and meta-analysis used 12 randomized controlled studies to investigate the efficacy and safety of paracetamol on knee OA pain and other joint associated pain. The investigators found high quality evidence that paracetamol showed a significant, but not clinically important decrease in short term pain associated with knee OA [99]. The authors concluded that because of the minimal benefit of paracetamol, recommendations for its use as a first line analgesic should be reconsidered.

Ibuprofen is another commonly prescribed drug for the treatment of knee OA. It has been shown to be effective and superior to paracetamol for the treatment of pain and inflammation in knee OA [100], [101]. A systematic review of commonly prescribed knee OA therapeutics also showed Ibuprofen had clinically significant improvements in pain and again outperformed paracetamol. The clinical benefits of Ibuprofen however, are curbed by the complication which can occur in the upper gastrointestinal from the use of non-selective non-steroidal anti-inflammatory drugs (NSAIDs). Treatment of patients with orally administered NSAIDs is associated with

a 3-5 fold increased risk of complications such as; peptic ulcer perforation, obstruction and bleeding [102].

NSAIDs are a class of non-selective cyclo-oxygenase inhibitors which possess potent analgesic, anti-inflammatory and antipyretic properties. Moreover, a range of mediators are responsible for driving knee OA associated inflammation and pain. Aside from cartilage-destructive enzymes and cytokines, leukotrienes and prostaglandins (PGs) are two types of mediators produced from the breakdown of arachidonic acid by three enzymes; 5-lipoxygenase, cyclooxygenase (COX)-1 and COX-2 [103]. Non-selective inhibition of cyclooxygenases in the knee joint, reduces inflammation because of a reduction of PGs which cause inflammation and pain in knee OA. However, non-selective inhibition also inhibits COX-1 in the gastrointestinal system, which produce PGs with various physiological functions. PGs in this case are essential for gastric mucosal defense and renal homeostasis. Also in the gastrointestinal system, COX-1 can synthesize, PGE2 and PGI2. Both molecules have a vasodilatory function and protect the gastrointestinal tract by reducing acid and pepsin, and increasing the production of mucus in the stomach [104].

To avoid the complication of orally administered NSAIDs, topical creams of the active drug have been developed. A topical cream containing 5% Ibuprofen investigated for the treatment of knee OA demonstrated both statistical and clinically relevant significance compared to placebo, additionally, and in contrast to orally administered NSAIDs, no drug-related adverse events were recorded [105]. Although the safety profile of topical NSAIDs is far superior to that of the oral route of administration, continued efficacy is compromised. A meta-analysis conducted on randomized clinical trials showed topical NSAID therapy can reduce pain and improve function only for the first two weeks of therapy and that after this period efficacy diminished [106].

Clinical toxicity in the gastrointestinal (GI) tract can increase patient morbidity and mortality rates especially in sub-populations such as the elderly, thus a NSAID without these side effects is highly desirable. Selective COX-2 inhibitors significantly decrease the adverse events associated with traditional non-selective NSAIDs in the upper GI tract [107,108]. However, although GI associated adverse events were reduced, selective inhibition of COX-2 caused other life threatening side effects on the renal and cardiovascular system. The use of selective COX-2 inhibitors increased the risk of myocardial infarction, stroke, heart failure and hypertension [109-112].

Opioids are very potent analgesics used in a wide variety of diseases. The use of opioids for the treatment of pain associated with knee OA can be used in patients no longer responding to first line treatments such as paracetamol and NSAID's. A meta-analysis of randomized controlled clinical trials showed that opioids had clinically significant decreases on OA-associated pain and significant but not clinically relevant improvements on function [113]. The benefits of opioids may however be limited by the adverse events associated with their use. The study reported nausea, constipation, dizziness, somnolence and vomiting as major adverse events associated with opioid use. Further to this, researchers also reported an average treatment discontinuation rate for toxicity at 25% and as high as 31% for stronger opioids.

Systemic pharmacotherapies administered orally are plagued by undesired side effect on patients suffering from knee OA. Topical formulations have an improved profile of adverse events but lack long term efficacy. A more direct route of administration and thus a more targeted approach to the treatment of knee OA is through an intra-articular injection. Intra-articular injection of therapeutics would by-pass first pass metabolism, which may enhance the side-effects profile of pharmacotherapies.

Intra-articular injection of corticosteroids is used to reduce inflammation and pain in knee OA. Studies have shown that although intra-articular injection of corticosteroids relieve pain associated with knee OA, the lowest efficacious dose should be used. This is because at higher doses they have deleterious effects on overall cartilage health, and can cause significant cartilage damage and chondrocyte toxicity [114]. Additionally the effectiveness in the medium to long term is almost non-existent. A recent Cochrane review of corticosteroids in knee OA showed considerable heterogeneity in the effectiveness and that any clinically significant effects experienced at 6 weeks, diminished greatly over time and were non-existent at 13 weeks [115].

Overall, the treatment of knee OA using pharmacological interventions produce relatively small clinical effects. They are usually only effective in the short to medium term at controlling pain [116]. This is because pharmacotherapies do not address the underlying disease progression and merely provide symptomatic relief of pain and inflammation.

Hyaluronic acid (Hyaluronan; HA) is an endogenous polysaccharide found in all tissues and body fluids of vertebrates. HA is a non-sulphated glycosaminoglycan with an anionic charge. HA polymers are comprised of repeating disaccharide units of glucuronic acid and N-acetyl glucosamine joined by alternating (β-1,3 and β-1,4) glycocidic linkages with no protein core.
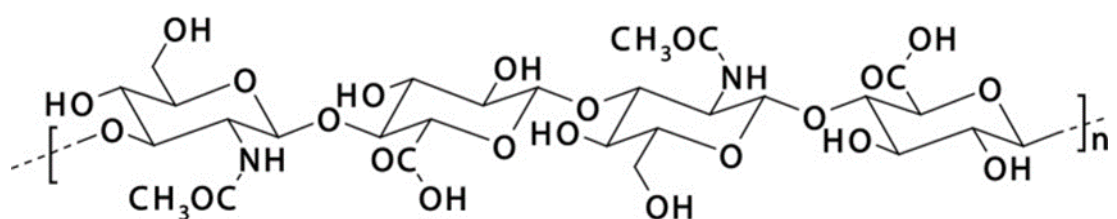
Figure 1.4 Structural Formula of Hyaluronic Acid

A figure depicting the structural formula of Hyaluronic acid. *adapted from [117].

HA is especially abundant in loose connective tissue and a major component of cartilage[118] and synovium[119]. In the knee joint, HA molecules are bound to proteoglycans and are an integral component of the extracellular matrix (ECM) and essential for cartilage integrity. Biosynthesis of HA occurs on the inner surface of the plasma membrane, after which the HA chain are extruded through to the extracellular space. Three HA synthases (HAS 1-3) are bound to the membrane and are responsible for HA synthesis [120,121].

Table 1.3 : Concentration of hyaluronan in tissues and tissue fluids

Tissue or Fluid	Concentration mg/l
Human umbilical cord	4100
Human synovial fluid	1420 – 3600
Human vitreous body	140 – 338
Human dermis	200
Human thoracic lymph	8.5 – 18
Human urine	0.1 – 0.5
Human serum	0.01 – 0.1 **

*Table has been re-produced from [122]; non-human data has been excluded.

**Value may fluctuate according to disease state[123]

By interacting with and absorbing into the articular cartilage surface, HA is the primary lubricating molecule in synovial fluid [124]. The viscoelastic properties of synovial fluid are dependent on HA molecular weight and concentration. Using radiolabeling of synovial fluid, the average molecular weight of HA found in normal synovial fluid of the knee is 6-7 x 10^6 Daltons [125] and compared to patients with pathological conditions of the knee, molecular weight decreased on average to 3-5 x 10^6 Daltons. Normal synovial fluid contains on average 4 mg/mL of HA but this can vary widely and will decrease with age [126]. In pathological conditions of the knee, concentration will decrease below 1 mg/mL HA [127]. At concentrations less than 1 mg/mL, HA molecular chains in solution behave as highly hydrated randomly kinked coils, which start to entangle. This is more commonly known as the entanglement point [128].
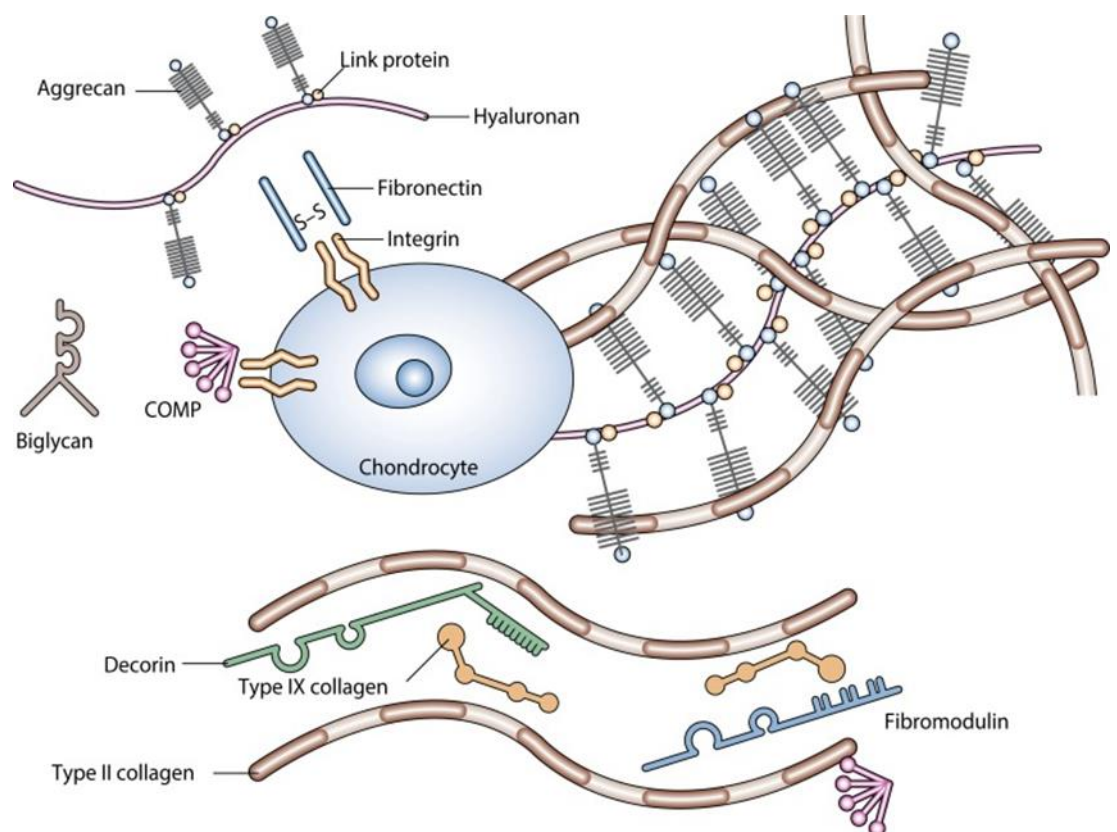
Figure 1.5 Molecular components found in articular cartilage

A schematic depicting the main articular cartilage components and structures. Hyaluronan has no protein core however it is anchored to the chondrocyte via the CD44 surface receptor. Link proteins connect aggrecan to Hyaluronan and further interactions with collagen contribute to the rigidity and viscosity found in vivo. *Image adapted from Chen et al. [129]

HA is an essential and highly viscoelastic molecule found in synovial fluid which greatly reduces shear stress in a normal functioning knee. HA has traditionally been considered to be only involved in processes such as of lubrication, as a structural molecule to support tissues and a general filling/bulking molecule [130]. These assumptions have since changed and aside from its viscoelastic properties, high molecular weight HA can decrease apoptosis, oxidative stress and necrosis [131]. High molecular weight HA can also inhibit the process of angiogenesis by excluding cells and other molecules which are pertinent to vascularisation such as endothelial cells [132,133]. Many studies have demonstrated HA can play a part in immunity. Following biosynthesis of HA, it is extruded through the plasma membrane into the extra cellular space where it is then anchored to the surface of the cell. This forms a peri-cellular halo around the cell, which can act as an exclusion zone to molecules, cells and cellular debris. The function of the peri-cellular halo is unclear, however it may promote cell proliferation by facilitating rounding during mitosis within a hydrated peri cellular zone. Many cell types produce a peri-cellular halo which can exclude immune cells, red blood cells and lymphocyte-mediated cytolysis [134-138, 139]. Studies have determined HA is the core component of the peri-cellular halo by way of HA-specific enzymatic digestion. The peri-cellular halo is maintained in a rigid formation around the cell by the virtue of anionic charge repulsion between the chains. These chains are then anchored to the surface of the cell by binding to specific HA-receptors i.e. CD44 [140, 141, 142].

High molecular weight HA can be endogenously catabolised in part by enzymes known as hyaluronidases (HYALs). There are currently six identified enzymes; HYALs 1-4, HYALP1 and PH20 [143]. The typical biological process of breaking down HA involves CD44, Hyal-1, Hyal-2, β-glucuronidase and β-N-

acetylglucosaminidase [144]. Turnover of HA in the knee occurs very regularly as the half-life is approximately 12 hours in synovial fluid and below five minutes in blood [118]. However in synovial fluid, HYAL activity is barely detectable and because HA undergoes regular cycles of degradation, other non-enzymatic catabolism must occur [145]. The knee joint undergoes a somewhat regular 12 hour cycle of high activity and low-no activity. This correlates to a cycle of hypoxia and re-oxygenation. Thus reactive oxygen species (ROS) have been implicated in the degradation high molecular weight HA. In the mornings and due to increased motor activity in the knee, the synovial fluid is re-oxygenated. Herein the excess oxygen is reduced to hydroxyl radicals (OH) by the presence of transition metals which therefore fragments the high molecular weight HA and leads to reduced viscosity [146]. It is postulated that a reduction in viscosity of the synovial fluid as a result of oxidant-mediated fragmentation, generates feedback which stimulates endogenous synoviocytes to produce higher molecular weight HA. Extracellular superoxide dismutase has a polycationic matrix-binding domain which can inhibit inflammation by significantly inhibiting oxidant-mediated fragmentation of HA [147]. Accumulation of fragmented HA as a result of impaired clearance and turnover, either from tissue injury or oxidant-mediated fragmentation, stimulates the expression of inflammatory genes in macrophages and results in persistent inflammation [148]. HA fragments can also induce inflammatory chemotactic mediators, inflammatory cytokines and inducible nitric oxide synthases in macrophages [149].

Viscosupplementation can be used to treat knee OA. This aims to replace lost synovial fluid with HA to reduce pain and increased mobility through rheological cushioning of the joint. However as the aforementioned highlighted, viscoelasticity of HA at high molecular weights, is not the only mode of action which can influence disease [150]. Commercially available HA viscosupplementation is derived from

rooster comb and is highly cross-linked and with a molecular of 6000KDa. They are commonly referred to as Hylans. A commonly prescribed Hylan, such as Hylan G-F 20 (Synvisc®) can be used in conjunction with NSAIDs for the treatment of knee OA pain [151]. Synvisc is administered to the knee via intra-articular injection. Originally Synvisc was administered by three weekly injections of 2mL, but more recently surgeons have adopted a single injection of 6mL (Synvisc-One®).

Patients suffering from chronic knee OA can benefit from Synvisc. In a randomised clinical trial comparing physiologically buffered saline and Synvisc, the Synvisc group had significantly more patients free from pain during weight bearing at 26 weeks. However, 13% of patients receiving the saline injections were also pain free during weight bearing. This suggests that the intra-articular injection caused a placebo effect [152]. Another study comparing Synvisc to an active control in clinical effectiveness for the treatment of knee OA, Synvisc was shown to be superior for decreasing pain on both the visual analogue scale (VAS) and the Western Ontario and McMaster Universities Arthritis Index (WOMAC). The superiority in clinical effectiveness was more evident in the later time points of 6 and 12 month follow up [153]. In a single-blind parallel control group study researchers compared changes in cartilage volume of Grade 2/3 knee OA patients treated with either 6 monthly intra-articular injections of Synvisc to patients receiving usual care for knee OA without injections. Using MRI to assess the effect of the intervention, they found reduced tibial cartilage loss and lower cartilage defect scores at two years follow-up [154]. In a recent systematic review of 14 meta-analysis' comparing intra-articular injection of HA with conservative pharmacological therapies for the treatment of knee OA, HA viscosupplementation was shown to improve pain and function for up to 26 weeks post treatment [155].

Overall HA viscosupplementation can provide pain relief and increased functionality in patients suffering from knee OA for up to 6 months post-injection. The viscoelastic properties of high molecular weight HA provide added rheological cushioning to synovial fluid in the knee. However as previously demonstrated, exogenous HA is short lived in the knee and has a half-life of approximately 12 hours. HA viscosupplementation must therefore have alternative mechanisms of action in the joint.

HA viscosupplementation may protect chondrocytes in articular cartilage by reducing apoptosis. An investigation into non-pathologically derived primary chondrocytes in culture, capitulated oxidant-mediated damage of articular cartilage in knee OA. They showed when chondrocytes were challenged with hypoxanthine (2 mM) and xanthine oxidase (20-60 mU), or with activated polymorphonuclear leukocytes (PMNs), high molecular HA treatment increased chondrocyte viability and thus played an anti-apoptotic role. HA binding to the CD44 receptor played a pivotal role in chondroprotection for oxidant-mediated damage [156]. Chondroprotective effects of HA viscosupplementation have also been demonstrated in rabbit models of knee OA [157,158]. Moreover interleukin 1-beta (IL-1β) is a major catabolic cytokine involved in the breakdown of cartilage. IL-1β stimulates the production of a range of matrix metalloproteinases (MMPs; i.e. MM1, 3 and 13), which catabolize the extracellular matrix in cartilage [159]. Both healthy and Osteoarthritic derived cartilage can be stimulated by IL-1β to produce cartilage destructive MMPs. This process however, can be interrupted by HA binding to CD44 on chondrocytes within the cartilage [160,161].

Proteoglycans and glycosaminoglycans (GAGs) are essential for cartilage integrity. Proteoglycans make up 10-15% of the wet weight in the extracellular matrix of articular cartilage [20], with aggrecan, biglycan, decorin, and fibromodulin the most

predominant, and having distinct functions in the tissue. Aggrecan is the principal large proteoglycan of cartilage and it propensity to aggregate with endogenous hyaluronan into much larger complexes is essential for the mechanical properties of cartilage [21]. Proteoglycan and GAG concentrations decline within cartilage during OA disease progression. In animal models of OA, intra-articular injection of HA has been shown to stimulate the synthesis of proteoglycans and thus preserve cartilage integrity [162,163]. In explant culture of osteoarthritic cartilage, Interleukin-1 can increase the expression of aggrecanase 1 (*ADAMTS4*). *ADAMTS4* plays a key role in aggrecan degradation. Treatment of cartilage explants with HA can inhibit accelerated aggrecanolysis in OA both by decreasing *ADAMTS4* expression and decreasing aggrecanase activity [164]. Similar results of increased aggrecan were observed in the joint fluid of human patients undergoing regular intra-articular injection of HA [165]. Decreased *ADAMTS4* gene expression in osteoarthritic chondrocytes with HA treatment is mediated by CD44 and Intracellular adhesion molecule 1 (ICAM-1) [166].

The binding of HA to chondrocytes through CD44 reduced the expression of IL-1β [160,161]. IL-1β can induce the secretion of MMPs which can cause extracellular matrix catabolism and a pro-inflammatory cascade of events in the joint [167]. Thus inhibition of IL-1β adds to the anti-inflammatory effect of HA in OA. Additionally, treatment of osteoarthritic-derived subchondral osteoblasts with HA can reduce the levels of pro-inflammatory Prostaglandin E2 (PGE2) and Interleukin-6 (IL-6) [168]. Moreover, during OA bone resorption causes the erosion of the subchondral bone. MMP-13 is plays fundamental role in bone resorption, as well as being a main catabolic enzyme for collagen type II in the cartilage matrix [169]. MMP-13 gene expression is decreased through HA binding to CD44 on subchondral bone osteoblasts [170].

Although HA can reduce pain, increase functionality and may reduce overall disease progression by binding to CD44, OA is a degenerative disease with poor prognostic outcomes. Due to inherent degenerative factors of OA and the limited capacity of conventional therapies to halt disease progression, artificial joint replacement surgery are commonly performed for a large proportion of OA patients.

Surgical interventions which involve knee arthroscopy, have been traditionally used for debridement of mechanical cartilage tares, lesions, defects and lavage of the knee. These are used in conjunction with systemic pharmacotherapies and other physical therapies. However the use of knee arthroscopy has greatly declined in the last decade following a landmark double blind placebo controlled trial of arthroscopic debridement and lavage for OA of the knee [171]. The authors found no clinically meaningful difference compared to the placebo group in the outcome of pain. A subsequent randomized trial also showed arthroscopy had no added benefit to physical and medical therapies [172]. Once patients have reached the end stage of OA a TKR is recommended. This is the final resort and it only considered when all conservative treatment options have been exhausted. A TKR is the final treatment option available for patients with knee OA and as such TKR has been used in observational studies as a measure of knee OA outcome [76]. TKR is not recommended for younger sufferers of knee OA as there is a limited useful lifetime of artificial prosthesis ranging between 10 and 20 years. However, the average time for revision surgery has been reported at 35 months and is most commonly caused by infection, instability and stiffness [173]. For these reasons TKR are often not recommended for patients under the age of 60.

1.5 Alternative biological therapies

There is a general consensus among surgeons that current non-surgical therapies are insufficient for younger OA sufferers who are not candidates for knee arthroplasties [174]. Although current therapies may provide temporary pain relief and increased mobility, there is a pressing need for treatments that slow or stop the degradation of cartilage. As a result of the treatment gap available for younger sufferers of OA, trial are intensifying for emerging alternative biological therapies. Some of these therapies include anti-cytokine therapy using monoclonal antibodies to inhibit key mediators in OA, autologous platelet rich plasma, autologous chondrocyte implantation and mesenchymal stem cell therapy.

Cytokines such as IL-1β and TNF-α play an integral role in degradation of cartilage and worsening prognosis of OA. Anti-cytokine therapies attempt to halt disease progression by targeting molecular mechanism in the breakdown of cartilage. Much like the administration of HA, anti-cytokine therapy is delivered via an intra-articular injection. Anti-cytokine therapy has low systemic bioavailability when administered orally. Thus intra-articular injection enables the active drug to have direct contact with the surface of cartilage which typically is non-vascularized [175]. The disadvantages however, are also typical and have a risk of infection, subjective injection technique, flares associated with repeat injections and rapid elimination of drug via lymphatic drainage.

It is firmly established that IL-1β can induce proteoglycan depletion in cartilage and thus contribute to cartilage erosion and OA progression [176]. Inhibition of IL-1β activity with Interleukin-1 Receptor antagonist (IL-1Ra) protein, traditionally used in rheumatoid arthritis (RA), can reduce the development of osteophytes and cartilage lesions dose-dependently in vivo [177] in animal models of knee OA. Initial human

trials on knee OA patients showed improvements in pain with minimal adverse events related to inflammatory reactions [178]. However in a randomized double blind placebo controlled study on knee OA patients, WOMAC assessment for pain showed there was no significant difference between placebo and treatment [179]. In another randomized, double blind and placebo controlled study, a monoclonal antibody which selectively binds the IL-1R type 1, which inhibits the activity of IL-1α and β was used to treat knee OA. The study was inconclusive for a difference in the WOMAC measures of pain, and showed that the treatment was clinically insignificant compared to placebo [180].

Similar to IL-1β, tumor necrosis factor (TNF-α) plays an essential role in the pathogenesis of OA. Serum concentration of TNF-α in patients with radiographic knee OA correlate to increased cartilage loss [181]. TNF-α can accelerate cartilage proteoglycan degradation in vivo by cleaving aggrecanase sites and inducing aggrecanase-generated aggrecan fragments in synovial fluid [182]. In a rabbit model for knee OA, the use of a chimeric monoclonal antibody for TNF-α, Infliximab, significantly decreased both TNF-α and nitric oxide content of synovial fluid and decreased the extent of cartilage lesions compared with saline injection [183]. A case report on a single patient suffering knee OA used Adalimumab, a fully human monoclonal antibody which neutralizes the biological function of TNF-α by blocking the TNF cell surface receptors. The study showed subchondral oedema that was visible on the initial MRI was almost completely resolved 6 months after treatment [184]. In a randomization, double-blind, placebo-controlled study with 16 patients, a single intra-articular injection of Infliximab was compared to methylprednisolone or placebo. The study showed no significant clinical difference between the treatment groups [185].

The approach of inhibiting catabolic cytokines such as IL-1β and TNF-α, for the treatment of knee OA showed limited success. Other strategies include regeneration of the articular cartilage using growth factors such as Transforming growth factor beta (TGF-β) and Insulin-like growth factor 1 (IGF-1). However rather than manufacturing these as recombinant proteins, growth factors can be derived from endogenous platelets. Platelet rich plasma (PRP) is an autologous platelet concentrate within a plasma suspension. PRP is prepared from blood and contains growth factors, cytokines, other biologically active molecules and may provide rapid healing and tissue regeneration when administered pharmacologically for the treatment of knee OA [186].

One of the major growth factors within PRP is TGF-β1. TGF-β1 contributes to the formation of cartilaginous tissue and increases chondrogenic potential of synovial mesenchymal stem cells [187]. Moreover, the extracellular matrix in articular cartilage is composed of type II collagen which regulated by chondrocytes. An investigation of articular cartilage derived chondrocytes, showed that TGF-β counteracts the catabolic activity of IL-1β on cartilage matrix and increased localisation of type II collagen in chondrocytes [188].

Another component of PRP which has an anti-catabolic effect and essential to articular cartilage integrity is IGF-1 [189]. IGF-1 can act synergistically act with other growth factors such as TGF-β1 and fibroblast growth factor (FGF), by increasing chondrocyte differentiation potential and increasing type II collagen mRNA expression [190]. Articular cartilage explants in culture stimulated with IL-1 and TNF-α increase degradation of proteoglycans, but IGF-1 can counteract this effect by promoting matrix formation by stimulating synthesis of aggregating proteoglycans [191,192]. In human knee OA-derived articular cartilage in culture, IGF-1 has a similar

effect. Although the degradation process cannot be stopped by IGF-1, it can greatly enhance the anabolism of proteoglycans [193].

Growth factors contained within PRP have a positive outcome on osteoarthritic cartilage. In vitro investigations of chondrocyte culture show that PRP can significantly increase the synthesis of collagens and proteoglycans without altering the cellular phenotype [194]. PRP may also be beneficial for the treatment of OA by acting on synoviocytes. Osteoarthritic derived-synoviocytes in culture treated with PRP increase secretion of HA [195]. In a rabbit model of knee OA, PRP was shown to significantly increase GAG synthesis in vivo and limit OA progression [196]. Similarly in a rabbit model targeting osteochondral tissue injury of the knee, showed 12 weeks post injury using micro-computed tomography and histology that neocartilage and newly formed bone was significantly higher in the PRP treated group compared to control [197].

The use of PRP for the treatment of knee OA in humans is underpinned by a large body of evidence at the basic science research level. PRP has been successfully used in a case where a loose chondral body was re-attached to the medial femoral condyle in a young adolescent patient [198]. A retrospective cohort study showed three weekly intra-articular injections of PRP significantly reduced the WOMAC scores in knee OA patients at 5 weeks compared to the same amount of injections of HA [199], others have shown similar results with longer follow up, but note that younger patients achieved better results [200]. It is difficult to ascertain why younger sufferers of symptomatic knee OA achieved better results than the older population. On one side it can be interpreted that they have a lower degree of cartilage degeneration. However it could also be interpreted as less potent growth factor concentration in the older population, or it could be a combination of both factors. In a double blind, randomized, placebo controlled trial of patients suffering from bilateral knee OA,

PRP significantly decreased the WOMAC scores compared to saline injections. The positive effects lasted up until 6 months, but worsened slightly compared to initial improvements. The investigators reported age did not influence outcome but that lower grade had a better prognosis than the higher grade OA [201]. Overall PRP treatment for knee OA provides relief from symptoms in the short to medium term, it may or may not provide more relief to younger sufferers of OA but certainly has improved prognostic outcomes for lower grade OA [202].

The use of alternative biological therapies, such as Adipose-derived mesenchymal stem cells (MSCs), for the treatment of OA is rapidly increasing as a result of a gap in healthcare options for middle age patients [174,203]. Current treatments do not provide a cure or halt OA disease progression but rather provide symptomatic relief in the short to medium term. The use of minimally processed autologous tissue, including adipose stromal vascular fraction therapy is exempt from regulation as a drug in many countries. This exemption has enabled rapid growth of adipose-derived autologous treatments, particularly for conditions such as knee OA.

1.6.1 Adipose tissue: A history of discovery

Adipose tissue is present in all mammals and can be divided into two types: brown adipose tissue (BAT) and white adipose tissue (WAT) based on their visual appearance. BAT is mainly found in newborns and hibernating mammals where it is principally required for non-shivering thermogenesis and is essential to avoid hypothermia [204,205]. The composition of BAT differs from WAT in that it contains a greater number of mitochondria per cell and each cell contains numerous lipid droplets [204]. As newborns develop, it is thought that WAT, the predominant type of adipose tissue and the focus of this thesis, replaces BAT. WAT is generally found in subcutaneous sites between the skin and the muscle but WAT deposits can also be present around organs such as the kidneys and heart [206]. For the purposes of this thesis, WAT will be referred to as adipose tissue.

Adipose tissue is considered the main energy source of the body where it is responsible for lipid synthesis and breakdown [207] whilst also providing thermal and mechanical insulation [54,208]. Consistent with this role in energy storage, up to 85% of the tissue weight of adipose tissue is comprised of triglycerides [207]. Historically, metabolic studies focused on understanding the regulation of the lipogenesis and lipolysis pathways. However, the first indication that adipose tissue was not just a simple energy storage organ stemmed from the discovery in 1987 that conversion of sex hormones into their active form was dramatically increased in obese women [209]. Another study in the same year illustrated a marked decrease in the endocrine factor, adipsin, in obese rodents [210]. This emerging view of adipose tissue as an endocrine organ was then solidified by a study in 1994 which identified and characterized leptin, an important hormone predominately produced by adipocytes, which regulates energy intake and expenditure [211].

These key discoveries have led to the view that adipose tissue is an important endocrine organ, which expresses and secretes an array of bioactive factors. Furthermore, adipose tissue can communicate extensively with other tissues and organs through the autocrine, paracrine and/or endocrine signaling activities of the secreted bioactive factors. Adipose tissue is now known to be involved in a multitude of processes including lipid metabolism, angiogenesis, insulin sensitivity, appetite and energy balance, blood pressure regulation and inflammation [208]. An increase in the incidence of human obesity and the discovery that adipose tissue signals to other tissues and systems through secreted factors has culminated in this tissue becoming a focus of many research groups.

Mature adipocytes are the characteristic cell type associated with adipose tissue. The adipocytes are surrounded by a connective tissue matrix which provides the structural framework to the tissue [212]. Adipose tissue is highly innervated and contains an intricate microvasculature network. In fact, adipose tissue contains such a dense network of capillary beds, that it has been reported that each adipocyte is in contact with multiple capillaries [213]. Apart from adipocytes, there are a number of other cell types associated with these capillary beds. Using the readily available collagenase enzyme, the connective tissue can be broken down, releasing the cells contained in adipose tissue. The digestion of this tissue and subsequent centrifugation results in floating adipocytes and a pellet containing a mixed population of cells, collectively termed the stromal vascular fraction (SVF). The SVF is comprised of white blood cells (leukocytes, T cells, B cells, mast cells, granulocytes and monocytes), endothelial cells, vascular smooth muscle cells/pericytes, hematopoietic (HSCs) and mesenchymal stem cells (MSCs) [214-216]. In 2007, Varma et al., performed a study to define the proportion of the different cell populations in cryopreserved human SVF samples using CD marker characterisation and flow cytometry [214]. This characterisation revealed that a large proportion (34.60

± 17.80%) of the SVF had MSC cell surface markers expressed on their surface and that collectively white blood cells comprise 31.06 ± 12.20% of the SVF [214]. The characteristics of each cell type present in the SVF as well as the adipocytes will be discussed in turn.

Mature adipocytes comprise a large proportion (approximately 50% of the total cellular content) of adipose tissue. Adipose tissue also contains preadipocytes. It is thought that these preadipocyte cells, in response to an increased need, can accumulate lipid resulting in the terminal differentiation of these cells into mature adipocytes [217]. Adipocytes have been shown to range in both size (25-190 μm in diameter) and volume [218]. Adipocytes store almost pure triglycerides in quantities of greater than 95% of their total cellular volume [219] and consequently are involved in regulating the lipogenesis and lipolysis pathways. During lipogenesis, adipocytes re-esterify free fatty acids and glycerol to produce triglycerides which are stored as excess energy in unilocular droplets inside the adipocytes [206]. Adipocytes also contain lipases that convert the stored triglycerides back into glycerol and fatty acids. These breakdown products are then transported via the blood to sites such as the liver and muscle to be used in fatty acid oxidation for energy production [206]. Consequently, adipocytes respond to acute changes in nutritional cues including hormonal (eg. insulin) and sympathetic (eg. adrenergic) stimulation [206]. This storage and release of energy for the body has long been considered the primary role of adipocytes. However, following the discovery of leptin production by adipose tissue, adipocytes have been shown to produce a number of cytokines, chemokines and other biologically active molecules, collectively referred to as adipokines.

1.6.2 The stromal vascular fraction

The immune system consists of lymphoid organs, cells, humoral factors and cytokines that work together to protect the host [220]. The immune system is a complex network consisting of multiple cell types and intricate positive and negative feedback loops, regulating the production, proliferation, activation and activities of immune cells [221]. Collectively, white blood cells comprise 31.06 ± 12.02 % of human SVF with differing levels of T cells, B cells, mast cells, granulocytes, monocytes and leukocytes present [214].

The characterisation of the SVF by Varma et al., (2007) revealed a population (10.30 $\pm$ 9.90%) of CD34+CD31−CD146+ cells [214]. Consistent with other studies, these cells were categorised as vascular smooth muscle cells (VSMC) and pericytes, collectively referred to as mural cells [222,223]. Pericytes and VSMC are thought to belong to the same cell lineage. However, they exhibit different morphologies and marker expression, as well as residing in different locations relative to the endothelium.

VSMC are highly specialised cells that constitute the main bulk of the vascular wall, the media, and are therefore physically separated from the basement membrane. There is significant heterogeneity between VSMC, however most VMSC exhibit a contractile phenotype and express specific contractile, signaling and cytoskeletal proteins [224,225]. The main function of these cells is to maintain the vascular tone and resistance [224] and consequently they control the diameter of the blood vessel to avoid hyper- or hypo-tension. However, these cells are also involved in repair of the blood vessel wall in response to environmental stimuli following vascular injury [224,226].

Pericytes are located within the endothelial basement membrane. In cases where there is no basement membrane, unique endothelial-pericyte cell contacts have been reported [227]. Pericytes cover between 10-50% of the abluminal vessel area of the endothelium depending on the type of vessel. Pericytes have several long processes

extending from their cell body that embrace the abluminal endothelium wall [228]. Consequently, as a single pericyte is commonly in contact with multiple endothelial cells, facilitating and integrating cell communication is thought to be the primary role of pericytes [229]. Furthermore, pericyte processes can extend to more than one capillary in the vasculature [228]. As a result, it is thought that pericytes may be responsible for integrating and coordinating endothelial cell responses in adjacent blood vessels through both cell-cell contact and paracrine signaling. Pericytes are also involved in regulating the development, stabilisation, maturation and remodeling of the vasculature and are thought to play a role in vasculature repair processes [229]. A study by Crisan et al., (2008) investigating the analysis and purification of cells that comprise blood vessel walls demonstrated a striking similarity in terms of CD marker profiles and differentiation potential between pericytes obtained from a variety of tissues and MSCs [222]. This led to the suggestion that MSCs are a subset of pericytes [230].

VSMCs and pericytes are able to differentiate into each other in conjunction with blood vessel growth and remodeling [231] and therefore, the distinction between pericytes and VSMC is not considered absolute. Instead, a "continuum of phenotypes ranging from the classical VSMC to the typical pericyte" is thought to exist throughout intermediate to small blood vessels [229]. It is likely that moderate levels of VSMC/pericytes are present in the SVF due to the nature of excising and digesting the fat and therefore the associated network of blood vessels.

The number of cells characterised in the SVF by Varma et al., (2007) as endothelial cells was relatively variable at $12.2 \pm 9.5\%$ [214]. These cells are a major cellular constituent of blood vessels and are located at the interface of the blood and underlying tissues. This positioning allows endothelial cells to control the trafficking of molecules and cells across the blood vessel walls. Furthermore, endothelial cells are actively involved in maintaining hemostasis, regulating vascular tone, immunity

and inflammatory processes. Endothelial cells achieve their functions through the use of both specialised transcellular systems and controlling cell-cell junctions [232,{Stevens, 2000 #1233]. Similar to VSMCs and pericytes, the most likely explanation that this cell type appears in the SVF is due to the disruption of the blood vessels during adipose tissue excision and digestion

Both HSCs and MSCs have been identified in the SVF. However, the characterisation by Varma et al., (2007) revealed that a relatively low percentage of the SVF was comprised of HSCs (5.97 ± 1.43%) in comparison to MSCs (34.6 ± 17.8%) [214].

1.6.3 Adipose-derived mesenchymal stem cells

MSCs are found in a variety of adult tissues including heart [233], synovial tissue [234], adipose tissue [235], skeletal muscle, spleen, thymus, brain, bone marrow, pancreas, liver, kidney and lung [236]. Prior to the Zuk et al., (2001) publication, bone marrow was considered the most accessible source of MSCs for research and therapeutic use [235]. However, as there are relatively low numbers of MSC in bone marrow [237,238], ex vivo expansion is necessary to increase cell numbers for research or therapeutic use. It now recognised that adipose tissue is a rich source of MSCs.

A number of studies on the comparison between MSCs derived from bone marrow and adipose tissue have been published [239]. These studies have demonstrated that bone marrow and adipose-derived MSCs are phenotypically similar but have some functional differences. In particular, they possess differences in their CD marker expression profiles, in vitro growth rates and differentiation capacities. Bone marrow-derived MSCs were found to express high levels of the stromal CD marker CD146 and readily differentiated into osteoblasts [240]. In contrast, adipose-derived MSCs expressed low levels of CD146 and readily differentiated into adipocytes [240]

and have a greater tendency to differentiate into muscle cells and cardiomyocytes [241]. Furthermore, microarray analysis revealed differences in gene expression levels with bone marrow-derived MSCs expressing high levels of gene involved in bone formation such as Wnt and MAPK signaling, whereas adipose-derived MSCs expressed genes responsible for fatty acid metabolism [240]. The functional differences observed between bone marrow and adipose-derived MSCs are likely due to the different microenvironment where these cells reside [242]. Despite these differences, the finding that adipose tissue contains MSCs at high frequency, coupled with the fact that large quantities of adipose tissue can be easily obtained via routine liposuction procedures, makes this tissue an attractive alternative source of cells for research and therapeutics.

The international Society for Cellular Therapy released a position statement in 2006 outlining the criteria for a cell to be designated a MSC [243]. Firstly, the cell has to display plastic adherence in vitro. Secondly, the cell must express CD73, CD90 and CD105 on its surface and lack expression of the hematopoietic surface markers, CD34 and CD45. Lastly, the cell must be capable of differentiating into various cells of the mesenchymal lineage. Differentiation is achieved by maintaining the MSCs in normal in vitro culturing conditions followed by treatment with media supplemented with defined chemical constituents optimized to turn these cells into cells of the desired lineage. Historically, as the name suggests, MSCs have been shown to differentiate into cells of mesenchymal origin: bone, cartilage and fat. MSCs have also been shown to have the capacity to differentiate into cells of non-mesenchymal origin such as neurons [244] and hepatocytes [245].

Historically, the focus of MSC research has been on their ability to differentiate into cells of mesenchymal origin. The central hypothesis of their therapeutic effect was that the cells would regenerate damaged tissue by engrafting and then differentiating into cells of that tissue type. This view emerged from the established function of

HSCs repopulating the cellular components of the blood following myeloablation of the bone marrow during cancer treatment regimes. However, the last decade has seen a drastic shift in focus in MSC research away from differentiation and to an increasingly clear understanding that autocrine, paracrine and endocrine effects are the primary drivers of the therapeutic effects of MSCs. It is now known that MSCs, through their various secreted factors, possess potent immuno-modulatory properties as well as angiogenic, anti-apoptotic and anti-scarring properties.

Cultured MSCs are usually found to be immuno-privileged after in vivo implantation. They express low levels of major histocompatibility complex (MHC) class I molecules and lack expression of MHC class II and co-stimulatory (CD40, CD80 or CD86) molecules [246,247]. Furthermore, MSCs do not evoke proliferation of allogeneic lymphocytes [246,248], nor are they subjected to cytotoxic T cell mediated lysis [248]. This immuno-privileged nature of MSCs is an attractive property for their therapeutic use, in particular for allogeneic transplantation. In animal models the use of xenogeneic transplants enables the cells and potentially their secretions to be readily tracked and identified. This approach has been widely adopted with the implant of human cells into animal models. The consequence of these allo and xeno studies is a reasonably clear understanding of the immuno-modulatory properties of MSCs in vitro and in vivo.

In vitro studies have demonstrated that MSCs have the capacity to modulate the activities of numerous immune cells including T cells, dendritic cells, B cells and natural killer cells. Collectively, these studies illustrated that MSCs have the ability to suppress immune cell proliferation, activation, maturation, secretion capabilities and their differentiation. The other important immuno-modulatory mechanism of action of MSCs is their ability to induce peripheral T cell tolerance by suppressing T cell proliferation and inducing T regulatory cell production [249]. The immuno-privileged nature of MSCs and their vast immuno-modulatory properties have made

them an attractive therapeutic to dampen immune-mediated disorders and transplantation rejection. Consequently, the immuno-modulatory properties of MSCs in vivo, in particular in models of autoimmunity, tumour immunity and alloreactive immunity to organ and stem cell transplantations, have been investigated. The results of these studies confirmed the in vitro data indicating that MSCs have the ability to modulate the immune response and effectively switch the response from tissue destruction to a protective response.

There are numerous routes for administration of MSCs to examine their effects for the treatment of a specific disease. However, site-directed and systemic delivery routes are the most commonly investigated. Numerous studies have shown that MSCs engraft following introduction at the site of injury or disease. For example, local injection of MSCs into infarcted hearts can regenerate the damaged tissue and restore function to the heart [250]. Additionally, Hofstetter et al., (2002) have demonstrated that delivery of MSCs into the spinal cords of recently rendered paraplegic rats promoted the regeneration and subsequent recovery of the injury [251]. However, where some sites for MSC administration may be easily accessible such as intra-articular injection into the knee joint, it may not also be possible or feasible to introduce cells to the site of injury, inflammation or disease. In these cases, intravenous (IV) introduction of MSCs is an attractive option providing the introduced cells travel to the specific site to be treated and do not just get trapped in the lungs. Animal models of myocardial infarction and brain injury in particular, have been utilised to investigate whether MSCs have the ability to home to sites of injury and inflammation following IV administration [252-255]. The results of these studies demonstrate that although a proportion of MSCs do get trapped in the lungs following IV administration, MSCs home to sites of injury and/or inflammation in the animal models investigated. Although the mechanism behind the ability of MSCs to home to sites of injury/inflammation is not well understood, it is likely that the

injured tissue expresses or secretes molecules that facilitate the trafficking, adhesion and infiltration of MSCs to damaged tissue. The ability of MSCs to home to sites of injury/inflammation enhances their attractiveness as a therapeutic for a variety of diseases.

The number of cells of each lineage in the body is controlled by maintaining the balance, or cell homeostasis, between cell proliferation and apoptosis [256]. Apoptosis, or programmed cell death, is essentially a regulated suicide pathway which is triggered by a variety of extrinsic and intrinsic stimuli [256] and references therein. Apoptosis can be induced to eliminate cells that have been produced in excess, cells that have not developed properly or cells that have sustained damage [256]. In vitro and in vivo studies have demonstrated that in response to tissue injury such as UV irradiation of cells or ischemic reperfusion injuries, MSCs secrete anti-apoptotic factors that reduce cell death in the surrounding areas [257,258]. These anti-apoptotic properties together with the angiogenic properties of MSCs, suggest that following in vivo implantation, MSCs are able to limit the field of injury and allow repair and regeneration of the damaged tissue.

There is now a wealth of literature published on the properties and mechanisms of action of MSCs in both in vitro and in vivo settings. The original view of the therapeutic potential of MSCs was based on the established ability of HSCs to repopulate the cellular components of the blood following myeloablation of the bone marrow during cancer treatment regimes. This led to a thorough investigation into the ability of MSCs to differentiate into different cell types and their subsequent therapeutic use. However, this ability of MSCs to differentiate into cells of a target tissue type is no longer considered to be the major mechanism of action of these cells therapeutically. Instead, the current view of the therapeutic effect of MSCs centers around their ability to home to the sites of injury/inflammation, secrete a variety of

factors which have immuno-modulatory, angiogenic, anti-apoptotic and anti-scarring properties, and their ability through their secretions to activate the endogenous cells to repair and regenerate the damaged tissue. Consequently, the secretions of MSCs are thought to be the main driver of their therapeutic effect.

The proteins secreted by a cell, tissue or an organism are an important class of proteins, which control and regulate a multitude of biological and physiological processes. The set of proteins secreted by a cell, tissue or an organism are collectively referred to as the secretome [259]. The secretome can contain proteins involved in determining, controlling or coordinating a number of biological processes. These include cell processes (cell growth, division, differentiation and apoptosis), development, the immune response (cytokines and anti-microbial agents), blood coagulation, tumorigenesis, and the extracellular matrix [260,261]. Furthermore, the secretome of a cell, tissue or organism can rapidly change in response to a given environment. It is now recognized that secretions are a major component of cell-cell signaling. It has recently been identified that extensive paracrine signaling occurs between cells through the horizontal transfer of microvesicles [262]. Microvesicles are circular fragments of membrane which are released from most cells through either a shedding vesicle via budding of the cell plasma membrane, or as exosomes derived from the endosomal membrane compartment [263]. Microvesicles can contain mRNA, microRNA, transcription factors and proteins from the cell of origin. These microvesicles can then mediate cell-cell communication through surface-expressed ligands that directly active the target cell, by transferring receptors between cells, delivery of proteins to the target cell or transfer of genetic material to the target cell [263]. Further research into the content and actions of these microvesicles is likely to reveal the extent of their influence on the functioning of recipient cells.

Inflammation is a complex, tightly regulated and highly orchestrated response by numerous cell types and their secreted factors to various stimuli including invading pathogens, cell or tissue damage, and irritants. The 5 classic signs of an inflammatory response are heat, swelling, redness, pain and loss of function. Mounting an inflammatory response is critical for host survival during pathogenic infections and injury. For example, inflammation is critical for wound healing as cytokines and growth factors regulate the recruitment, proliferation and differentiation of a variety of cells in conjunction with promoting the synthesis and degradation of the extracellular matrix [264]. Furthermore, blocking inflammation with glucocorticoid treatment inhibits wound healing [265].

Normally, the inflammatory process is tightly regulated and involves a balance between the components which initiate and maintain inflammation (pro-inflammatory or Th1 response) and the mediators which shut the inflammatory process down (anti-inflammatory or Th2 response) [266]. The most important factor regulating Th1 and Th2 responses is the cytokine milieu of the immune response. There are complex positive and negative feedback loops governing the production of secretions from the cells of the immune system. But an appropriate immune response to invading pathogens/tissue injury involves a fine balance between the Th1 and Th2 responses, thereby promoting removal of the invading pathogen/damaged cells and repair of the injured tissue with minimal detrimental impact to the host. In general, the cytokine network is self-regulating due to the existence of opposing cytokines, soluble cytokine receptors, and antagonists binding to receptors thereby blocking the actions of that particular cytokine [267]. However, in some cases an imbalance between Th1 and Th2 develops, resulting in a state of chronic inflammation involving uncontrolled inflammation and consequently tissue damage.

Cytokines have been defined as "regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of cytokines include numerous effects on cells of the immune system and modulation of inflammatory responses". It is now recognised that an inflammatory response is initiated, maintained and resolved by the complex interplay of cytokine networks [268]. Cytokines are 8 – 60 kDa polypeptides, proteins or glycoproteins that can act at the autocrine, paracrine and/or endocrine level [269,270]. There is a significant level of redundancy in the biological activities of each of the cytokines [271,272]. In some cases, physiological levels of cytokines are very low but increase dramatically in response to tissue disease, injury or repair [269]. Generally, cytokines are defined as pro- or anti-inflammatory based on their primary and downstream activities during the response to invading pathogens, disease or tissue injury. The balance between these pro- and anti-inflammatory cytokines during inflammation is imperative for mounting an appropriate immune response without unnecessary uncontrolled inflammation resulting in detrimental effects to the host. Interferon gamma (IFN-γ), IL-1β and tumour necrosis factor alpha (TNF-α) are considered to be the major pro-inflammatory cytokines, with IL-8 and IL-12 also playing important roles. In contrast, Interleukin 1 receptor antagonist (IL-1Ra), IL-4, IL-10 and IL-13 are considered to be the main anti-inflammatory cytokines. Under certain circumstances, cytokines may display both pro- or anti-inflammatory activities. IL-6 is one of the cytokines which exhibits this dual role.

Chemokines are a family of small proteins, generally 8-10 kDa in size, characterized by the conservation of four cysteines in their protein structure [273,274]. They are divided into sub-families based on the relative position of their cysteine residues. Chemokines, or chemotactic cytokines, direct leukocyte movement in development, homeostasis, and during inflammation. They are essential for inflammation as their

primary role is to attract immune cells to the site of infection or injury. Chemokine production is dramatically increased during infection and in inflammatory states in response to pro-inflammatory cytokine release by a variety of cell types. This produces a concentration gradient of chemoattractant molecules that directs the immune cells to the site of infection/inflammation. Furthermore, the presentation of chemokines by endothelial cells stimulates rolling and migration of the leukocytes through the endothelium [275]. However, a role for chemokines in other aspects of the inflammatory process, such as fibrosis, tissue remodeling and angiogenesis, has also been described [276]. Over 40 chemokines have been identified to-date [273].

During the inflammatory response, growth factors are produced by a variety of cell types. The release of growth factors during infection/inflammation results in the recruitment/activation of cells required for tissue remodeling. Fibroblasts, epithelial cells and vascular endothelial cells in particular, are recruited to the site of infection/inflammation [277]. Here they commence the repair/tissue remodeling phase by proliferating, synthesising extracellular matrix components, promoting initial scar tissue formation, stimulating new capillary formation and driving tissue remodeling [278]. Therefore, growth factors are involved in regulating inflammation whilst stimulating tissue repair.

Pericytes are highly specialised cells that belong to the same cell lineage and reside in the blood vessel wall. Pericytes have been implicated in repair of the blood vessel wall in response to environmental stimuli following vascular injury [224,229,245]. Therefore, it is not surprising that these cells are capable of secreting large quantities of growth factors including bFGF, PDBG-bb, and VEGF [279,280]. IL-6 production by pericytes [281] has also been demonstrated. Furthermore, a study by Edelman et al., (2007) demonstrated that pericytes possess the ability to produce pro-inflammatory cytokines such as TNF-α and IL-1β [281]. Given that MSCs are thought to be a subset

of pericytes, it is feasible that pericytes contain the same secretion capabilities as MSCs.

MSCs have a broad range of therapeutic properties, which include their ability to modulate the immune system as well as their trophic functions, specifically, angiogenic, anti-apoptotic and anti-scarring actions. As discussed above, MSCs exert their therapeutic effects through their ability to home to sites of inflammation, embed and control the micro-environment via paracrine signaling. MSC secretions include, pro- and anti-inflammatory cytokines, chemokines and growth factors, such as VEGF, bFGF, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-6, MCP-1, MIP-1α, MIP-1β, RANTES, eotaxin, IL-8, inducible protein 10 (IP-10) and IL-1Ra see [282] and references therein.

1.6.4 MSC therapy in osteoarthritis

One of the first sources of MSCs sources to be isolated came from bone marrow. Bone marrow and adipose tissue are the two most commonly used tissue targets as a source of MSCs. In a comparative analysis between adipose and bone marrow-derived, bone marrow-derived MSCs may be inferior to adipose-derived MSCs, in terms of clinical feasibility. It was shown adipose tissue had the highest concentration of MSCs and a greater proliferative capacity when compared to bone marrow-derived MSCs [239]. In addition, access to adipose tissue is minimally invasive and does not require drilling into the bone, which makes adipose derived-MSCs a favourable source of MSCs in emerging alternative regenerative therapies.

Although MSCs can differentiate into mesodermal lineages, differentiation is not the primary mode of action and *in vitro* animal models and human data have demonstrated that a complex set of secretions drive anti-inflammatory and regenerative effects that have been observed [242]. MSCs secrete trophic factors with substantial potential in regenerative medicine. Transplanted MSCs exert their therapeutic effects through secreted trophic signals [283]. The majority of transplanted MSCs become trapped in the liver, spleen and lungs with less than 1% reaching the target tissue. The Mammalian MSC secretome has been found to contain growth factors, chemokines, angiogenic, anti-inflammatory, pro-inflammatory and pleiotropic cytokines and a plethora of bioactive cell signaling molecules [284]. Thus, the proteomic profiling of MSC conditioned culture medium for clinically relevant factors is imperative as these will become more and more relevant in human regenerative medicine.

In an investigation of the MSC secretome, MSCs stimulated with TNF-α secreted anti-inflammatory factors which in turn could be used to modulate inflammatory markers in human OA-derived cartilage and synovium. In synovium, the MSC

secretions reduced gene expression of IL-1β, MMP-1 and MMP-13. In cartilage the MSC secretions reduced expression of aggrecanase-2 (ADAMTS-5) and decreased the production of catabolic NO by cartilage explants [285]. Neocartilage formation is stimulated by the trophic effect of MSCs acting on chondrocytes and not by differentiation into chondrocytes. This is evident in pellet co-cultures of MSCs and chondrocytes in which cartilage matrix production increased by chondrocytes as measured by increased GAGs [286]. MSCs also have a chondroprotective role when chondrocytes are exposed to camptothecin-induced apoptosis. Chondrocytes become increasingly hypertrophic with increased pathology. The MSC secretome in co-culture of MSCs and osteoarthritic chondrocytes, decreased gene expression of both hypertrophic and fibrotic markers [287].

Primary culture of MSCs from adipose tissue involves the enzymatic digestion of lipoaspirate to obtain the SVF. To further support the notion that the mode of action of MSCs is secretion driven, investigators have shown that using conditioned culture medium alone, without the addition of cells can reduce inflammation in mouse model of OA. Secretions harvested from SVF/adipocyte cultures reduced the clinical arthritis score in a collagen antibody-induced arthritis model [288].

Moreover in a hemi-meniscectomy model of OA, human MSCs injected into rats inhibited OA progression by increased expression of rat type II collagen and promoting meniscal regeneration as observed through histopathological analyses. The investigators reported however, that injected human MSCs could not be detected 7 days post injection [289], this may have been a result of the host immune system recognising the cells as foreign to the system and clearing them.

In a caprine model of OA, injury was induced by complete excision of the medial meniscus and resection of the anterior cruciate ligament unilaterally. MSCs were injected 6 weeks post-injury and although no effect was observed on the anterior

cruciate ligament, MSCs stimulated regeneration of the meniscal cartilage and delayed OA progression by reducing articular cartilage degeneration, osteophytic remodeling and subchondral bone sclerosis [290]. In the adult mini pig, investigators created partial cartilage defects in the medial femoral condyle. Autologous MSCs were injected into the knee and 12-weeks post-injection showed neocartilage formation occurred using histopathological observation. An important limitation of this study as concluded by the authors, was that regeneration may have been limited by a dose-dependent response to the number of MSCs injected [291].

Early autologous cell therapy fell within a medical exemption in many countries and was free from regulation as a drug. This enabled the rapid growth of therapies such as adipose-derived autologous treatments especially for orthopedic diseases such as knee OA [292]. The adipose SVF secretome contains bioactive molecules which are anti-inflammatory, anti-fibrotic and immunomodulatory [293,294]. The secretions alone, without the inclusion of cells could reduce the clinical arthritis score in a mouse model of collagen antibody-induced arthritis model [288]. MSCs within the SVF may communicate, stimulate or mobilize endogenous cell populations [294,295]. Moreover, when the SVF is co-cultured with natively isolated adipocytes, the secretion profile is enhanced, with respect to an increase in known anti-inflammatory cytokines and significantly higher amounts of growth factors in OA as a result of cellular cross-talk [296].

Initial trials using adipose derived SVF indicated good clinical feasibility because of the minimal occurrence of adverse events and ease of isolation of adipose tissue. A single arm safety and feasibility trial using just the SVF showed by injecting approximately 14 million nucleated cells into the knees of OA patients, significant reductions in pain at 3 months and 1 year follow-up were observed using WOMAC

[297]. However in the absence of a suitable control group the placebo effects cannot be ruled.

The SVF with the addition of adipocytes was used in one of the earliest double blind placebo controlled clinical trials for the treatment of knee OA with adipose derived cell populations. The study proved inconclusive on the primary outcome of pain [298]. However significant difference were observed in the secondary biomarker outcomes which further support the paracrine mechanism of action for MSC-SVF therapy. The treatment group significantly decreased serum levels of Macrophage migration inhibitory factor (MIF), a classic pro-inflammatory cytokine observed and correlated with severe knee OA [299,300] and stabilized C-terminal crosslinking telopeptide of type II collagen (CTX-II) in the treatment group but continued to increase in the placebo group, a urinary biomarker used to assess cartilage degradation [301]. The investigators reported an average of 55 million nucleated cells isolated from 400 grams of fresh lipoaspirate was used in the treatment. However the MSC population make up less than 10 percent of freshly isolated SVF from adipose tissue [302].

There is obvious heterogeneity between trial designs, but both studies showed a positive outcome on the treatment of knee OA. Nonetheless, autologous SVF therapy, may ultimately become redundant in the coming years. This is because of a lack of control on the amount/dose of MSCs being injected in the SVF suspension and because it is the MSCs which will provide the therapeutic effect, more advanced isolation methods are required. Additionally in the interest of commercialization, SVF therapy requires a multistep labor-intensive process which may pose the risk of bacterial contamination [298] and cannot be sold as a reproducible and batch controlled off-the-shelf product thus making it difficult to commercialize.

Culture purified MSCs, whereby the SVF is cultured in vitro, has particular advantages in that the process of culture can be standardized, cells can be batch

tested for quality, clinical-grade MSCs can be produced in line with current good manufacturing practices which ensure the safety, quality and molecular characteristics of MSCs [303]. The process of manufacturing off-the-shelf MSC therapeutics for the treatment of knee OA, is firmly hinged on the immune-privileged status of MSCs which means they will not be rejected by the host upon transplantation [304].

The fundamental feature of Adipose-derived MSCs, is that they are immune-privileged i.e. they are hypo immunogenic. MSCs do not express major histocompatibility complex class II antigens on the cell surface, thus allowing MSCs to evade the host immune system [243]. In addition to evading the host immune system, MSCs are immunomodulatory and thus can suppress the immune system. MSCs secrete cytokines which suppress the proliferation of peripheral blood mononuclear cells in culture and inhibit monocyte differentiation toward immature dendritic cells via induced expression of indoleamine 2,3-dioxygenase (IDO) and increased secretion of Interleukin-10 (IL-10) and IL-6 [293]. MSCs can also lower the activation of lymphocytes from a range of stimuli suppress the proliferation of both subsets of T-cells via the production of PGE2 [305,306]. Moreover Galectin-1 is highly expressed in MSCs, can be detected on the cell surface and can be secreted by MSCs [307]. Galectin-1 can reduce the proliferation of peripheral blood mononuclear cells and modulates the anti-inflammatory cytokines which are secreted by the them, such as interferon-gamma (IFN-ɣ) [308].

Allogeneic transplantation of MSCs for the treatment of knee OA will likely be the model In the future. As with pharmacological interventions the effect of MSCs is dose dependent; especially for achieving neocartilage formation and halting knee OA progression humans. In a trial of four geriatric patients with severe knee OA, cultured MSCs were injected at a dose of 9 million cells unilaterally into the more

severely affected knee whilst the healthier knee served as a control. After 5 year follow-up walking time, stair climbing, gelling pain, patella crepitus, flection contracture and the visual analogue score on pain were all better than baseline and that the healthier control continued to deteriorate until it became the worse knee. The authors concluded intervention earlier in age and severity of disease progression may have produced a better outcome [309].

In phase 1/2 proof of concept clinical trial of grade 3-4 knee OA patients with a mean age > 60 years, Jo *et al.,* showed using a dose-escalation design of cultured expanded adipose-derived MSCs could successfully regenerate cartilage and halt disease progression in high the high dose group. The study consisted of three dose groups; 1×10^7, 5×10^7 and a high dose group of 1×10^8 MSCs. At 6 month follow-up only the high dose group significantly decreased the primary outcome of pain as measured by WOMAC achieving a mean reduction of 36%. Additionally in the high dose group at 6 month follow-up, significant reduction of cartilage defects were observed by MRI in both the medial and lateral knee compartments of the femoral and tibial condyles. Further evidence of cartilage defect reduction was supported through second look arthroscopy, which showed cartilage defects in the medial compartment decreased significantly by 64% and regeneration and neo-formation of hyaline-like cartilage were observed. No patients in the high dose group experienced any serious adverse events [310].

1.6.5 Techniques for measuring cellular secretions

The ability to identify and measure the quantity of bioactive factors secreted by cells is an important component in understanding cell-cell communication and the role these bioactive factors play in the therapeutic effect of cellular therapy. Cytokines secreted by MSCs in soluble MSC conditioned medium are present in low picogram per milliliter concentrations and therefore be easily masked by serum proteins contained in cell growth media at the point of quantification. Classical proteomic identification such as mass spectrometry based techniques are therefore less desirable as they are critically sensitive to high abundance proteins contained in serum. Whilst there are new techniques such as selected reaction monitoring (SRM, also known as multiple reaction monitoring) being developed and validated for the quantitation of secreted proteins, the most commonly employed approach to measure bioactive factors is immunoassays such as direct enzyme-linked immunosorbent assays (ELISAs).

SRM is a targeted quantitative mass spectrometry technique. In contrast to conventional mass spectrometry techniques designed to detect all peptides in a given sample, SRM aims to detect only the peptide/s of interest. The peptide mixture is analysed using a scanning mass spectrometer and restricting the acquisition mass range to the mass to charge ratio of the ion/s of interest (Lange et al., 2008). Whilst SRM is a promising technique, developing these assays for target analytes requires significant optimization and subsequent validation to identify suitable transitions (pairs or peptide precursor ion and fragment ion mass masses) unique to the peptide/protein of interest (lange et al., 2008). Advantages – does not require antibodies, therefore could be applied to any protein even those where antibodies are unavailable or difficult to obtain.

Direct ELISAs rely on antibodies specific for the target analyte. In the case of a sandwich ELISA, the analyte is 'sandwiched' between a primary antibody used for

its capture and an enzyme-linked secondary antibody, which serves as the detection antibody. Addition of a substrate for the enzyme conjugated to the secondary antibody allows for quantitation of the amount of target analyte present through either colorimetric or fluorescent detection means. ELISAs are typically designed to Limitations of such immunological techniques however, are that only defined analytes which have been chosen by the user are detected. Thus the involvement of molecules not used in the assay are omitted.

However, in recent years, multi-plex immunoassays have been developed to enable the measurement of multiple analytes of interest in a complex sample within a single assay. This approach utilizes Luminex xMAP technology, which involves a set of uniquely coloured beads that may also be magnetic. Antibodies to the analytes of interest are conjugated to a unique coloured bead. The secondary antibody and a reporter ion are subsequently added. These complexes are analysed using a flow cytometry fitted with two lasers. The first laser detects the uniquely coloured bead and therefore the antibody to the target analyte of interest. The second laser detects and quantifies the amount of reporter ion bound to the complexes. This approach enables up to 100 proteins to be quantified in a single sample in a single assay. Multiple companies now offer disease or pathway specific multi-plex panels. This technology therefore has many advantages over the conventional single-plex ELISA approach. In particular, multi-plex assays allow for large numbers of proteins to be detected in a small sample volume (50 μL) in a more cost and time efficient manner.

All data on cytokine concentrations are reported using the multiplex Luminex® technique (Bio-Plex). The two Bio-Plex panels chosen for use in the experiments encompass a range anti-inflammatory, proinflammatory and pleotropic cytokines, growth factors, chemokines, anti-catabolic and catabolic enzymes that are known to be critical in both MSC mode of action and the molecular pathological progression of OA disease. However our work presents a targeted approach to quantifying cytokines

in the MSC secretome and thus does not encompass the secretome in its entirety. There may be cytokines or molecules which contribute to the measured outcomes and may also be important in explaining the observed effects. Henceforth the term "MSC secretome" in this thesis will be used to describe defined critical molecules measured rather than the true secretome produced by MSCs.

1.7 MSCs and Hyaluronan: Evidence for synergy?

Culture expanded MSC therapy for the treatment of knee OA is a safe and effective therapy and as evidenced by the landmark proof of concept clinical trial by Jo *et al.,* the efficacy of MSC therapy is dose dependent. Culture expanded MSCs have particular advantages over autologous SVF therapy in that they will allow for more streamlined commercialization strategies and for the manufacture of off-the-shelf therapeutics. Future preparations of MSCs therapeutics for the treatment of knee OA may consist of HA as a possible suspension medium. This may be because HA has shown efficacy in the treatment of such a disease and researchers may perceive a synergistic relationship between the two therapies achieving a two prong approach of viscosupplementation as well as molecular regeneration.

HA shows great potential as an adjunct to MSC therapy for the treatment of knee OA. Not only for its ability to provide rheological cushioning, but also the potential mechanistic biochemical relationship it forms with MSCs. The relationship with high molecular weight HA and MSCs is founded in CD44, the primary HA receptor which is abundantly expressed on the surface of MSCs [311]. It has been proposed that the cross-talk between CD44 and HA may promote rapid resolution of immune responses [312] and thus enhance the anti-inflammatory effect of MSCs in knee OA. However as discussed previously, HA is not inert but rather an active dynamic molecule involved in apoptosis, oxidation, angiogenesis and chondroprotection [131, 132,133, 156-158]. Importantly, HA is a ubiquitous molecule which is found throughout the human body. It is safe to assume that adult MSCs found in niches, especially around vascularized tissue regularly interact with HA *in vivo*. Therefore it stands to

reason that *ex vivo* interaction may be advantageous, in that MSCs cultured in HA would better reflect the *in vivo* environment from which they have been isolated from.

Without full consideration for possible molecular interactions, surgeons have experimented with combinations of HA and MSC therapy. Lee *et al.* used a porcine model of OA creating large cartilage defects in the articular cartilage of the medial femoral condyle without penetration to the subchondral bone. MSCs were suspended in HA and then injected into the injured knee and compared to HA alone. Upon sacrifice of the animals at 6 and 12 weeks, investigators observed through histopathological observation that injection of MSCs suspended in HA homed to the site of injury, adhered, proliferated and lead to neocartilage formation [291]. One limitation of this study as concluded by the authors was that regeneration may have been limited by a dose-dependent response to the number of MSCs injected. Similarly in a model of seven month old guinea pigs with spontaneous knee OA, HA was injected either with fluorescently labelled (carboxyfluorescein diacetate succinimidyl ester) MSCs or alone. Animals were sacrificed at five weeks, the labelled MSCs had migrated to the site of injury and cartilage repair was observed in the MSC-HA group but not in the HA alone group. Immunohistochemical examination of the cartilage showed type II collagen accumulated around the transplanted MSCs [313]. In a caprine model of OA, unilateral resection of the anterior cruciate ligament and excision of the medial meniscus was used to destabilise the knee joint. Six weeks after injury, 10 million cultured MSCs transduced to express green fluorescent protein were injected into the damaged knee suspended in HA and HA alone was used as a control. Strikingly animals treated with MSCs suspended in HA showed marked regeneration of the medial meniscus but lacked repair of the anterior cruciate ligament. Other hallmark histopathological observations of articular

cartilage degeneration, ostephytic remodelling and subchondral sclerosis all decreased in the MSC treatment compared to HA alone [290].

Aside from animal models of OA, surgeons have also experimented with a combination of MSCs and HA in humans. In a trial of 35 patients with symptomatic knee OA as a result of cartilage defects, were injected with 1×10^7 cultured MSCs followed immediately by a 2 mL injection of HA and then two more subsequent injections of HA administered fortnightly. At an average of 24 month follow-up, the study patients showed significant increases in function and pain, as measured by the visual analogue scale [314]. Moreover, in a patients undergoing partial meniscectomy, allogeneic cultured MSCs were injected into the operated knee using HA as a vehicle. The study was a randomized, double-blind, controlled study which measured meniscal regeneration, pain and safety. After a single injection of 50×10^6 allogeneic MSCs, patients showed a significant reduction in pain as measured by visual analogue scale and significantly increased meniscal volume by 24% at 12 month follow-up compared to no change in the HA alone group, as determined by quantitative MRI [315].

1.8 Purpose of the study and research aims

There is currently a treatment gap in the therapies available for younger sufferers of knee OA. Current therapies provide short to medium term symptomatic relief, but cannot halt disease progression and thus patients who are not candidates for total knee replacement, generate economic and healthcare burdens for governments around the world. End stage knee OA inevitably results in the need for prosthetic interventions, which also have a limited lifespan and often require revision surgery. MSC therapy for the treatment of knee OA is emerging as an alternative biological therapy, which can significantly improve pain, function, slow disease progression, regenerate articular and meniscal cartilage, reduce inflammation and modulate the immune system. Investigators have combined MSC therapy with HA for the treatment of knee OA and have shown there are minimal adverse events associated with the combination. However, all studies thus far have been uncontrolled and no comparison on the efficacy is made between MSC suspended in HA compared to MSCs suspended in an inert solution such as saline. Therefore it is imperative to explore the effects of HA on the efficacy of MSC therapy for the treatment of knee OA. Moreover, MSC mode of action is secretion driven and therefore understanding the effects HA can have on the MSC secretome will be of clinical value for future investigations.

In this study we sought to assess the feasibility of a range of preparations in which adipose-derived mesenchymal stem cell therapy could be administered in combination with HA by assessing MSC surface markers, differentiation capability and the secretome.

Culture expanded MSC therapy for the treatment of knee OA is advantageous compared to other preparations and likely to be the approach of future investigations.

However if HA was to be used in combination with MSC therapy, practical considerations need to be made for the concentration which would be used. Therefore we sought to test the effect of a range of HA concentrations on MSC growth-kinetics *in vitro* and the effect of concentration on the secretome.

To better understand what effects HA would have on MSC binding to cartilage *in vivo*, we sought to develop a quantitative explant model using equine articular to assess MSC adherence and proliferation to articular cartilage in culture.

Finally, changes in the MSC secretome resulting from a combination with HA *in vitro* are not of high clinical significance. We therefore sought to test the secretions from MSC grown in HA compared to standard growth medium on live human cartilage and synovial explants from patients undergoing total knee replacement as a result of end stage OA.

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2

Methods

2.1 Methods

Methods used in this work are summarise in Table 2.1. Detailed descriptions for each method can be found in the corresponding chapter.

Methods	Refer to Chapter
Isolation of human adipose-derived mesenchymal stem cell populations	3, 4 & 5
Propagation of adherent adipose-derived mesenchymal stem cells	3
Standardised enumeration of MSCs using flow cytometry	3,4
SVF seeded with hyaluronan treatment	3
Purified adherent-MSC hyaluronan treatment	3
Morphology & differentiation assessment	3
Immunophenotypic characterisation	3
Secretome analysis using Bio-Plex	3, 4 & 5
MSC growth kinetics assessment	4
Cartilage sectioning method	4
Qualitative cartilage-adherence time-course of MSCs	4
HA media viscosity assessment using falling-ball viscometry	4
Qualitative assessment of MSC dispersion on cartilage	4
Quantitative assessment of MSC adherence & proliferation using the ex vivo cartilage assay	4
Culture of osteoarthritis-derived explants	5
Histopathological scoring methods	5

2.2 Statistical methods

In chapter 3 we compared the concentration of cytokines in the conditioned medium of samples treated with HA against standard controls which were not. For each experiment a different donor was used and repeated for a total of 3 donors (n = 3). Within each donor sample 4 flasks were used per treatment from which the conditioned media samples were measured i.e. 4 technical replicates. Across the technical replicated the intra-assay variability was within normal limits. However the inter-assay variability was substantially heterogeneous. Stromal vascular fraction (SVF) and MSC culture can produce a variable cytokine profile, as measured in our lab using the targeted Bio-Plex technique. Variability is less pronounced in later passages as the milieu becomes less heterogeneous and dominated my multiplying MSCs. The variability may arise from a multitude of factors such as sample preparation, donor variation or cell viability. Therefore to account for biological variability between donor samples (inter-assay variation), the data was normalized to fold change of concentration (pg/mL) of HA treated over control. The average of the fold changes was then determined and graphed with the upper and lower confidence intervals set at 95%. This allowed significance to be determined numerically as cytokines with a fold change less than one indicated a decrease in concentration with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment.

In chapter 4 the cytokine data again applied the same statistical method as described above. However, this chapter utilized fluorescence rather than concentration values to directly compare each of the HA concentrations back to the control. The change to analyzing fluorescence data was applied following a publication by Breen et al. which showed fluorescence values rather than concentration resulted in greater statistical power than traditional concentration values [316].

MSC growth kinetics were tested on a range of surfaces known to produce different cell binding coefficients. In each experiment an independent donor cell line was used for a total of three donors (n=3). To control for intra-assay variability, 5 technical replicates were used for each of the conditions tested. Each of the conditions was compared to the control media condition using a two tailed t-test with the α-threshold for statistical significance set at 0.05 if assumption for normal distributions were met. Conservative multiple test corrections using the Bonferroni method were applied to all p-values, thus controlling false positive discovery rate {Haynes, 2013 #1202}. In cases were the data was not normally distributed, the Mann Whitney test for non-parametric data was applied. Any outliers were controlled using the Grubb's test within GraphPad Prism, with the α-level set at 0.05.

Chapter 5.5.7 details the statistical methods used in the pilot study for Hyaluronan-mediated stem cell secretions in osteoarthritis.

3

Alterations in the secretome of clinically relevant preparations of adipose-derived mesenchymal stem cells co-cultured with Hyaluronan

Prologue

Initial inspiration for this chapter came from a meeting of orthopaedic surgeons discussing the application of MSC therapy for the treatment of knee Osteoarthritis. Doctor Donald Kuah, an orthopaedic surgeon administering MSC therapy for patients with knee OA asked the question, can my patients who are currently being treated with Synvisc, also receive MSC therapy? Research into any previous work pertaining to the question of a MSC/HA combination showed there was a gap in the literature. At the same time our lab had just published a study demonstrating MSC secretions alone could be of clinical benefit in arthritis, therefore we sought to investigate the effects of HA on the MSC secretome as a preclinical tool to measure cytokine changes in a range of clinically relevant MSC preparations. I would like to thank Dr Kuah, for providing the Synvisc for our experiments and a clinical perspective on the study. Also Dr Edmond Breen for providing me with statistical support and review of the manuscript and Dr Benjamin Herbert, for helping me to coordinate resources, aiding in the experimental design and final review of the manuscript

The results of this research were reported in the form of a peer-reviewed journal article in *Stem Cells International*.

Contributions to publication statement

The concept of this publication was developed in partnership with my supervisors Benjamin Herbert and Donald Kuah, an orthopaedic surgeon using both Hyaluronan and MSC therapy for the treatment knee Osteoarthritis. Dr Edmond Breen provided statistical support throughout all projects. Peter Succar performed all of the experimental work, data analysis and the preparation of the manuscript. All authors reviewed the manuscript and thus contributed to the final publication.

Table 3.1: Author contribution matrix for Publication.

	Peter Succar	Edmond J Breen	Donald Kuah	Benjamin R Herbert
Experiment Design	●			●
Provision of Study material	●		●	
Data Collection	●			
Data Analysis	●	●		
Manuscript	●	●	●	●

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3.1 Alterations in the secretome of clinically relevant preparations of adipose-derived mesenchymal stem cells co-cultured with Hyaluronan.

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3.2 Disclosures

Benjamin Herbert is A/Prof Translational Regenerative Medicine at the University of Sydney. He is also a co-founder, shareholder and consultant to Regeneus Ltd. Peter Succar is a PhD student and a casual employee at Regeneus Ltd. Edmond J Breen is the Head of Bioinformatics at the Australian Proteome Analysis Facility (APAF) at Macquarie University and a consultant to Regeneus Ltd. Dr Donald Kuah is a sports physician specialist and one of the founding practitioners at Sydney Sports Medicine Centre in Olympic Park. He is a licenced provider of HiQCell, an autologous adipose-derived cell therapy commercialised by Regeneus.

3.3 Abstract

Osteoarthritis (OA) can be a debilitating degenerative disease and is the most common form of arthritic disease. There is a general consensus that current non-surgical therapies are insufficient for younger OA sufferers who are not candidates for knee arthroplasties. Adipose-derived mesenchymal stem cell (MSCs) therapy for the treatment of OA can slow disease progression and lead to neocartilage formation. The mechanism of action is secretion driven. Current clinical preparations from adipose tissue for the treatment of OA include autologous stromal vascular fraction (SVF), SVF plus mature adipocytes and culture-purified MSCs. Herein we have combined these human adipose-derived preparations with Hyaluronan (Hylan G-F 20: Synvisc®) *in vitro* and measured alterations in cytokine profile. SVF plus mature adipocytes showed the greatest decreased in the pro-inflammatory cytokines IL-1β, IFN-ɣ and VEGF. MCP-1 and MIP-1α decreased substantially in the SVF preparations but not the purified MSCs. The purified MSC preparation was the only one to show increase in MIF. Overall the SVF plus mature adipocytes preparation may be most suited of all the preparations for combination with HA for the treatment of OA, based on the alterations of heavily implicated cytokines in OA disease progression. This will require further validation using *in vivo* models.

3.4 Introduction

Osteoarthritis (OA) can be a debilitating degenerative disease and is the most common form of arthritic disease. Onset is usually in the third and fourth decade of life and has a gradual worsening prognosis over time.

OA is most prevalent in weight bearing joints; the highest incidence of the disease occurs in the knee. It is classified as idiopathic and or secondary. Primary clinical intervention currently involves limited systemic pharmacotherapy such as analgesics, non-steroidal anti-inflammatories (NSAIDS). Controlling mechanical overload through weight loss and supporting braces can be used autonomously or in combination with physical therapy. Viscosupplementation can also be used to treat OA. This aims to replace lost synovial fluid in an OA knee with Hyaluronan (HA) to reduce pain and increase mobility by lubricating the joint. Hyaluronan is an endogenous polysaccharide found in all tissues and body fluids of vertebrates. HA is especially abundant in loose connective tissue and is a major component of Cartilage [118] and the synovium [119]. Aside from its rheological properties, high molecular weight HA can decrease apoptosis, oxidative stress and necrosis [131].

Arthroscopic surgical interventions are used for the debridement of mechanical cartilage tears, lesions and defects, as well as cartilage re-surfacing or microfracture to stimulate fibrocartilaginous repair. However, due to inherent degenerative factors of OA and the limited capacity of conventional therapies to halt disease progression, arthroplasties (artificial joint replacement surgery) are commonly performed for a large proportion of OA patients. The useful lifetime of an artificial knee joint is between 10 and 20 years. Average time for revision surgery has been reported at 35 months and is most commonly caused by infection, instability and stiffness [173]. For these reasons arthroplasties are often not recommended for patients under 60. There is a general consensus that current non-surgical therapies are insufficient for younger

OA sufferers who are not candidates for knee arthroplasties [174]. Although current therapies may provide temporary pain relief and increased mobility, there is a pressing need for treatments that slow or stop the degradation of cartilage.

The use of alternative biological therapies, such as mesenchymal stem cells (MSCs), for the treatment of OA is increasing as a result of a gap in healthcare options for middle age patients [174,203]. MSCs can differentiate into mesodermal lineages making them an attractive source for cell based therapies in musculoskeletal conditions such as OA [317]. However, differentiation is not the primary mode of action and *in vitro* animal models and human data have demonstrated that a complex set of secretions drive the anti-inflammatory and regenerative effects that have been observed [242]. Thus, the secretome of MSCs has become of particular interest and multiplex cytokine analyses are used to assess cell populations and the 'cytokine profile' is predictive of *in vivo* effect [288]. Proinflammatory cytokines such as Interleukin-1β (IL-1β) play a pivotal role in the pathogenesis of OA. IL-1β can dramatically increase the expression of matrix metalloproteinases, which contribute to cartilage degradation in OA. Chondrocytes stimulated with IL-1β increase secretion of Nitric oxide and Prostaglandin E2, which are key mediators essential to inflammation in OA [318].

The role of inflammation in the pathophysiology of OA has shifted from originally being considered a by-product of the disease to the current understanding as a key driver of disease progression [319]. MSCs have an anti-inflammatory effect and are able to stimulate cartilage proliferation via paracrine signaling [286]. As such, regenerative therapies using MSCs are emerging as a suitable treatment for degenerative musculoskeletal defects.

Surgeons have experimented with possible combination therapies; most notably Lee *et al.* showed in a porcine model using histopathological observation that injection of bone marrow-derived MSCs suspended in Hyaluronan (Hylan G-F 20: Synvisc®) could home to the site of injury, adhere, proliferate and lead to neocartilage

formation [291]. One limitation of this study as concluded by the authors was that regeneration may have been limited by a dose-dependent response to the number of MSCs injected.

One of the first MSCs sources to be isolated came from bone marrow, however as shown by Kern *et al.* in a comparative analysis between adipose and bone marrow-derived, these MSCs may be inferior to adipose-derived MSCs, in terms of clinical feasibility. It was shown adipose tissue had the highest concentration of MSCs and a greater proliferative capacity when compared to bone marrow-derived MSCs [239]. The above components combined with minimal invasiveness, spearhead adipose tissue as a favorable source of MSCs in emerging regenerative therapies.

Hyaluronan (HA) shows great potential as an adjunct to adipose-derived MSC regenerative therapy for the treatment of OA. Not only for its ability to provide rheological cushioning, but also the mechanistic biochemical relationship it forms with MSCs. The relationship with high molecular weight HA and MSCs is founded in CD44, the primary HA receptor and abundantly expressed on the surface of MSCs. It has been proposed that the cross-talk between CD44 and HA may promote rapid resolution of immune responses [312] and thus enhance the anti-inflammatory effect of MSCs in OA.

In this study we sought to assess the feasibility of a range of preparations in which adipose-derived mesenchymal stem cell therapy could be administered in combination with HA by assessing the secreted soluble protein fraction produced by MSCs i.e. the secretome. We employed a multiplex analyses of 48 cytokines involved in OA to assess the suitability of conditions. These conditions include autologous therapies such as the stromal vascular fraction (SVF), the SVF co-injected with autologous mature adipocytes and cultured allogeneic preparations such as culture purified adipose-derived MSCs. As adipose-derived MSC therapy

moves closer to mainstream medicine, secretome data will be a valuable assessment

tool to inform animal studies and human clinical trials in years to come.

3.5 Materials and Methods

3.5.1 Isolation of human adipose-derived mesenchymal stem cell populations

This research was approved by the Macquarie University human research ethics committee (Ref #: 5201100385). Human lipoaspirate was obtained from patients undergoing elective cosmetic liposuction surgery. All patient demography was concealed as per instructions of the human research ethic committee. The lipoaspirate was digested as previously described [296]. Briefly, 200mL of fresh lipoaspirate was mixed and enzymatically digested in pre-warmed (37°C) saline containing 0.5 mg/mL collagenase (Lomb Scientific, USA). The lipoaspirate was then incubated in a 37°C water bath for 30 minutes and mixed periodically to circumvent layer separation. The digested lipoaspirate was then passed through an 800 μm mesh to exclude undigested tissue clumps. Finally, the suspension was centrifuged at 1500 x g for 5 minutes to obtain the pelleted stromal vascular fraction cells (SVF) and floating adipocytes. At this point, the SVF was either used for experimentation or propagated to obtain an adherent purified adipose-derived mesenchymal stem cell (MSC) population.

3.5.2 Propagation of adherent adipose-derived mesenchymal stem cells

To obtain a population of adherent MSCs, the SVF pellet was transferred into three T175cm2 flasks containing Standard culture media which consisted of Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, USA) supplemented with 10% foetal

bovine serum (FBS; Bovogen, Australia) and 1% Penicillin-Streptomycin solution (Invitrogen, USA). Flasks were incubated for 72 hours at 37°C with 5% carbon dioxide. To prevent iron toxicity to adherent MSCs; the flasks were washed using DMEM with no additives to wash away non-adherent cells and the media replaced with fresh standard culture media. Once the monolayer of MSCs reached 80% confluency, cells were passaged with enzymatic digestion using TrypLE express (Invitrogen, USA) and then re-suspended in standard culture media. Cells from each flask were then transferred to three new T175cm^2 flasks now termed passage 1. Media changes were performed every 72 hours.

Standardised enumeration of the SVF, dependant on nucleated cell clusters, was achieved with Trucount tubes (Becton Dickinson, USA) containing sheath fluid (isoflow; Becman Coulter, USA) and nucleic acid dyes; propidium iodide (10ug/mL; Sigma, USA), Syto11 (1uM; Invitrogen, USA) and a defined bead number. The combination of a charged (propidium iodide; Pi) and a cell permeate (Syto11) dye allows for discriminant cell population gating, which are based on the principal of Pi membrane exclusion and thus viability can be calculated from defined bead populations run as standards. Samples were all run on FACSCalibur flow cytometer (Becton Dickinson, USA).

3.5.3 SVF seeded with hyaluronan treatment

Following on from the digestion of the fresh lipoaspirate, the pelleted SVF was resuspended in standard culture media. Viable SVF cells were all seeded at two million nucleated cells per T175cm^2 flask with or without the addition of adipocytes. Flasks were always seeded in either two conditions; the control, which consisted of standard culture media or treatment, which consisted of HA media (standard culture

media; hyaluronan (6% (v/v))). In each condition, three subset arms were tested; SVF and mature adipocytes, SVF alone and mature adipocytes alone. Adipocytes were used at 2.5 mL (suspended in saline) and flasks were normalised to 20 mL total containing 17.5 mL media and 2.5 mL saline equivalent (with or without adipocytes). Flasks were incubated for 72 hours at 37°C with 5% carbon dioxide.

3.5.4 Purified adherent-MSC hyaluronan treatment

Seeding experiment – Once the flasks reached 80% confluency at passage 2, cells were stripped off the plastic and enumerated. Viable MSCs were then seeded at 1.1 million cells per T175cm^2 flask. Total media contained within the flasks was standardised to 20mL. Flasks were seeded with standard culture media or HA media for treatment flasks. This method approximates the intra-articular co-injection of HA and MSCs. After 24 hours of incubation, conditioned media was collected from all flasks. Cells were then stripped off the plastic and a portion was used for immunophenotypic characterisation.

Media change experiment – Once the flasks reached 80% confluency at passage 2, cells were stripped off the plastic and enumerated. Cells were seeded at 1.1 million cells per T175cm^2 and incubated in standard culture conditions. On the third day the media was changed with either standard culture media in control flasks or HA media for treatment flasks. After three days, conditioned media was collected from each of the flasks. Cells were then stripped off the plastic and a portion was used for immunophenotypic characterisation.

3.5.5 Morphology & differentiation

Morphology – prior to harvesting the purified adherent-MSCs were imaged under a Zeiss Primo Vert inverted light microscope (Zeiss, Australia) using an eye-piece attached, Canon EOS 5D Mark II Digital SLR camera (Canon, Australia).

Differentiation – adipogenic and osteogenic differentiation media formulations were used as previously described [98] with slight modification. In brief, cells received media changes twice weekly for 28 days. Upon termination of the differentiations, monolayers were washed in PBS and fixed in 4% paraformaldehyde for 1 hour. To confirm the multi-potency of our MSCs, differentiated adipocytes and osteocytes were challenged with Oil Red O (Sigma, Australia) and Alizarin Red (Sigma, Australia) respectively.

The adipogenic differentiation was washed with milliQ water, incubated with 60% isopropanol, stained using three parts filtered Oil Red O stock (0.3% w/v) with two parts milliQ water for five minutes at room temperature and washed with tap water.

The osteogenic differentiation was washed with milliQ, stained with 2% Alizarin red S solution for two minutes at room temperature and washed three times with milliQ water.

All differentiations were visualised using an Olympus IMT-2 inverted microscope (Olympus, Australia) and imaged with a mounted Scion VisiCapture Firewire camera (Scion Corporation, USA).

3.5.6 Immunophenotypic characterisation

MSC characterisation can be achieved through adherence to cell culture flasks, differentiation and immunophenotype. A portion of the endpoint cells collected were used for MSC immunophenotypic characterisation. Cells from either control or treated flasks were diluted in their respective media (standard culture media or HA media) and centrifuged at 2000 x g for 5 minutes. The cells were washed in PBS and resuspended in PBS with 2% FBS. The cells were stained with the following antibodies, which were all sourced from BD Biosciences: CD34 (#550619), CD45 (#557059), CD73 (#550257), CD90 (#555596), CD105 (#560839), IgG1κ isotype control (#551436). Cell were incubated with each of the antibodies for 45 minutes and then washed with ice cold PBS, centrifuged at 2000 x g for 5 minutes and resuspended in 1x FACS Lysing Solution (Becton Dickinson, USA). The cells were all stained with phycoerythrin (PE) conjugated antibodies and thus were resuspended in Syto11 (1uM) and isoflow to achieve contrast fluorescence. Stained and unstained control cells were analysed using a FACSCalibur flow cytometer (Becton Dickinson, USA). CD44 has long been recognized as an established receptor for HA [320], therefore in addition to the immunophenotypic MSC characterisation above, CD44 (#550989) was also tested using the same protocol.

3.5.7 Secretome analysis

The conditioned media was collected from every flask in this study, centrifuged at 5000 x g for 5 minutes and stored at -80°C. Upon thawing, the samples were filtered through 0.2 mm Nanosep MF Centrifugal Devices with Bio-InertH Membrane (Pall Scientific, USA). Filtrates (50 μL) were analysed using either the Bio-Plex Pro

Human Cytokine 27-plex or the Bio-Plex Pro Human Cytokine 21-plex assay (Bio-Rad, USA), according to the manufacturer's instructions. The washing steps were performed using the Bio-Plex Pro II magnetic wash station and the data was acquired using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, USA). The average concentration of each cytokine in the conditioned medium samples was calculated from four technical replicates in cultured MSC experiments. To account for biological variation in the concentration of secreted cytokines and, in the stromal vascular fraction, the variable concentration of MSCs, the data was normalised to fold change of concentration (pg/mL) of HA treated over control ($n=3$). The fold change was then averaged and graphed with the upper and lower confidence intervals set at 95% as error bars. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment. Any cytokines with a fold change clear of the axis at 1 were reported as significant (determined numerically).
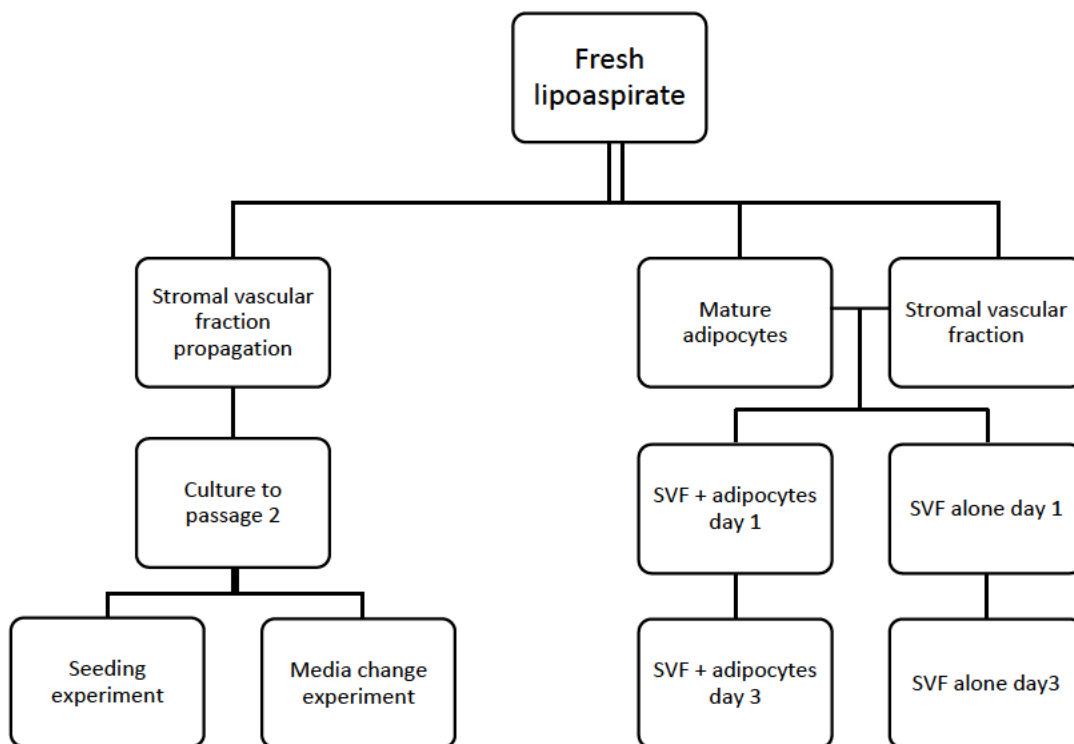
3.5.8 Experimental schematic

Figure 3.1 Schematic representation of the experimental workflow used in this investigation

3.6 Results

3.6.1 Multipotent MSC validation

Previously published data from our lab demonstrated methods used within this study to both isolate and culture/propagate the SVF and obtain adherent-MSC populations [98,296]. These were in accordance with the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy statement [243]. Herein we have supplemented media used to grow MSCs with HA media in an attempt to quantify the effects on the secretome.

Cultured MSCs were tested for immunophenotypic expression with and without HA media treatment. The MSC profile was maintained for the duration of the study. Monolayers from both the control and HA treated MSCs presented with equivalent differentiation potential. Figure 3.2 shows no detectable difference in morphology and differentiation potential between MSCs cultured in standard culture media and HA media. The differentiated MSCs were tested with either Alizarin Red S, which stains positive for calcium deposits confirming osteogenic potential or Oil Red O, which stains positive for lipid accumulation confirming adipogenic potential. The consistency in the take up of both stains used to test differentiated cell monolayers indicates the differentiation potential is maintained.

In addition to differentiation potential, immunophenotypic expression on the control and HA treated MSCs is depicted in figure 3.3. The presence of CD73, CD90, CD105 and the absence of hematopoietic markers CD34 and CD45 indicated the presence of replicative and self-renewal capacity of MSCs of both the control and the HA media treated. CD44 is a major receptor for HA, as such it was also tested. The number of CD44$^+$ cells in the control and the HA media treatment showed no major quantifiable difference in the tested replicates.
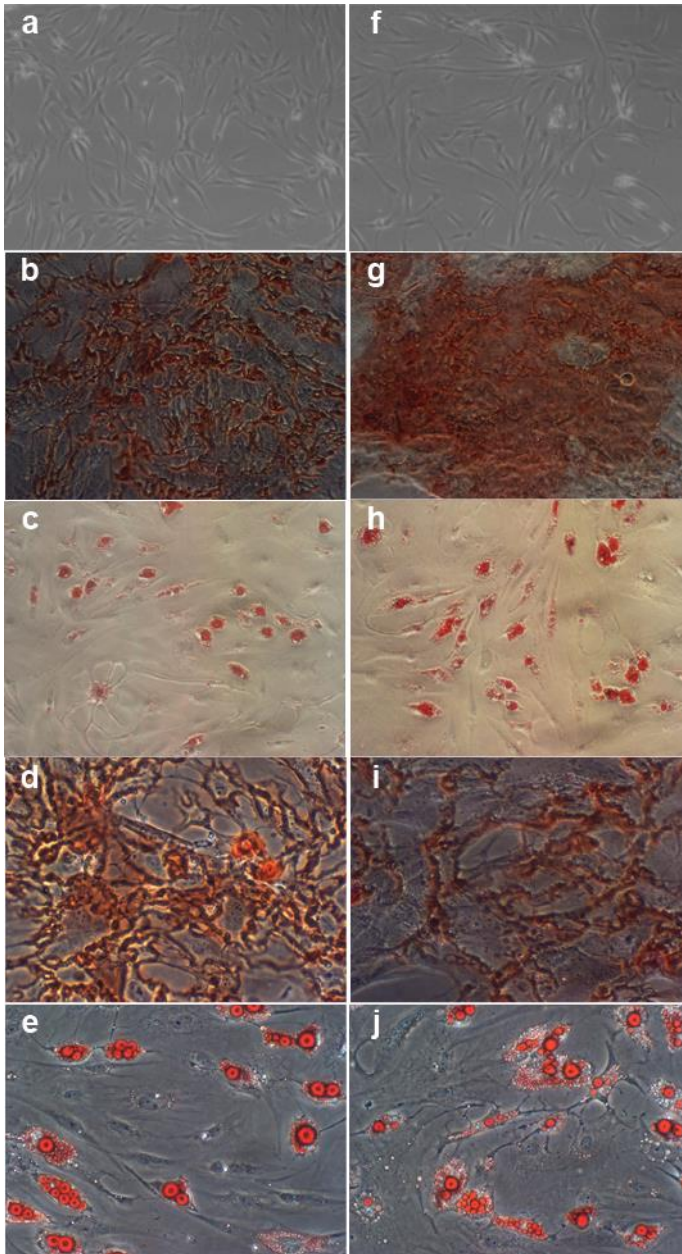
Figure 3.2 Morphology & differentiation potential of control and treated MSCs.

MSCs cultured in either standard culture media or HA media. No detectable differences were seen in the morphology of control (a) MSCs and HA treated (f) monolayers. Osteogenic differentiation of control MSCs (b) and of the HA treated (g) at 10x magnification. Adipogenic differentiation of control (c) and of the HA treated (h) at 10x magnification. At 20x magnification no detectable differences in Alizarin Red S staining were observed in the osteogenic differentiation of control MSCs (d) and HA treated (i). At 20x magnification no detectable differences in Oil Red O staining were observed in the adipogenic differentiation of control MSCs (e) and HA treated (j).

Figure 3.3 Immunophenotypic expression of control and HA treated MSCs.

Immunophenotypic expression of MSCs cultured in either standard culture media or HA media. Each of the histograms depicts the IgG iso-type control (---), control media (---) and HA media (---). Maintenance of MSC profile was confirmed with the lack expression in the haematopoietic markers CD34 (a), CD45 (b) and positive expression of CD73 (c), CD90 (d) and CD105 (e) in both the control and HA treated MSCs. CD44 (f), the major receptor for HA, showed no major increases in immunophenotypic expression.

3.6.2 Alterations of secretion profile in HA media treatment

In this study we sought to profile changes in the secretome to ascertain the effects of combining HA with MSCs. Changes in the secretions profile can be an indication of positive or negative effect in the context of OA i.e. increase in the anti-inflammatory cytokines may be considered positive or an increase in pro-inflammatory cytokines may be negative. All the cytokines were represented as a percentage of the control i.e. fold change (fold Δ). The average fold change of three biological replicates for each of the cytokines was plotted as an average ± the upper and lower confidence intervals at 95%. The horizontal axis was set at one (i.e. no change), any point with error bars that cleared one, were considered a significant difference from the control.

Cytokines	Fold Change from control (CI = 95%)						Role in OA
	SVF day 1	SVF day 3	SVF + adipo day 1	SVF + adipo day 3	MSC Seeding	MSC Media	
Pro-inflammatory							
MIF	0.94	0.93	0.89	0.99	*1.49*	0.79	Induces the release of proinflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-6, IL-8 [321] and MIF levels in synovial fluid correlate to OA severity [322]
TNF-β	1.00	*1.26*	1.03	1.28	*1.52*	1.18	TNF beta stimulates cartilage matrix breakdown [323]
IL-1α	1.21	*1.35*	0.98	1.26	1.34	N.D.	Highly expressed in OA-derived human articular cartilage [324]
IL-1β	1.03	1.12	*0.59*	0.89	1.00	0.94	Induces inflammatory reactions and catabolic effects independently as well as in combination with other mediators in OA with respect to the articular cartilage [325]
IL-12	*0.67*	0.86	*0.49*	0.78	1.00	*0.90*	Expressed by infiltrating macrophages & synovial cells in OA [326]
IL-17	*0.70*	1.17	0.83	0.90	0.96	0.09	inhibits the synthesis of proteoglycans in OA and upregulates catabolic enzymes which break down cartilage [325]
IFN-γ	0.96	0.86	0.89	*0.80*	0.99	0.93	Stimulate cartilage breakdown by production of enzymes via IL-1 β [327]

Anti-inflammatory / Dual role	IL-10	0.90	0.85	*0.40*	*0.61*	1.05	*0.86*	Expressed in chondrocytes and involved in collagen and aggrecan synthesis. Inhibition of catabolic enzymes and apoptosis of chondrocytes [325]
	IL-2	0.81	2.35	*0.69*	*0.74*	1.23	1.35	Elevated levels are found in OA synovial fluid and increased levels correlate to increase severity [328]
	IL-6	0.93	0.84	0.47	0.94	1.11	*0.84*	Catabolises aggrecan via aggrecanase activity [329] Involved in the synthesis of tissue inhibitor of metalloproteinases known to stop cartilage breakdown [330]
Chemokines	GROα	1.02	0.89	0.79	0.87	*0.94*	*1.12*	Constitutively secreted by chondrocytes and secretion of this chemotactic protein is increased in stimulated osteoarthritic chondrocytes and synovial fibroblasts [331]
	MIP-1β	0.92	0.83	0.86	*1.17*	1.16	1.14	Present in significantly higher levels in OA synovial fluid compared to normal synovial fluid [332]
	RANTES	0.78	0.99	0.94	1.31	1.09	*0.67*	Secreted by IL-1 stimulated osteoarthritic chondrocytes and TNFα stimulated synovial fibroblasts [331]
	MCP-1	0.79	*0.65*	*0.75*	0.90	0.95	*0.91*	Constitutively expressed in osteoarthritic chondrocytes and increased secretion in IL-1 stimulated osteoarthritic chondrocytes [331]
	MCP-3	0.83	1.07	*0.82*	0.95	0.97	N.D.	Activates immune cells such as monocytes, T lymphocytes, basophils and eosinophils [333]
	MIP-1α	0.83	*0.67*	0.82	0.90	1.04	0.95	Expressed by osteoarthritic derived chondrocytes and production is enhanced by TNFα stimulated chondrocytes [334]

Growth Factors								
	HGF	0.69	*0.83*	0.83	1.56	1.42	0.95	Promotes osteophyte formation via MCP-1 mediated infiltration of immune cells into OA affected joint [335]
	FGF-β	0.89	1.21	*0.79*	0.98	*1.26*	0.84	Potentiates articular cartilage resurfacing and may stimulate expression of MMP-13 [336,337]
	β-NGF	1.02	0.92	*0.73*	0.86	*0.78*	0.86	Higher expression is found in osteoarthritic derived chondrocyte compared to healthy chondrocytes [338]
	GM-CSF	0.84	*1.82*	0.76	0.91	1.38	N.D.	key mediator in inflammation and arthritic pain [339]
	VEGF	0.71	0.82	*0.51*	*0.71*	1.00	*0.84*	expressed in osteoarthritic-derived chondrocytes and shown to increase osteo-chondral angiogenesis in OA patients [340]
	G-CSF	0.97	0.96	*0.51*	0.80	1.06	0.84	Can significantly increase nitrite levels in cartilage when combined with IL-1β explants [341]

Table 3.2 Summary of Cytokines across all conditions

A summary of cytokines across all the conditions treated with HA. The data is represented as fold change in concentration compared to control. A value more than one indicates the HA treatment increased the secretion of that cytokine in that condition. ***Bold*** values represent a significant fold change from control determined numerically using confidence interval set at 95%. Red values were only detected in one of the three biological replicates for that condition.

3.6.3 Stromal vascular fraction

The stromal vascular fraction (SVF) alone prepared from fresh lipoaspirate via enzymatic digestion is currently used in autologous therapy for the treatment of OA. When the SVF alone was combined with HA, pro-inflammatory cytokine IL-1α (figure 3.4) increased 21% on the first day and significantly on the third day of culture by 34%. IL-12 decreased significantly on the first day by 33% (figure 3.4) but later recovered on the third day of culture with a 15% decrease. IL-17 decreased significantly by 30% (figure 3.4) when combined with HA on the first day of culture but on the third day increased by 17%.

FGF-β is one of six detected growth factors across the range of conditions tested. On the third day of culture, the SVF increased FGF-β secretion by 20% on the third day (figure 3.6). Also on the third day of culture the SVF decreased secretion of GM-CSF significantly by 81% (figure 3.6). VEGF also decreased on the first and third day culture by 21% and 19% respectively but not significantly (figure 3.6). Chemokines are chemotactic cytokines responsible for homing and immune cell recruitment. RANTES in the first day of culture in the SVF decreased by 23% but not significantly (figure 3.7). The SVF on day one decreased secretion of MCP-1 by 21% and then significantly on the third day by 36% (figure 3.7). MIP-1α decreased by 17% on the first day of SVF culture and then significantly on the third day of culture by 33% (figure 3.7).
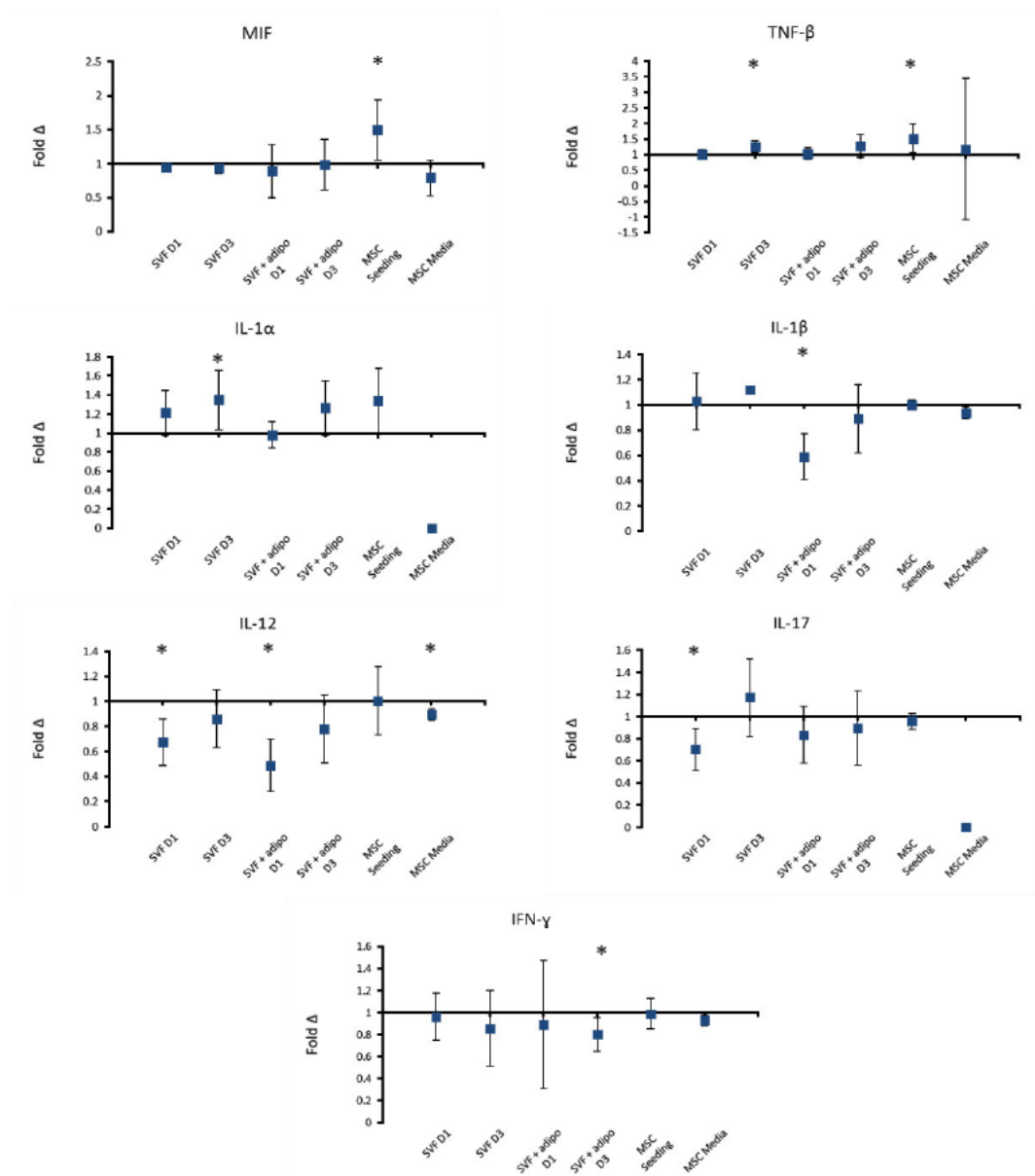
Figure 3.4 fold change in concentration of pro-inflammatory cytokines detected across experiments

Average fold change in concentration (n=3) ± upper & lower confidence intervals at 95% (y-axis). Conditions (x-axis): Stromal vascular fraction on day 1 (SVF D1), stromal vascular fraction on day 3 (SVF D3), stromal vascular fraction plus mature adipocytes on day 1 (SVF + adipo D1), stromal vascular fraction plus mature adipocytes on day 3 (SVF + adipo D3), Seeding experiment with purified MSCs (MSC seeding) and media change experiment with purified MSCs (MSC media). Conditions were all cultured in either standard culture media or HA media. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment. Macrophage migration inhibitory factor (MIF), Tumor necrosis factor-beta (TNF-β; lymphotoxin-alpha), Interleukin-1α (IL-1α), interleukin-1β (IL-1β), Interleukin-12 (IL-12), Interleukin-17 (IL-17) and Interferon-gamma (IFN-γ). IL-1α was not detected in the MSC media condition. IL-17 was only detected in one biological replicate in the MSC media condition. IL-1β was only detected in one biological replicate in the SVF condition on day 3.

3.6.4 Stromal vascular fraction plus adipocytes

Aside from the SVF, another by-product of the enzymatic digestion of adipose tissue is mature adipocytes. A combination of the SVF and adipocytes can be used to treat knee OA. This therapy is available commercially for use in the autologous setting and has been used in a placebo control trial. In this study, when SVF plus adipocytes were combined with HA and in the first day IL-β decreased significantly by 41% but then later recovered on the third day to just a 11% decrease (figure 3.4). Also another very significant decrease was observed on the first day of culture, the SVF plus mature adipocytes decreased the secretion of IL-12 by 52% when combined with HA, this later recovered on the third day to just a 22% decrease (figure 3.4). Anti-inflammatory cytokine IL-10 decreased significantly on the first (60%) and on the third day (40%) of the SVF plus adipocytes combined with HA culture (figure 3.5). The SVF plus adipocytes combined with HA also decreased secretion of IL-2 significantly on the first day by 31.5% and again on the third day significantly by 26% (figure 3.5).

Growth factor secretions were also measured from the SVF plus adipocytes when combined with HA. B-FGF on the first day of culture decreased significantly by 21% but later recovered by the third day culture to no change from the control (figure 3.6). β-NGF decreased significantly on the first day of culture by 27.5% but on the third day of culture was not significant and only decreased by 14% (figure 3.6). VEGF secretion by the SVF plus adipocyte preparation when combined with HA decreased significantly by 50% and 29% on the first and third day of co-culture respectively (figure 3.6). G-CSF also decreased significantly on the first day by 50% but then later recovered on the third day to decrease secretion not significantly by 20% (figure 3.6).

MIP-1β is one of six detectable chemokines across all the preparations. Secretion of MIP-1β by the SVF plus adipocytes when combined with HA decreased on the first day but on the third day increased significantly by 16.5% (figure 3.7). MCP-1 decreased significantly on the first day when combined with HA by 25%, on the third day and recovered to just a 10% decrease, it was however only detected in one of three biological replicates (figure 3.7). Finally MCP-3 decreased significantly by 18% on the first day when combined with HA but on the third day recovered with no observable change (figure 3.7).
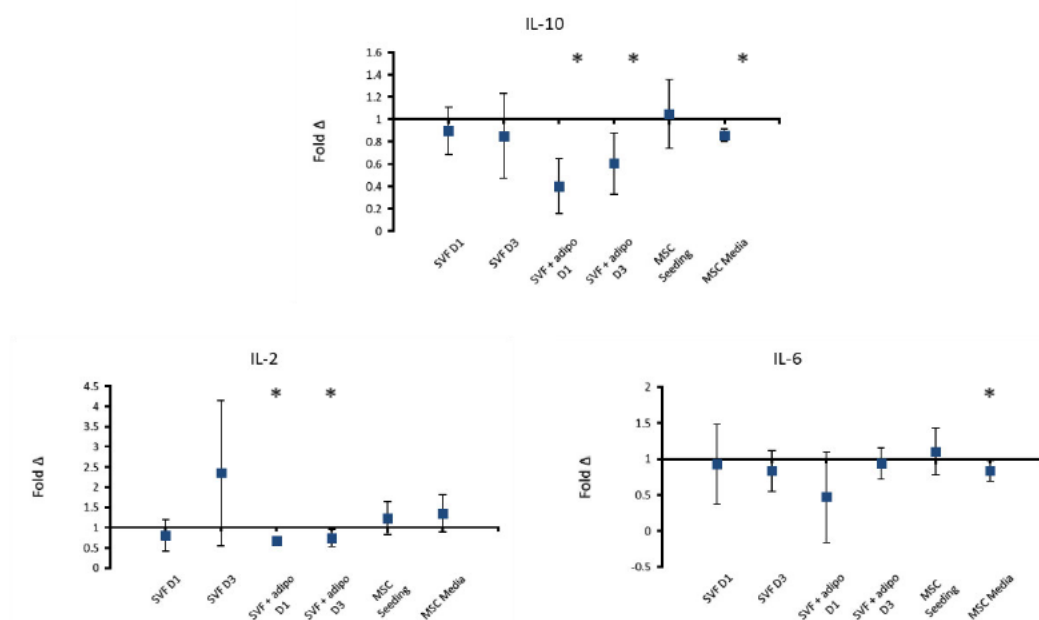
Figure 3.5 fold change in concentration of anti-inflammatory & dual role cytokines detected across experiments

Average fold change in concentration (n=3) ± upper & lower confidence intervals at 95% (y-axis). Conditions (x-axis): Stromal vascular fraction on day 1 (SVF D1), stromal vascular fraction on day 3 (SVF D3), stromal vascular fraction plus mature adipocytes on day 1 (SVF + adipo D1), stromal vascular fraction plus mature adipocytes on day 3 (SVF + adipo D3), Seeding experiment with purified MSCs (MSC seeding) and media change experiment with purified MSCs (MSC media). Conditions were all cultured in either standard culture media or HA media. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment. Interleukin-10 (IL-10), Interleukin-2 (IL-2) and Interleukin-6 (IL-6).

3.6.5 Culture purified MSCs

The final preparation tested in this study and a likely future therapeutic in MSC therapy is the culture purified and expanded MSC population. In the MSC seeding experiment the combination with HA saw a significant increase in a major pro-inflammatory cytokine MIF by 49% but then a non-significant decrease after 3 days in the MSC media experiment of 21% (figure 3.4). TNF-β secretion by purified MSCs in the MSC seeding experiment increased significantly by 52% when combined with HA but on the third day recovered to a 17% increase (figure 3.4). IL-1α increased in the MSC seeding experiment by 34% but not significantly (figure 3.4). There was no detectable IL-1α in the MSC media experiment.

IL-6 is considered a dual role cytokine and in the MSC seeding experiment increase by 10.5% but then decreased significantly in the MSC media experiment by 16% (figure 3.5).

Culture purified MSCs in combination with HA also secrete appreciable amounts of growth factors. FGF-β increase significantly in the MSC seeding experiment by 25.5% but decreased in the MSC media by 16.5% (figure 3.6). In the MSC seeding experiment β-NGF decreased significantly by 21.5% but in the MSC media experiment decreased non-significantly by 14% (figure 3.6).

Chemokine secretion by culture purified MSCs in combination with HA were also measured. GROα decreased significantly in the MSC seeding experiment by 6% but inversely significantly increased in the MSC media experiment by 12% (figure 3.7). RANTES secretion did not change in the MSC seeding experiment, however in the MSC media experiment it decreased by 33.5% when combined with HA (figure 3.7). MCP-1 showed no appreciable change in secretion in the MSC seeding experiment but decreased significantly by 9.5% in the MSC media experiment when combined with HA (figure 3.7).
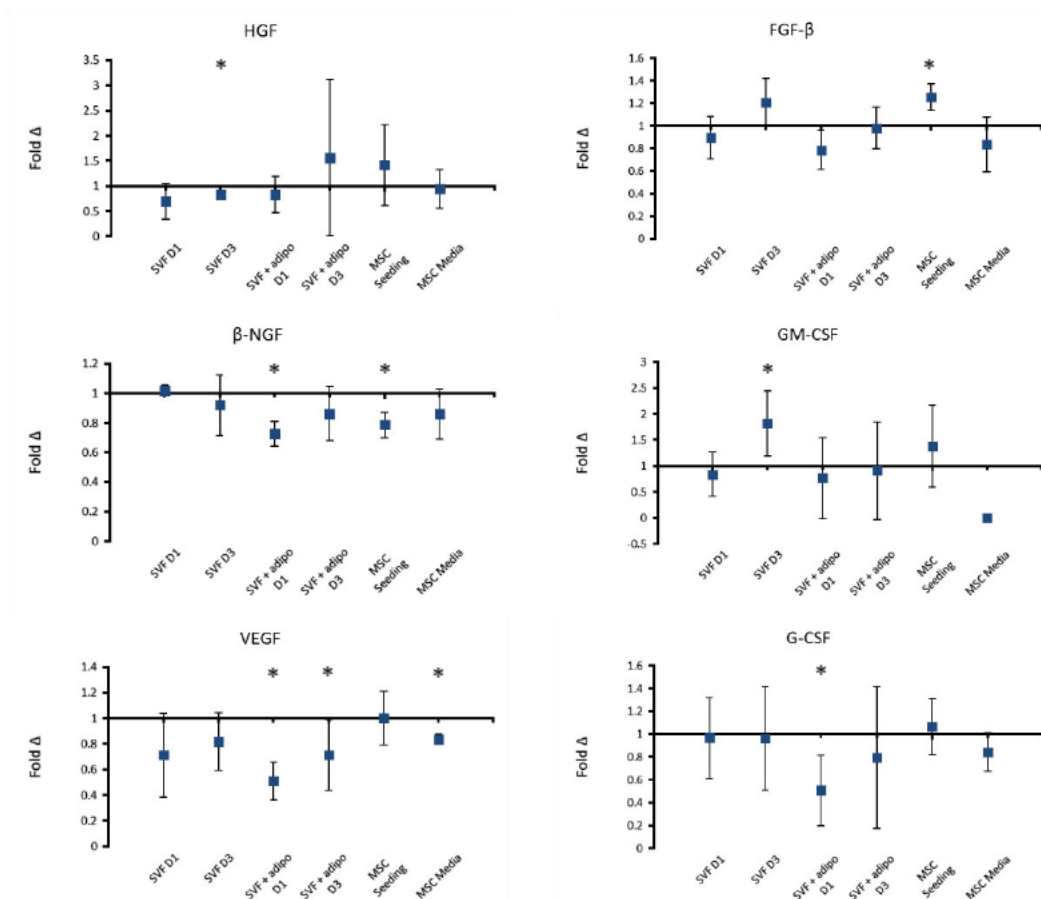
Figure 3.6 fold change in concentration of growth factors detected across the experiments

Average fold change in concentration (n=3) ± upper & lower confidence intervals at 95% (y-axis).
Conditions (x-axis): Stromal vascular fraction on day 1 (SVF D1), stromal vascular fraction on day
3 (SVF D3), stromal vascular fraction plus mature adipocytes on day 1 (SVF + adipo D1), stromal
vascular fraction plus mature adipocytes on day 3 (SVF + adipo D3), Seeding experiment with
purified MSCs (MSC seeding) and media change experiment with purified MSCs (MSC media).
Conditions were all cultured in either standard culture media or HA media. Cytokines with a fold
change less than one indicate a decrease in concentration with HA media treatment, a fold change
greater than one indicates an increase in the cytokine with HA treatment. Hepatocyte growth factor
(HGF), Fibroblast growth factor-basic (FGF-β), Nerve growth factor-beta (β-NGF), Granulocyte
macrophage colony-stimulating factor (GM-CSF), Vascular endothelial growth factor (VEGF) and
Granulocyte colony-stimulating factor (G-CSF). GM-CSF was not detected across all biological
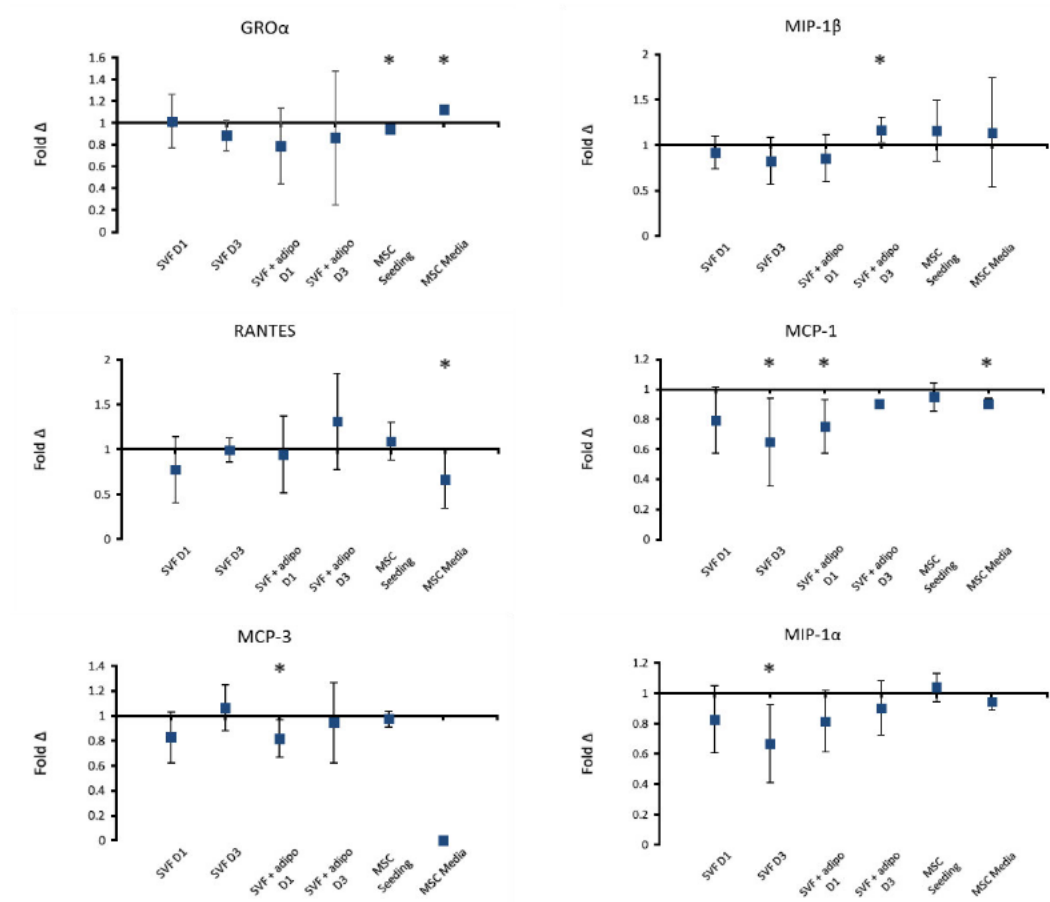replicates in the MSC media condition.

Figure 3.7 fold change in concentration of chemokines detected across the experiments

Average fold change in concentration (n=3) ± upper & lower confidence intervals at 95% (y-axis). Conditions (x-axis): Stromal vascular fraction on day 1 (SVF D1), stromal vascular fraction on day 3 (SVF D3), stromal vascular fraction plus mature adipocytes on day 1 (SVF + adipo D1), stromal vascular fraction plus mature adipocytes on day 3 (SVF + adipo D3), Seeding experiment with purified MSCs (MSC seeding) and media change experiment with purified MSCs (MSC media). Conditions were all cultured in either standard culture media or HA media. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment. Chemokine ligand-1 (CXCL-1) aka growth-regulated peptide alpha (GROα), CC-Chemokine ligand-3 (CCL-3) aka Macrophage inflammatory protein-1-alpha (MIP-1α), CC-Chemokine ligand-4 (CCL-4) aka Macrophage inflammatory protein-1 beta (MIP-1β), CC-Chemokine ligand-5 (CCL-5) aka Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), CC-Chemokine ligand-2 (CCL-2) aka Monocyte chemotactic protein-1 (MCP-1) and CC-Chemokine ligand-7 (CCL-7) aka Monocyte chemotactic protein-3 (MCP-3). MCP-3 was not detected across all biological replicates in the MSC media condition. MCP-1 was only detected in one of three biological replicates in the SVF + adipo D3 condition.

3.7 Discussion

Biological therapeutics such as MSC therapy are gaining acceptance for the treatment of inflammatory conditions, including musculoskeletal ailments such as OA.

It is likely MSC therapy will be administered with viscosupplementation such as HA, which has extensive evidence for therapeutic efficacy in OA. In this study we used a number of adipose MSC preparations that are currently in commercial use or undergoing clinical trial for the treatment of OA. We co-cultured the different cell preparations with HA to investigate the effects on the secretome. We found that MSCs maintained differentiation potential when co-cultured with HA. These results are consistent with [291] although enhanced adipogenic potential was observed in the HA treated MSCs (data not shown). Immunophenotypic expression was characteristic of multipotent mesenchymal stem cells following co-culture, additionally, no observable changes could be observed in the expression of the hyaluronan receptor CD44.

Changes in the cytokine profile may be an indication of therapeutic efficacy reflective of combining HA and MSCs. We therefore profiled pro-inflammatory cytokines in an attempt to measure the effects of HA on MSCs either in SVF, SVF plus mature adipocytes and culture MSC populations. Significant reductions in a well-established pro-inflammatory cytokine, interferon gamma in the SVF plus adipocyte preparation (figure 2.1) suggests this is a favorable preparation, although the fold change of all other preparations trended downward also. Interleukin 12 also trended downward for all preparations and significantly for the SVF, SVF plus adipocytes and the MSC media. Interleukin 12 is expressed in the OA milieu [342] by infiltrating immune cells such as mature dendritic cells [343] however MSCs are known to inhibit the maturation of dendritic cells [344] and thus a combination with HA for

this cytokine may be desirable. The MSC seeding preparation was intended to model co-intra-articular injection of purified MSCs with HA. The significant increase in macrophage migration inhibitory factor (MIF) (figure 2.1) may contraindicate the combination of purified MSCs with HA. Animal models have shown a correlation with reduced severity of OA and depletion of MIF [345]. MIF acts in local tissue to increase neutrophil and macrophage migration to regions of inflammation. Recent investigations have shown that TNF-β (lymphotoxin-alpha) initiates an inflammatory response in human chondrocytes as a consequence of increase NF-κB signaling [346]. Figure 2.1 shows there is both a significant increase of TNF-β in both the SVF alone and MSC seeding preparations, although the SVF plus adipocytes increased but not significantly, further supporting the SVF plus adipocytes as an ideal preparation. The secretion of Interleukin-1 alpha (IL-1α) trended upward for most preparations however only significantly for the SVF alone preparation. Interleukin-1 family of cytokines have long been implicated in degradation of the knee joint cartilage in OA [324], pending *in vivo* trials, this increase may amplify endogenous interleukin-1 secretion in the OA joint much to the detriment of MSC therapy. Interleukin-1 beta (IL-1β) is heavily implicated in cartilage catabolism [347]. Collagen-induced arthritis models in mice have shown HA alone can reduce IL-1β mRNA expression in articular cartilage [348]. Here we have shown minimal changes in purified MSC secretion of IL-1β when combined with HA, however when the SVF plus mature adipocytes were exposed to HA there was a significant 41% (figure 2.1) decrease in secretion of IL-1b as opposed to an increase in secretion when the SVF alone was combined with HA. This may contraindicate the use of the SVF alone in combination with HA but encourage the addition of mature adipocytes.

Anti-inflammatory cytokines and growth factors play a crucial role in the regenerative potential of MSCs. As such, a decrease in anti-inflammatory cytokines

as a result of the combination with HA may not be desirable in the context of trophic efficacy. Interleukin-10 decreased significantly in both the SVF plus adipocytes and in the cultured MSC media preparation (figure 2.2). Interleukin-10 secretion by MSCs has been proposed to be a cytokine involved in the inhibition of immune cell, Th17, differentiation [349]. Therefore a downward trend in the preparation when combined with HA, as seen in figure 2.2, may not be desirable for MSC therapy as it may hinder the immunomodulatory capacity. Basic fibroblast growth factor (FGF-β) increased significantly in the cultured MSC seeding preparation (figure 2.3). Which could suggest increased proliferative capacity of MSCs as FGF-β is a potent mitogen. It may also play a synergistic role in regenerating cartilage in OA with MSCs. Animal models of cartilage defects have shown recombinant human FGF-β alone potentiates articular cartilage resurfacing after just 6 weeks [336]. However other studies suggest endogenous FGF-β stimulates the expression of matrix metalloproteinase-13 [337], an enzyme which degrades type II collagen present in articular cartilage. Granulocyte macrophage colony-stimulating factor (GM-CSF) significantly increased in the SVF alone preparation (figure 2.3). This change may negatively affect MSC therapy for the treatment of OA as GM-CSF is a key mediator in inflammation and arthritic pain [339]. Animal studies of experimental osteoarthritis using knockout mice showed the development of OA was strictly dependent on GM-CSF [350]. Nerve growth factor-beta (β-NGF) showed a downward trend in most preparations and significantly in the SVF plus adipocytes and the cultured MSC seeding preparations (figure 2.3). β-NGF in human osteoarthritic-derived chondrocytes shows increased mRNA expression when compared to chondrocytes from healthy donors [338] suggesting the combination of HA with MSCs might be beneficial with decreased overall levels of β-NGF. Vascular endothelial growth factor (VEGF) is expressed in osteoarthritic-derived chondrocytes and shown to increase osteo-chondral angiogenesis in OA patients [340]. Animal models have shown

intra-articular injection of recombinant VEGF causes cartilage degradation as in OA [351]. VEGF showed a downward trend across all preparations and decreased significantly in the SVF plus adipocyte and cultured MSC media preparations when combined with HA (figure 2.3). Colony stimulating factors are pivotal in influencing cartilage metabolism, a combination of IL-1β and granulocyte colony stimulating factor (G-CSF) significantly increased nitrite levels by cartilage explants [341]. In the SVF plus mature adipocytes preparation G-CSF secretion decreased 50% when combined with HA, the other preparation showed no appreciable changes. This again re-iterates the SVF plus mature adipocyte preparation as an ideal combination with HA.

Chemokines are chemoattractant cytokines that play a pivotal role in regulating migration and infiltration of immune cell populations. In a study of OA-derived chondrocytes it was found that Monocyte chemotactic protein-1 (MCP-1) induced matrix metalloproteinase-3 expression and inhibited proteoglycan synthesis [334]. MCP-1 in OA-derived sub-chondral bone marrow stromal cells also showed constitutive expression [352]. MCP-1 secretion decreased across all preparations and significantly for the SVF alone and SVF plus mature adipocytes by 35% and 25% respectively. This suggests a combination of these preparations with HA may synergistically work to reduce immune cell recruitment. Macrophage inflammatory protein-1-alpha (MIP-1α) showed increased expression in OA-derived bone marrow stromal cells when challenged with IL-1β [352]. Therefore modulating levels of MIP-1α is desirable for the treatment of OA. Secretion of this chemokine decreased across both the SVF alone and SVF plus adipocyte preparation but not appreciably in the purified MSC preparations.

3.8 Conclusion

In this study we investigated a range of adipose-derived mesenchymal stem cell preparations, some of which are currently used for autologous therapy and some which will become important for future off-the-shelf allogeneic preparations. As the mode of action of MSCs is driven by the secretion of immuno-modulatory and trophic factors, we assessed changes in the secretion of cytokines in the conditioned media when cells were co-cultured with HA. When co-cultured with HA, SVF plus mature adipocytes showed the greatest decrease in the pro-inflammatory cytokines IL-1β, IFN-ɣ and the growth factor VEGF, which has been identified as a negative influence in OA. Two chemokines, MCP-1 and MIP-1α decreased substantially in the HA SVF preparations, with and without adipocytes, but not the purified MSCs. Both the SVF alone and purified MSC populations also had small but significant increases in TNF-β when co-cultured with HA. There was an increase in TNF-β in the HA co-culture of SVF and adipocytes, but this was not significant.

The purified MSCs co-cultured with HA was the only preparation to show increased MIF, a major pro-inflammatory cytokine, which is correlated with OA severity. The increased concentration was observed at the initial stage of cell seeding and MIF was below the control concentration by the media change at day three. It is unknown what effect a transient spike of MIF may have on OA pathology and this would be ideally tested in an animal model.

As shown in previous *in vitro* and *in vivo* studies from our group, the mixed population of cells in adipose SVF plus adipocytes produces a distinct and therapeutically superior cytokine profile. In this study the SVF plus mature adipocytes preparation appears to be most suited of all the preparations for combination with HA.

3.9 Acknowledgments

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4

Priming adipose-derived mesenchymal stem cells with Hyaluronan alters growth kinetics and increases attachment to articular cartilage

Prologue

Our previous findings on the alterations of the MSC secretome co-cultured with HA, concentrated on clinically relevant preparations. In this investigation we sought to refine our understanding on the effects of the MSC/HA combination. We moved to culture purified MSCs to determine how HA would affect the physical attributes of growth kinetics, especially with respect to cartilage. Dr Michael Medynskyj, a former member of the Herbert lab was now conducting research at Regeneus Pty Ltd. Dr Medynskyj was working with cartilage shavings and experimenting with MSC growth kinetics. To improve consistency, together we decided to utilise a hole punching tool, purchased from the local hardware store to create cartilage discs. The absolute size of the hole punch proved problematic, either being too small or too large to create a seal at the bottom of a 96-well plate. I then worked closely with Walther Adendorff at the Macquarie Engineering & Technical Services unit to design and manufacture a custom cartilage sectioning tool measured to the exact size of a 96-well plate. This tool proved invaluable to the success of a reproducible cartilage disc assay utilised in testing MSC interactions with cartilage explants. I would like to thank Tony Batterham for allowing me to work at the equine centre to harvest the joints from horses and Mark Molloy for agreeing to become my official supervisor at Macquarie University when we relocated our lab during my PhD. I would also like to thank Dr Edmond Breen for the continued statistical support and review of the manuscript and Dr Benjamin Herbert, for helping me to coordinate resources, aiding in the experimental design and final review of the manuscript

The results of this research were reported in the form of a peer-reviewed journal article in *Stem Cells International*.

Contributions to publication statement

The concept of this publication was developed in collaboration with Dr Michael Medynskyj. Dr Benjamin Herbert provided the conceptual foundation and assisted in the coordination of this study. Dr Edmond Breen provided statistical support throughout all projects. Peter Succar performed all of the experimental work, data analysis and the preparation of the manuscript.

Table 4.1: Author contribution matrix for Publication.

	Peter Succar	Edmond J Breen	Michael Medynskyj	Tony Batterham	Mark Molloy	Benjamin Herbert
Experiment Design	●		●			●
Provision of Study Materials	●			●		
Data Collection	●					
Data Analysis	●	●				
Manuscript	●	●			●	●

Citation

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4.1 Priming adipose-derived mesenchymal stem cells with Hyaluronan alters growth kinetics and increases attachment to articular cartilage

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4.2 Disclosures

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4.3 Abstract

Biological therapeutics such as Adipose-derived Mesenchymal stem cells (MSC) therapy are gaining acceptance for the treatment of inflammatory degenerative conditions, such as knee osteoarthritis (OA). Some reports of OA-patients show reductions in cartilage defects and regeneration of hyaline-like cartilage with MSC-therapy. Suspending MSCs in hyaluronan is common practice in animal models, treatment of human OA, usually without supporting data. Herein we sought to elucidate the effects of different concentrations of hyaluronan on MSC growth kinetics and evaluate MSC secretome. Using a range of hyaluronan concentrations, we measured MSC adherence and proliferation on both culture plastic surfaces and a novel cartilage-adhesion assay. We employed time-course and dispersion imaging techniques to assess MSC binding to cartilage. Additionally, 48 cytokines were profiled to assess the MSC-hyaluronan combination on the MSC-secretome. Hyaluronan has profound dose-dependent effects on early growth kinetics of MSCs at concentrations up to the hyaluronan entanglement point at 1 mg/mL. At higher concentrations viscosity effects outweigh any benefit of additional HA. The cartilage-adhesion assay highlighted for the first time that hyaluronan-primed MSCs can increase cell attachment to cartilage and that the presence of hyaluronan did not. Our time-course suggested patients undergoing MSC-therapy for knee OA, could benefit from joint immobilisation for up to 8 hours post-injection to enable MSCs to thoroughly adhere in the joint. Additionally, concentration of HA was also shown to greatly affect the dispersion of MSCs on cartilage. Our results should be considered in future trials with MSC-therapy using hyaluronan as a vehicle, for the treatment of OA.

4.4 Introduction

Osteoarthritis is a degenerative disease characterised by degradation of cartilage and inflammation of the synovium [353]. Arthritic-degeneration and associated pain leads to reduced mobility, decreased economic contribution and a significant healthcare burden of developed nations [1]. Conventional treatments of OA for middle-age sufferers are targeted at relieving pain using analgesics and non-steroidal anti-inflammatories [354]. Others, include intra-articular injection of exogenous preparations of hyaluronan (HA) as a viscosupplementation therapy [355]. End stage treatment of OA involves surgical realignment or total knee replacement (TKR) with artificial prostheses. Increasing lifespan has exacerbated the problem and widened the treatment gap for middle aged sufferers who are not suitable candidates for TKR [174].

Recently, adipose-derived mesenchymal stem cells (MSCs) have shown promise as an OA therapeutic. Numerous *in vitro* studies have demonstrated MSCs can differentiate into mesodermal cell types that form cartilage, bone and fat. However, differentiation of implanted cells *in vivo* is a rarely documented mode of action. Perhaps differentiated allogeneic cells are not immune privileged and would be removed by the host immune system. Paracrine effects, both anti-inflammatory and trophic, via the secretion of a complex mixture of cytokines is currently a more accepted and well-studied mode of action [242]. The trophic action of cytokines act to stimulate and mobilise endogenous MSCs to repair and regenerate tissue.

MSCs can improve function and pain in patients suffering from knee OA with minimal adverse events. Patients show a consistent reduction in the size of cartilage defects by the regeneration and neo-formation of hyaline-like cartilage [310]. Manufacture of cell therapeutics for OA may involve the use of HA, particularly as a vehicle. The rationale for this is that HA is a core component of the extracellular

matrix, endogenously abundant in the knee joint [118,119] and approved for the treatment of knee OA.

Suspending MSCs in HA is common practice in rodents [313], larger animals [291,290] and for the treatment of OA in humans [314,315]. MSCs and HA are safe and are used independently in humans, however there are few studies which explore the effect of HA on MSC growth-kinetics, function and tissue binding, especially with respect to cartilage.

Therefore our study sought to address alterations of MSC-HA growth-kinetics and whether changes would persist in HA-primed cells both on plastic and articular cartilage. We employed a novel cartilage-disc assay in a time-series to determine adherence-time and assessed the physical dispersion of MSCs over cartilage shedding light on the biological relevance of the physio-chemical phenomena of HA-entanglement point. As the mode of action of MSCs is secretion-driven, the cytokine profile was measured to assess clinical feasibility of the MSC-HA combination.

4.5 Materials and Methods

4.5.1 MSC growth kinetics

Adherence. This experiment was undertaken on *standard* 96-well plates and CellBind 96-well plates (*High*-adherence*;* Corning, Australia) to test the effect of HA on different binding chemistries. Cells were seeded into 96-well plates at 5×10^3 cells/well using a combination of media preparations (see supplementary figure 4.1). A standard curve was seeded ranging from 1×10^3 to 16×10^3 cells/well. After 24 hours all wells were washed in PBS and 100 µL of control media added. A further 10 µL of Cell-Counting Kit-8 (water soluble tetrazolium salt, WST-8; CCK-8) reagent was added and following a four hour incubation at 37°C, the absorbance measured at 450 nm (5 technical-replicate; n = 3). Cell number was extrapolated from the standard curve using a trend line with a polynomial of two. Combinations of the media preparations *(- +)*, *(+ -)* and *(+ +)* were compared back to cells grown in control media and seeded in control media *(- -)* using a Mann Whitney test (non-parametric test).

Proliferation. This experiment was similar to the *Adherence* protocol with minor differences. Cells were seeded into 96-well plates at 2×10^3 cells/well (5 technical-replicates; *n = 3)*. The standard curve was seeded on the second day, 24 hours before the 3 day endpoint.

4.5.2 MSCs kinetics on equine articular cartilage explants: *ex vivo* cartilage assay

All horses used in this study were due to be sacrificed at a commercial abattoir to be processed and sold as dog food (Kankool Pet Food, Australia). The fetlock joint from mature horses was cut out by sawing 15 cm below and above the joint. The fetlock was then shaved, scrubbed and soaked in iodine bath for a minimum contact time of 5 minutes and then frozen for later use.

4.5.3 Cartilage sectioning

The frozen fetlock joint was thawed at 37 degrees for 1-2 hours until the fetlock joint became flexible. Once thawed, the joint was dis-articulated in a sterile hood (see supplementary figure 4.2). The third metacarpal was obtained from the fetlock joint and the distal end was soaked in PBS for 5 minutes. The articular cartilage was then perforated using a custom designed hollow cylindrical instrument measuring 6.45 mm in diameter equal to a *standard* 96-well plate. The perforated discs were then sliced off with a scalpel and stored in the correct orientation (Joint side facing up) in a 96-well plate with 200 µL of DMEM and frozen for later use.

4.5.4 Cartilage-adherence time-course of MSCs

Cartilage discs were used to plug *ultra-low* adherence 96-well plates. Passage 2 cells were seeded onto the cartilage discs at a density of 5×10^3 cells/disc and incubated at $37°C/5\%$ CO_2. At each time points (1, 2, 3, 4, 8 & 24 hours), cartilage discs were washed twice in PBS, fixed and washed again. Cartilage discs were stained (see supplementary methods) and imaged using confocal microscopy.

4.5.5 HA media viscosity assessment

Falling-ball viscometry was used to determine the viscosity (adapted from Eguchi and Karino, 2008 [356]) of HA media relative to control. Briefly, a 5 mL serological pipette was filled with pre-warmed media. A bio-silicate sphere was dropped and the time measured between two defined points. The data was expressed as a mean (5 technical-replicates) flow-rate (millimetres/second) ± standard deviation.

4.5.6 MSC dispersion on cartilage with increasing concentration of HA

The media formulations used were control media, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, 3mg/mL, 4mg/mL and 5 mg/mL HA media. MSCs were seeded onto the cartilage-discs at a density of 5×10^3 cells/disc. Following a 24 hour incubation, cartilage discs were washed twice in PBS, fixed and washed again. Cartilage discs were stained (see supplementary methods) and imaged using confocal microscopy.

4.5.7 MSC adherence & proliferation on cartilage

MSCs were treated with either control or 1 mg/mL HA media for three days. *Ultra-low* adherence 96 well plates were plugged with cartilage-discs. The conditions tested included; MSCs grown in control and seeded in control *(- -)*, grown in control and seeded in 1 mg/mL HA media *(- +)*, grown in 1 mg/mL HA media and seeded in control *(+ -)* (primed) and grown in 1 mg/mL HA media and seeded in 1 mg/mL HA media *(+ +)*.

Adherence. MSCs were seeded onto cartilage discs at a density of 5×10^3 cells/disc. After 24 hours cartilage-discs were removed to a new 96-well plate and washed twice with PBS. Fresh culture media (200 µL) was added to discs followed by 20 µL of CCK-8 and incubated for four hours at 37°C. Colour-developed wells were read at an absorbance of 450 nm (5 technical-replicates; n = 3).

Proliferation. This experiment was undertaken in the same way as the *adherence* experiment, however the cells were cultured for 3 days and seeded at a density of 2×10^3 cells/disc.

4.5.8 Secretome analysis

Conditioned media was collected from every flask in this study *(n=3)*, centrifuged at 5000 x g for 5 minutes and stored at -80°C. Filtrates (50 µL) were analysed using both the Bio-Plex Pro-Human Cytokine 27-plex and Bio-Plex Pro-Human Cytokine

21-plex assay (Bio-Rad, USA), according to the manufacturer's instructions (see

supplementary methods).

4.6 Results

4.6.1 MSCs grown in control media and seeded in HA media

HA concentrations were compared to control i.e. **(- -) v (- +)**. In *standard* 96-well plates, the highest cell proliferation was observed for 1 mg/mL HA ($p < 0.07$). In *high*-adherence 96-well plates the 2 mg/mL HA decreased adherence by 45% ($p < 0.05$) (figure 4.1).
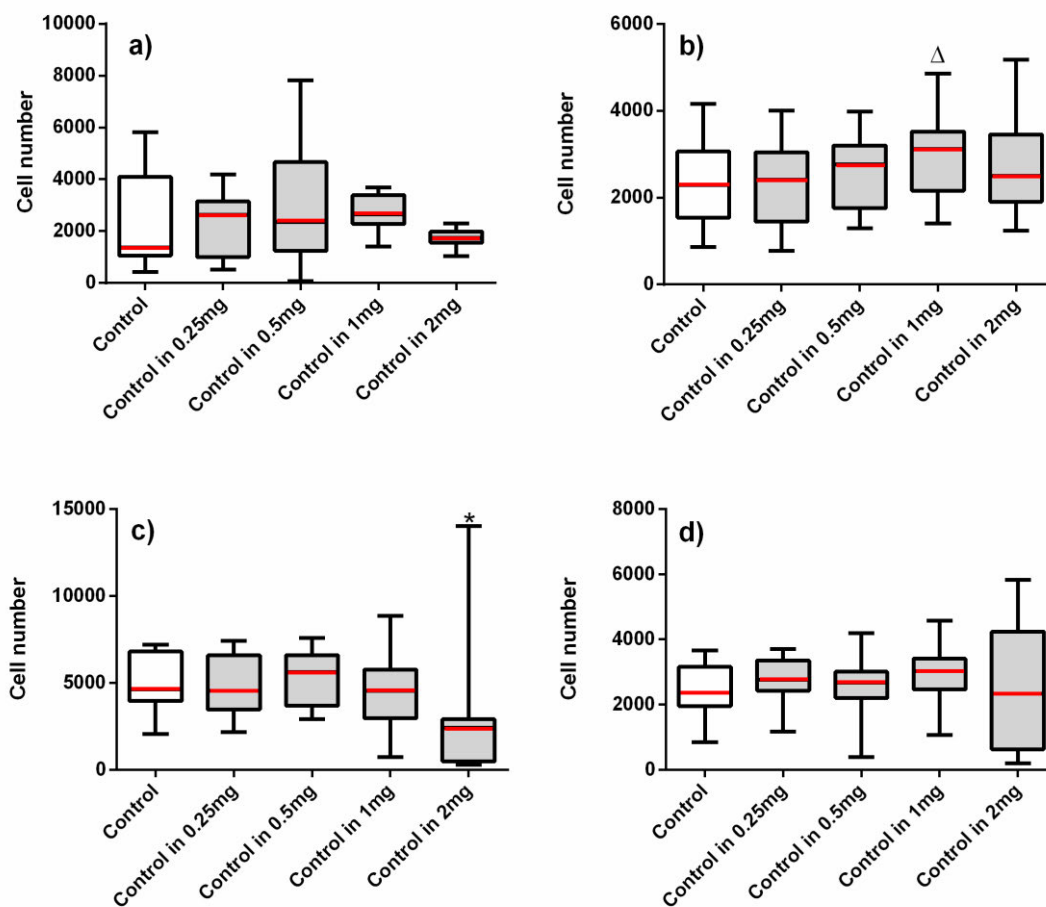
Figure 4.1 MSCs grown in control media and seeded in HA media

MSCs expanded until passage 2 in control media. Cells from flask (A) were then stripped and cell suspensions were counted using the standard enumeration technique. Cells were seeded into 96-well plates in either control media (control) *(- -)* or HA media *(- +)* containing a series of concentrations ranging from 0.25 to 2 mg/mL of HA to make up the five conditions *(x-axis)*. Conditions were tested using five technical replicates per 96-well plate and run in biological triplicate *(n=3)*. Wells were assayed at the endpoint using cell counting kit-8 with standard curves run on every 96-well plate seeded 24 hours prior to endpoint and absorbance read at a wavelength of 450nm *(y-axis)*. All conditions were compared back to the control using a t-test *(Δ p-value = 0.07, * p-value < 0.05)*. **a)** MSCs seeded in *standard* 96-well plates for 24 hours (adherence) **b)** MSCs seeded in *standard* 96-well plates for three days (proliferation) **c)** MSCs seeded in *high*-adherence 96-well plates for 24 hours (adherence) **d)** MSCs seeded in *high*-adherence 96-well plates for three days (proliferation).

4.6.2 MSCs grown in HA media and seeded in control media (priming)

MSCs grown in HA media (B-E) were compared to control **(- -) v (+ -)**. In *standard* 96-well plates, HA primed conditions showed peaked adherence for 0.5 mg/mL and 1 mg/mL HA ($p < 0.05$). Inversely, in *high*-adherence 96-well plates, adherence decreased with increasing concentration of HA and significantly for 2 mg/mL HA ($p < 0.05$). However in *high*-adherence 96-well plates, proliferative effects of HA priming recovered with 0.5, 1 and 2 mg/mL HA significantly ($p < 0.05$, < 0.001 and < 0.01 respectively) increased (figure 4.2).
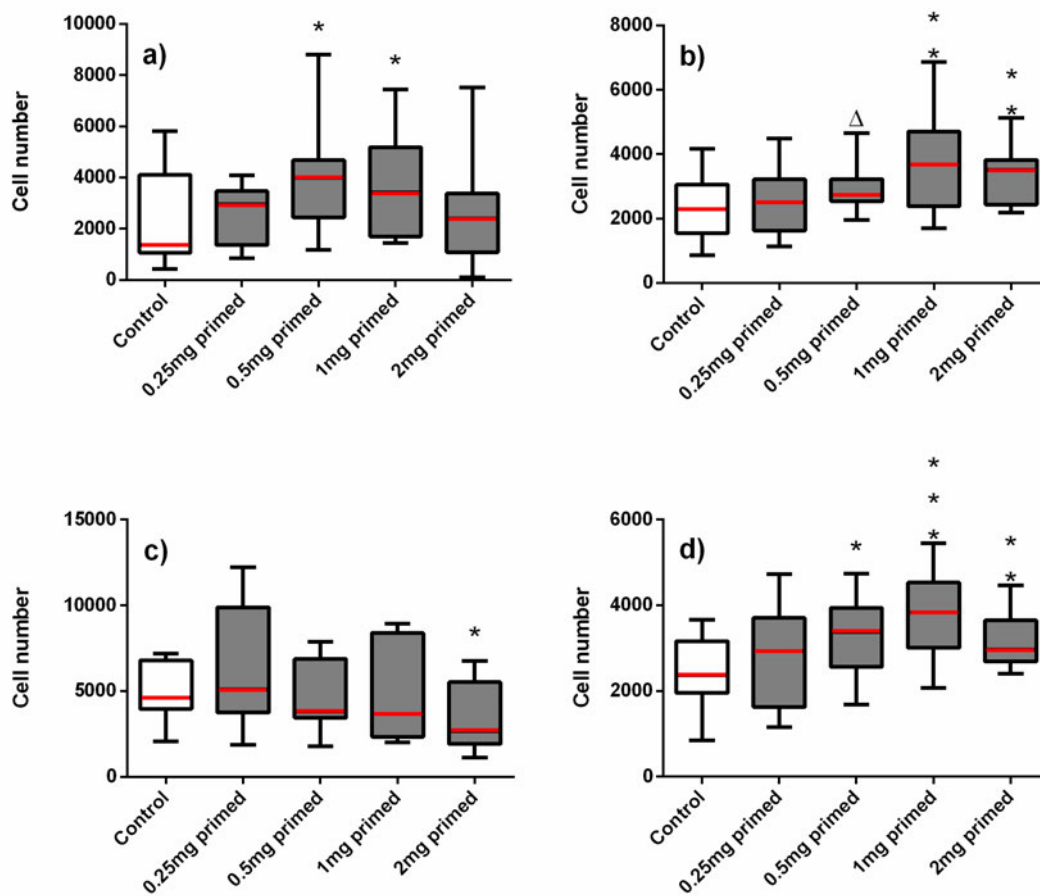
Figure 4.2 MSCs grown in HA media and seeded in control media (primed)

MSCs expanded until passage 2 in control media. Cells were then stripped and cell suspensions were counted using the standard enumeration technique. Cells grown control media, flask (A), were seeded in control media (control) *(- -)* and MSCs grown in flasks (B-E) HA media, were seeded into 96-well plates in control media (HA primed) *(+ -)*, to make up the five conditions *(x-axis)*. Conditions were tested using five technical replicates per 96-well plate and run in biological triplicate *(n=3)*. Wells were assayed at the endpoint using cell counting kit-8 with standard curves run on every 96-well plate seeded 24 hours prior to endpoint and absorbance read at a wavelength of 450nm *(y-axis)*. All conditions were compared back to the control using a t-test *(Δ p-value = 0.1, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001).* **a)** MSCs seeded in *standard* 96-well plates for 24 hours (adherence) **b)** MSCs seeded in *standard* 96-well plates for three days (proliferation) **c)** MSCs seeded in *high*-adherence 96-well plates for 24 hours (adherence) **d)** MSCs seeded in *high*-adherence 96-well plates for three days (proliferation).

4.6.3 MSCs grown in HA media and seeded in HA media

MSCs grown in HA media (B-E) were compared to control **(- -) v (+ +)**. In *standard* 96-well plates, adherence showed a dose-dependent trend with a peak at 1 mg/mL HA ($p < 0.001$) and decreased in 2 mg/mL HA. Similarly proliferation with 0.5, 1 and 2 mg/mL HA increased, but peaked at 1 mg/mL HA ($p < 0.05$, < 0.01, and < 0.01 respectively). Inversely in *high*-adherence 96-well plates, adherence decreased with increasing concentration of HA and significantly for 2 mg/mL HA ($p < 0.01$). However proliferation again increased with 0.5 and 1 mg/mL HA ($p < 0.01$, < 0.001 respectively) (figure 4.3).
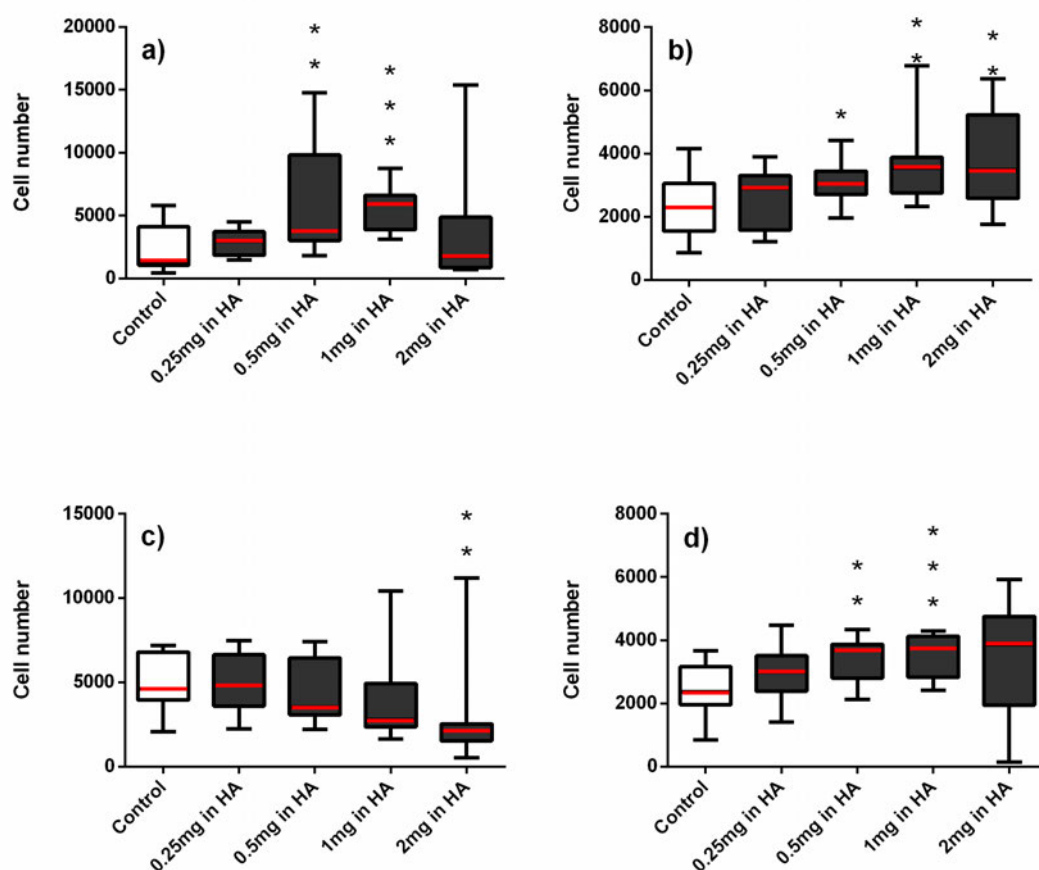
Figure 4.3 MSCs grown in HA media and seeded HA media

MSCs expanded until passage 2 in control media. Cell were then stripped and cell suspensions were counted using the standard enumeration technique. Cells grown control media, flask (A), were seeded in control media (control) *(- -)* and MSCs grown in flasks (B-E) HA media, were seeded into 96-well plates in HA media with the same concentration of HA *(+ +)* ranging from 0.25 to 2 mg/mL of HA to make up the five conditions *(x-axis)*. Conditions were tested using five technical replicates per 96-well plate and run in biological triplicate *(n=3)*. Wells were assayed at the endpoint using cell counting kit-8 with a standard curves run on every 96-well plate seeded 24 hours prior to endpoint and absorbance read at a wavelength of 450nm *(y-axis)*. All conditions were compared back to the control using a t-test *(* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001).* **a)** MSCs seeded in *standard* 96-well plates for 24 hours (adherence) **b)** MSCs seeded in *standard* 96-well plates for three days (proliferation) **c)** MSCs seeded in *high*-adherence 96-well plates for 24 hours (adherence) **d)** MSCs seeded in *high*-adherence 96-well plates for three days (proliferation)

4.6.4 Time course of MSC grown on cartilage

Ultra-low adherence 96-well plates were plugged with cartilage-discs and MSCs seeded in control media to assess adherence-times from one to 24 hours (figure 4.4). The one hour time point a), only a handful of cells adhered to cartilage. At higher magnification g) MSCs appeared spherical with extensions clearly visible. From three to four hours (c-d), MSCs covered the surface with overlapping flat cell bodies. Overlapping between cells e) was greatly reduced by eight hours and cell began to display h) spindle morphology. At 24 hours f) MSCs adhered to cartilage and dissipating membrane stain indicated possible doubling i) typical fibroblastic-like MSC morphology and organisation can be observed.
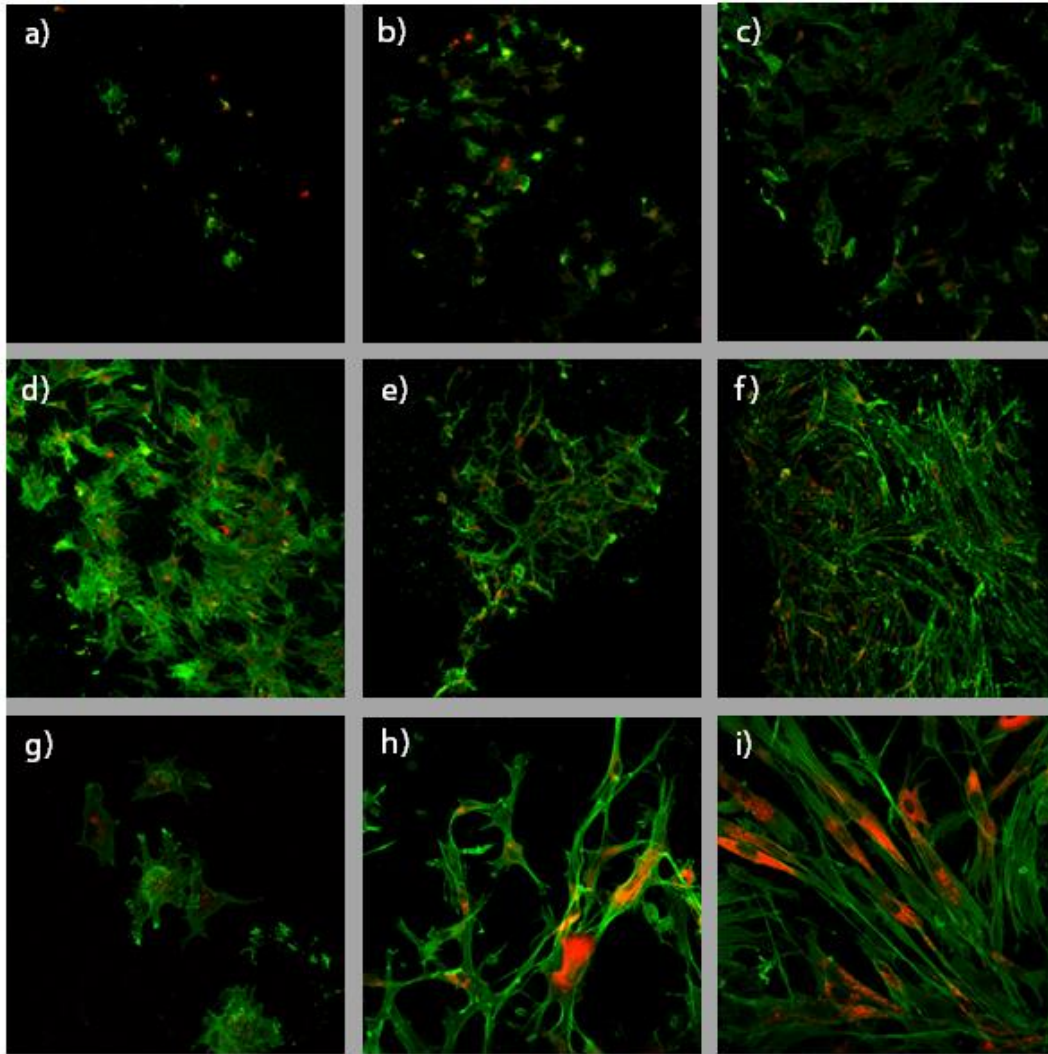
Figure 4.4 Cartilage adherence time course of MSCs

MSCs were cultured until the second passage before use in this experiment. Prior to seeding cells for the time course, the monolayer was washed in PBS and then stained using CM-DiI (orange colour) membrane dye. Cartilage discs were used to plug the bottom of a 96-well place in the correct orientation. Cells were then stripped using TrypLE, counted using the standard enumeration technique and then seeded onto the cartilage discs at a density of 5×10^3 cells/disc and the 96-well plate was then incubated at 37°C and 5% CO_2. At each of the time points (1, 2, 3, 4, 8 & 24 hours), the cartilage discs were washed in PBS and then fixed in 4% paraformaldehyde and then washed in PBS. Cartilage discs with attached cells were then permeabilised in 0.1% Triton X-100, washed in PBS, blocked in 1% Bovine serum albumin in PBS before being stained with F-actin-specific Alexa Fluor 488-phalloidin (green). All images were taken using the OLYMPUS FLUOVIEW FV1000 IX81 inverted confocal microscope. **a)** one hour time point at 10x magnification **b)** two hour time point at 10x magnification **c)** three hour time point at 10x magnification **d)** four hour time point at 10x magnification **e)** eight hour time point at 10x magnification **f)** 24 hour time point at 10x magnification **g)** one hour time point at 40x magnification **h)** eight hour time point at 40x magnification **i)** 24 hour time point at 40x magnification.

4.6.5 MSC dispersion on cartilage with increasing concentration of HA and viscosity of media formulations

Ultra-low adherence 96-well plates were plugged with cartilage-discs and MSCs were seeded suspended in either control or HA media (0.5-5 mg/mL HA). At lower concentrations of HA, MSCs uniformly dispersed across the cartilage surface. As concentration increased to 3 mg/mL dispersion was maintained but with reduction in adhered cells. Singular colonies were observed at highest concentrations 4 mg/mL and 5 mg/mL HA (figure 4.5).

The flow-rate of the sphere in the falling-ball test was measured to show viscosity of HA media formulations relative to control (see supplementary figure 4.3). The control displayed the highest flow-rate, which decreased with increasing concentrations of HA. The flow-rate for 0.5 mg/ml and 1mg/ml HA was 57% and 51% of the control respectively. Flow-rate of 2 mg/mL HA dropped to 20% of control and then a plateau observed for higher concentrations.
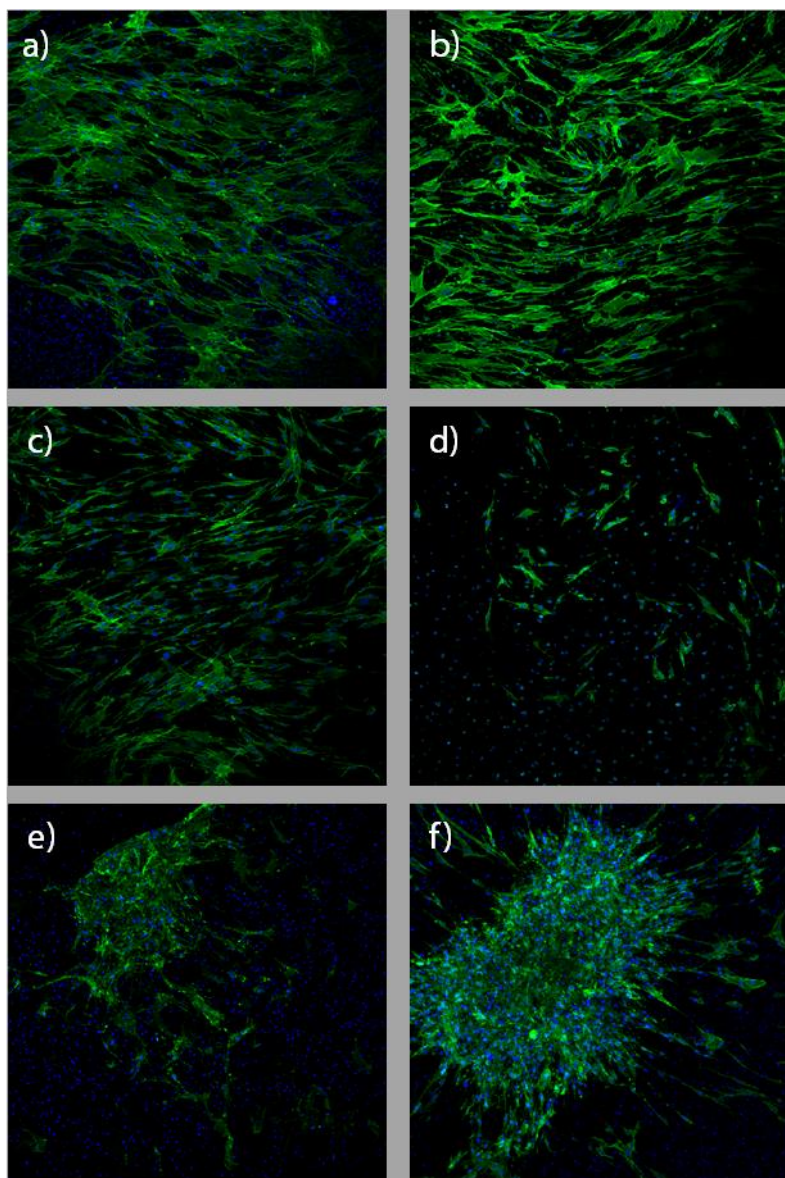
Figure 4.5 MSC dispersion on cartilage with increasing concentration of HA

MSCs were cultured until the second passage before use in this experiment. Cartilage discs were used to plug the bottom of a 96-well place in the correct orientation. Media formulations were then added to each well. The media formulations used were **a)** 0.5 mg/mL HA media **b)** 1 mg/mL HA media **c)** 2 mg/mL HA media **d)** 3 mg/mL HA media **e)** 4 mg/mL HA media and **f)** 5 mg/mL HA media. MSCs were then stripped using TrypLE, counted using standard enumeration technique and then seeded onto the cartilage discs at a density of $5x10^3$ cells/disc. The 96-well plate was then incubated for 24 hours at 37°C and 5% CO_2. After 24 hours the cartilage discs were washed in PBS and then fixed in 4% paraformaldehyde. Cartilage discs were washed in PBS and then stained in using Hoechst 33342 (blue) and again washed five times in PBS. Cartilage discs were then permeabilised in 0.1% Triton X-100, washed in PBS, blocked in 1% Bovine serum albumin in PBS before being stained with F-actin-specific Alexa Fluor 488-phalloidin (green). All images were taken using the OLYMPUS FLUOVIEW FV1000 IX81 inverted confocal microscope at 10x magnification.

4.6.6 MSC adherence & proliferation on cartilage

The 1 mg/mL HA formulation was tested for all conditions (figure 4.6). *Ultra-low* adherence 96-well plates were plugged with cartilage-discs and cells seeded at either 5×10^3 cells (*adherence*) or 2×10^3 cells (*proliferation*) per well. In the adherence experiment, a peak was observed in the primed *(+ -)* condition ($p < 0.05$). A similar peak was observed for the primed condition in the proliferation experiment ($p = 0.1$).
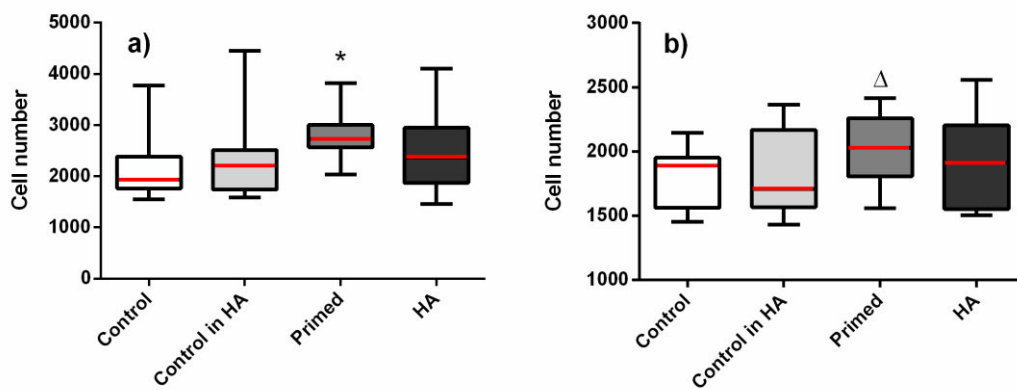
Figure 4.6 Treated MSCs seeded onto articular cartilage explants for 24 hours and three days

MSCs expanded until passage 2 in control media. On the third day flasks were treated with either control media or 1 mg/mL HA media for another three days. Cells were then stripped and cell suspensions were counted using the standard enumeration technique. Cells were seeded into *ultra-low* adherence 96-well plates plugged with *articular cartilage explants* in the correct orientation. Conditions *(x-axis)* tested were cells grown in the control flask and seeded in control media (**Control**) *(- -)* , grown in control media and seeded in 1 mg/mL HA media (**Control in HA**) *(- +)*, grown in 1 mg/mL HA media and seeded in control media (**Primed**) *(+ -)*, grown in 1 mg/mL HA media and seeded in 1 mg/mL HA media (**HA**) *(+ +)* and articular cartilage explants alone (no cells seeded to serve as a blank absorbance). Conditions were tested using five technical replicates per 96-well plate and run in biological triplicate *(n=3)*. Wells were assayed at the endpoint using cell counting kit-8 and absorbance of the resulting media alone read at a wavelength of 450nm *(y-axis)*. All conditions were compared back to the control using a t-test *(Δ = 0.19, * p-value < 0.05)*. **a)** MSCs seeded for 24 hours (adherence) into *ultra-low* adherence 96-well plates plugged with articular cartilage explants **b)** MSCs seeded for three days (proliferation) into *ultra-low* adherence 96-well plates plugged with articular cartilage explants.

4.6.7 HA media alters the cytokine secretion profile of MSCs

MSCs were treated for three days in either control or HA media formulations. Changes in concentration were observed in 28 of the 48 measured cytokines (see Supplementary figures 4.4 – 4.7 and supplementary table 4.2). MSCs treated with 1 mg/mL HA, increased secretion for Interleukin-1β (IL-1β) and Macrophage migration inhibitory factor (MIF) (figure 4.7). Anti-inflammatory cytokine Interleukin-1 receptor antagonist (IL-1ra) secretion increased dose-dependently and was significant at 2 mg/mL HA. Interleukin-6 (IL-6), a dual role cytokine, increased in the 0.25 mg/mL HA concentration but then decreased with increasing concentration of HA. Fibroblast growth factor-basic (FGF-β), increased dose-dependently and was significant at 2 mg/mL HA. Vascular endothelial growth factor (VEGF) decreased with increasing concentration of HA and was significant at 2 mg/mL HA.
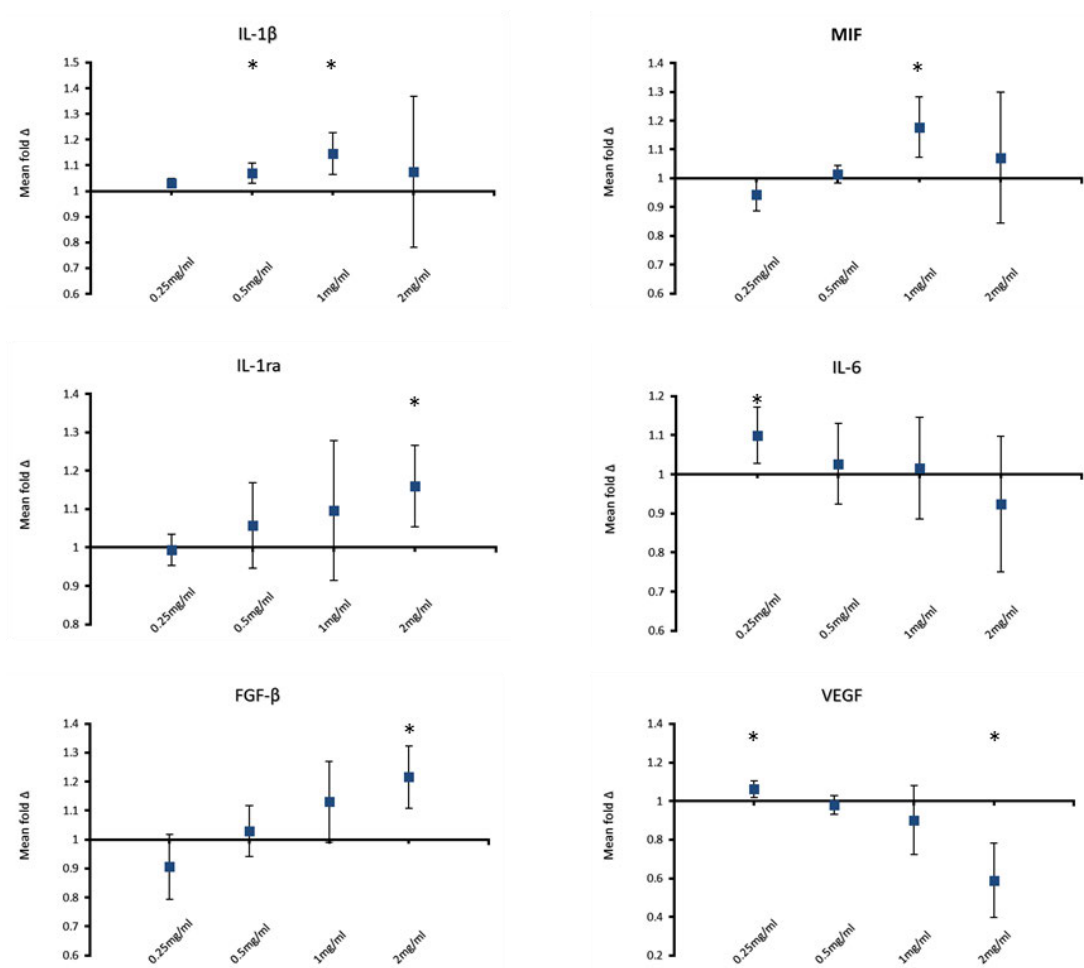
Figure 4.7 fold change of cytokine secretion by MSCs with HA treatment

Conditions were all cultured in either control media or HA media for three days after the media change. Conditions *(x-axis)* were grown in flasks (B-E) HA media, ranging from 0.25 to 2 mg/mL of HA. These were all compared back to the control condition (cells grown control media, flask (A)). Average fold change in fluorescence *(n=3)* ± upper & lower confidence intervals at 95% *(y-axis)*. Cytokines with a fold change less than one indicate a decrease with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment (* cytokines with a fold change clear of the axis at 1 reported as significant). Interleukin-1β (IL-1β), Macrophage migration inhibitory factor (MIF), Interleukin-1 receptor antagonist (IL-1ra), Interleukin-6 (IL-6), Fibroblast growth factor-basic (FGF-β), Vascular endothelial growth factor (VEGF). See supplementary data for complete list of significantly changed cytokines and numerically tabulated summary.

4.7 Discussion

Biological therapeutics such as MSC therapy are gaining acceptance for the treatment of inflammatory conditions, including musculoskeletal ailments such as knee osteoarthritis (OA). This is most likely due to the well documented treatment gap for middle-age sufferers of OA who are not candidates for total knee arthroplasty [174]. The safety of allogeneic MSC therapy will continue to drive the universal donor model because an "off-the-shelf" therapeutic is desirable for scalability and cost. The manufacture of cell therapeutics for the treatment of OA will likely involve the use of agents that can enhance MSC function. The use of HA is attractive as an adjunct, as it is a core component of the extracellular matrix, endogenously abundant in the knee joint [118,119] and approved for the treatment of OA. It is important to understand growth kinetics of MSCs with respect to HA. Already there have been case reports [357] and randomised, double blind, placebo controlled studies [315] where MSCs were either suspended in HA as a vehicle or as a combination for the treatment of OA. These studies did not document any exploration of positive or negative interactions between MSCs and HA [358]. Therefore we tested different concentrations of HA in culture media to assess adherence and early proliferation kinetics of MSCs on both plastic culture surfaces and equine articular cartilage explants in an *ex vivo* model. Seeding MSCs onto equine articular cartilage was an attempt to simulate early cell behaviour following an intra-articular injection of this combination. We hypothesised that the high viscosity of HA may interfere with cell binding and thus to avoid this problem, we explored whether MSCs could be primed with HA to improve growth kinetics or adherence. We employed a novel cartilage disc assay in a time series to determine MSC adherence to cartilage. In the same cartilage assay the physical dispersion of MSCs was assessed using a concentration series of HA media over the

cartilage shedding light on the biological relevance of the entanglement point of HA chains.

An allogeneic preparation of MSCs and HA may be developed in a number of ways; with HA only present in the final cellular product, used as a priming agent during culture but not in the final formulation or both. Here we tested three representative conditions and found that cells grown in standard culture and seeded in HA (- +) show some increases in adherence and proliferation. However, greater differences were observed in the (+ -) & (+ +) conditions, where HA as a culture supplement exhibited dose-dependent increases in cell number. It is well known that HA molecules behave in solution as highly hydrated randomly kinked coils, which start to entangle at concentrations of less than 1 mg/mL (entanglement point) [128]. The peak seen in the HA dose required for optimal cell attachment, is centred on this entanglement point of the HA chains. In this study, for the first time we have attributed biological significance to the physio-chemical phenomena of the entanglement point seen in HA chains. At this concentration of HA, all HA molecules are connected via a meshwork and behave like a weak and elastic gel which mimics properties of soft tissue [359].

In contrast to the standard plates, the *high*-adherence plates decreased the number of adherent cells in the presence of HA, and for the HA primed cells. According to the manufacturer, the surface is treated (non-biologically) to improve cell attachment by incorporating more oxygen into the cell culture surface by increasing surface area and rendering it more hydrophilic. Increasing concentrations of HA decreased the number of attached cells within the first 24 hours significantly. HA is a regulator of water homeostasis in the body [118], therefore it is reasonable to suggest HA interfered with the treated surface, an interference which persisted in the primed conditions.

The constraint of viscosity made it unclear whether increasing HA concentration further would amplify the effects seen in early time points. Viscosity-induced heterogeneity can be seen in the variance of the ranges observed for the 2 mg/mL HA concentration across all the growth kinetics experiments when cell were seeded in HA. Previous investigations of porcine bone marrow-derived MSCs showed using 4 mg/mL HA (*Mw 800KDa*) was optimal at 7 days [360] but no changes in two days. It is counterintuitive to expect optimal growth kinetics at 7 days since HA half-life in the knee joint is reported to be less than 24 hours [361].

Our study was concerned with high molecular weight HA, as it is more applicable as an OA therapeutic [362]. The study of low molecular weight HA found no difference in cell adherence or proliferation between 0.5, 1 & 2 mg/mL concentrations in the early time points. Another adhesion study using the same high molecular weight HA also found a decrease in synovial MSC adhesion with increasing concentrations beyond the entanglement point [363]. We employed a modified falling-ball test to show relative changes in viscosity from control media. The biggest change in viscosity was seen at concentrations above the entanglement point. The flow rate of which decreased by 31% between 1 & 2 mg/mL concentrations and then a plateau. This further explains decreases seen in cell attachment for concentrations higher than 1 mg/mL as a viscosity constraint in the early phase.

Increased attachment and proliferation on culture surfaces enabled the initial experiments to be performed under controlled conditions, but may not reflect cartilage binding. We therefore employed a novel equine-derived cartilage explant culture model *ex vivo* in an attempt to better mimic true architecture of the target tissue in OA, cartilage. Shimaya *et al.* had shown previously that synovial MSCs will increase adherence to an osteochondral defect with 10 mM magnesium *ex vivo* [364]. However, inconsistencies can be observed in defects created and further limitations

of subjectivity can arise when using an image analysis technique to quantify cell adherence. Baboolal *et al.* utilised a similar technique with osteochondral plugs embedded in agarose to test MSC adherence to cartilage. However, MSCs were labelled with iron-oxide micro-particles and semi-quantitative image analysis techniques were used to report cell adhesion [363]. Herein we have described a reproducible, consistent and quantitative method for cell attachment to biologically inactive cartilage in isolation. This enabled the testing of various cell and HA formulations without any modulatory signalling from the cartilage because it was not viable. Some considerations for our technique revolved around the plug integrity in the well i.e. if the cartilage disc curled and the well no longer sealed, the replicate was discarded. For this reason, all conditions were run using five technical replicates. The use of *ultra-low* adherence plates and repeated washing limited non-specific binding of cells to plastic and therefore gave a true indication of cell adherence to the cartilage.

Growth kinetics on the cartilage surface employed the optimal concentration of 1 mg/mL HA for all the conditions on *ultra-low* adherence plates plugged with the cartilage discs as the new surface. Interestingly, if the cells were seeded in the presence of HA (- + & ++), cell adherence did not improve which suggests the HA may interfere with MSCs binding to cartilage. Only the primed MSCs adhered (p < 0.05) and proliferated (p = 0.1) more than the control. Therefore it is reasonable to expect that the use of HA as a vehicle for MSCs during intra-articular injection for knee OA, will interfere with cell attachment to cartilage. Indeed this was consistent with previous investigations, where high molecular weight HA in OA-derived synovial fluid interfered with MSCs binding to cartilage, an effect which could be overcome by hyaluronidase (enzyme used to break down HA) treatment [363]. Furthermore it was shown in a caprine model using a collagen II-induced arthritis,

that the half-life of HA was an average of 11.5 hours and 20.8 hours in the non-challenged knees [365].

We showed MSCs substantially adhere after three hours but do not regain fibroblastic morphology until eight hours after seeding. For surgeons injecting allogeneic MSCs into humans, this may indicate the need for an extended un-weighted rest period of 8-24 hours post injection to allow MSCs to thoroughly adhere to the cartilage in the joint. Although, our model does not take into account the internal pressures of the knee joint, endogenous HA concentration or the catabolic milieu of an OA affected knee. Previous investigations of cell attachment to human arthritic cartilage showed synovial MSCs can adhere within 10 minutes [364], however the cell dose used was more than 200 times greater per square millimetre of cartilage and lacked of washing steps to account for loosely bound cells. In light of this, the model cannot take into account internal shearing forces of the joint which may contribute to cell detachment. Also no effort was made to show morphology of cells was fibroblastic and therefore they may have been in the flat bodied and in the initial attachment phase observed in this study. Furthermore, OA-derived synovial fluid was shown to inhibit MSC binding to cartilage [363], thus an extended rest period beyond 24 hours may be required for thorough adhesion.

Concentration of HA as a vehicle for intra-articular injection of MSCs is important for dispersion and internal cartilage coverage. The dispersion assay with increasing concentration of HA indicated the higher (4 & 5 mg/ml HA) concentrations would form single aggregate colonies. A randomised, double-blind, controlled study used intra-articular injection of allogeneic bone marrow-derived MSCs with HA as a vehicle at a concentration of 10 mg/mL showed a reduction in pain in patients with OA changes using a visual analogue scale [315]. However, only some patients had

increased meniscal volume, which may be *de novo* tissue regeneration. According to our dispersion data, it is likely the injected cell suspension did not disperse throughout the joint. Furthermore, the endogenous concentration of HA found in post-mortem synovial fluid was reported at 1-4 mg/mL of HA [366] and this can decrease with joint pathologies as reported by Dahl *et al.* to 0.17-1.32 mg/mL [125]. It is unclear if the lack of dispersion and therefore close proximity of MSCs would have an effect on tissue regeneration or migration of MSCs *in vivo*. However, MSCs grown in hanging drop culture as spheroids express higher levels of anti-inflammatory TNF alpha stimulated gene 6 (TSG-6) and stanniocalcin-1 (STC-1) compared to monolayer culture [101] which suggests altered immunomodulatory capacity of MSCs.

Culturing MSCs with HA can alter the cytokine secretion profile. Investigations of the secretome of MSCs when combined with HA have been shown to increase the secretion of Macrophage migration inhibitory factor (MIF) [311]. MIF secretion by MSCs in this study peaked significantly only for the 1 mg/mL treated cells. MIF is considered to be a pro-inflammatory mediator especially in OA and acts in local tissue to increase neutrophil and macrophage migration to regions of inflammation. Our previous investigations also showed increased secretion of MIF by MSCs cultured with HA [311]. Vascular endothelial growth factor (VEGF) decreased with increasing concentration of HA and significantly for the 2 mg/mL concentration. This was consistent with our previous investigations of HA and MSC co-culture [311]. VEGF may play an active role in the pathogenesis of OA. Ludin *et al.* found that if exogenous VEGF was injected into the knees of mice, the joint presented with synovial hyperplasia and cartilage degradation as typically seen in OA disease [351]. Thus a consistent decrease in the secretion of VEGF by MSCs in the presence of HA may suggest therapeutic synergy between the two.

Basic fibroblast growth factor (FGF-β) increased with increasing concentrations of HA. FGF- β is a potent mitogen on MSCs and the increased secretion may help to explain the increase in proliferative capacity of the cells [340,367]. HA may therefore have an agonistic role in potentiating the secretion of FGF- β by MSCs but this has not been shown. Additionally, an increase in secretion of FGF-β may contribute to increase therapeutic efficacy of MSCs for the treatment of OA. A cartilage defect model in rabbit showed exogenous human FGF-β alone could potentiate articular cartilage resurfacing within 6 weeks of the injury [336].

4.8 Conclusion

Allogeneic preparations of MSCs will become an increasingly attractive therapeutic in the treatment of knee OA. These preparations are increasingly seen to be tested with HA as a vehicle without consideration for the likely interactions. Here we have shown HA can have a profound dose-dependent effects on early growth kinetics of MSC and these effects were founded on the physio-chemical phenomena of the HA entanglement point. The use of our *ex vivo* cartilage model further emphasises the limitation of *in vitro* experimentation but also highlighted for the first time that HA-primed MSCs can increase cell attachment to cartilage and that the presence of HA did not. Our time course study also suggests patients undergoing MSC therapy for knee OA, could benefit from being immobilised and unweighted for 8 hours post injection to allow MSCs to thoroughly adhere in the joint. Additionally, concentration of HA was also shown to greatly affect the dispersion of MSCs. These factors should be considered in future trials with respect to the culture media for MSCs and where HA is being considered as a component of vehicle for the treatment of OA.

4.9 Acknowledgments

4.10 References

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4.11 Supplementary materials

4.11.1 Isolation of human adipose-derived mesenchymal stem cell (MSC) populations

This research was approved by the Macquarie University human research ethics committee (Ref #: 5201100385). Human lipoaspirate was obtained from patients undergoing elective cosmetic liposuction surgery. The lipoaspirate was digested as previously described. Briefly, 200mL of fresh lipoaspirate was mixed and enzymatically digested in pre-warmed (37°C) saline containing 0.5 mg/mL collagenase (Lomb Scientific, USA). The lipoaspirate was then incubated in a 37°C water bath for 30 minutes and mixed periodically to circumvent layer separation. The digested lipoaspirate was then passed through an 800 μm mesh to exclude undigested tissue clumps. Finally, the suspension was centrifuged at 1500 x g for 5 minutes to obtain the pelleted stromal vascular fraction cells (SVF) and floating adipocytes.

4.11.2 Propagation of adherent adipose-derived mesenchymal stem cells

To obtain a population of adherent MSCs, the SVF pellet was transferred into three T175cm^2 flasks containing Standard culture media (control media) which consisted of Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, USA) supplemented with 10% foetal bovine serum (FBS; Bovogen, Australia) and 1% Penicillin-Streptomycin solution (Invitrogen, USA). Flasks were incubated for 72 hours at 37°C with 5% carbon dioxide. To prevent iron toxicity to adherent MSCs; the flasks

were washed using DMEM with no additives to wash away non-adherent cells and the media replaced with fresh standard culture media. Once the monolayer of MSCs reached 80% confluency, cells were passaged with enzymatically using TrypLE express (Invitrogen, USA) and then re-suspended in standard culture media. Cells from each flask were then transferred to three new T175cm^2 flasks now termed passage 1. Media changes were performed every 72 hours. Once all the flasks were confluent, the cells were stripped, counted and frozen in 90% FBS and 10% DMSO firstly in isopropanol containing vessel for gradual cooling in a -80°C freezer and then transferred to liquid nitrogen for long term storage.

4.11.3 Standardised enumeration

Standardised enumeration of cells was achieved with Tru-Count tubes (Becton Dickinson, USA) containing sheath fluid (isoflow; Becman Coulter, USA) and nucleic acid dyes; propidium iodide (10 µg/mL; Sigma, USA), Syto11 (1 µM; Invitrogen, USA) and a defined bead number. The combination of a charged (propidium iodide; Pi) and a cell permeate (Syto11) dye allows for discriminant cell population gating, which are based on the principal of Pi membrane exclusion and thus viability can be calculated from defined bead populations run as standards. Samples were all run on FACSCalibur flow cytometer (Becton Dickinson, USA).

4.11.4 MSC growth kinetics

MSCs were all used from frozen preparations at passage one and were cultured until the second passage before use in this experiment. The flasks were left in control media for another three days after seeding them in the second passage. On the third day each of the flasks were treated with a range of media formulations supplemented with high molecular weight HA (*Mw 2600 KDa)* (Lifecore Biomedical, USA) for another three days (see supplementary figure 1). These were control media, control + 0.25 mg/mL HA (0.25 mg HA media), control + 0.5 mg/mL HA (0.5 mg HA media), control + 1 mg/mL HA (1 mg HA media) and control + 2 mg/mL HA (2mg HA media). Another three days after each of the flask had been treated, the conditioned media from each was collected, centrifuged at 5000 RCF for 5minutes and frozen for later analysis. The cells were then stripped using TrypLE, made up in a final volume of 5 mL of control media and then the cells were counted using the standard enumeration technique.

Adherence. This experiment was undertaken on *standard* 96-well plates and CellBind 96-well plates (*High*-adherence; Corning, Australia) to test the effect of HA on different binding chemistries. Once a cell number was achieved for each of the flasks, the cells would be seeded into a 96-well plate at 5×10^3 cells/well using a combination of media preparations (see Supplementary figure 1). Alongside these test wells, a standard curve was seeded ranging from 1×10^3 to 16×10^3 cells/well. After 24 hours all the wells were washed twice with PBS and 100 µL of fresh control media added. A further 10 µL of CCK-8 reagent was added and following a four hour incubation at 37°C, the absorbance measured at 450 nm. An average of five technical replicates for each media preparation per biological replicate (*n=3*). Cell number was extrapolated from the standard curve. All combinations of the media

preparations *(- +)*, *(+ -)* and *(+ +)* were compared back to the cells grown in control media and seeded in control media *(- -)* using a t-test (two-sided). All calculations were done using Microsoft excel 2013.

Proliferation. This experiment was undertaken on *standard* 96-well plates and *high-*adherence 96-well plates. This arm of the experiment was undertaken in a similar way to the adherence experiment. Once the cell number was achieved for each of the flasks, the cells were seeded into a 96-well plate at $2x10^3$ cells/well using a combination of media preparations (see Supplementary figure 1). The standard curve would be seeded 24 hours (on the second day) before the endpoint At the 3 day time point, all the wells were washed twice in PBS, replaced with 100 µL of fresh control media followed by the addition of 10 µL of CCK-8 reagent and at the conclusion of a four hour incubation at 37°C, the absorbance measured at 450 nm. An average of five technical replicates for each media preparation per biological replicate (*n=3*). Cell number was extrapolated from the standard curve. All combinations of the media preparations *(- +)*, *(+ -)* and *(+ +)*, were compared back to the cells grown in control media and seeded in control media *(- -)* using a t-test (two-sided). All calculations were done using Microsoft excel 2013.

Left column flow text:
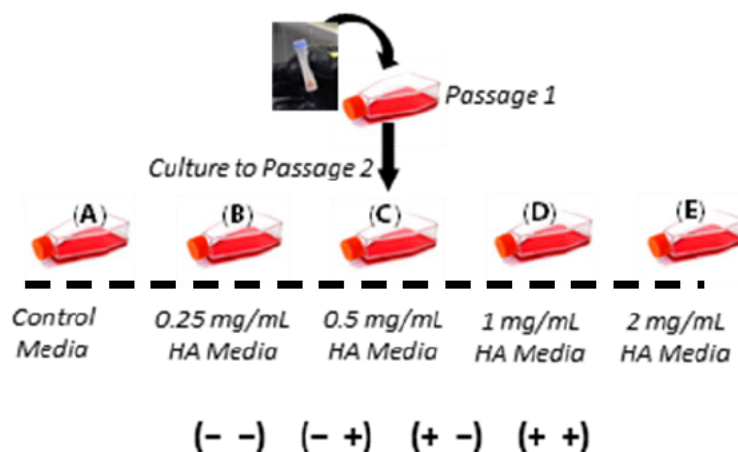
MSCs frozen at passage 1 were thawed

Cells are put into culture until confluent monolayer is achieved

Cells passaged at a ratio of 1:3

After reaching passage 2, flasks are kept in control media for three days

Media is changed into either control media or media containing Hyaluronan for another three days

Cells are seeded into 96-well plates in a range of media formulations

Diagram labels: Passage 1 • Culture to Passage 2 • (A) Control Media • (B) 0.25 mg/mL HA Media • (C) 0.5 mg/mL HA Media • (D) 1 mg/mL HA Media • (E) 2 mg/mL HA Media

(– –) (– +) (+ –) (+ +)

Legend:

(– –) *Cells grown in control media and seeded into test wells with control media*

(– +) *Cells grown in control media and seeded into test wells with HA media*

(+ –) *Cells grown in HA media and seeded into test wells with control media (primed)*

(+ +) *Cells grown in HA media and seeded into test wells with HA media*

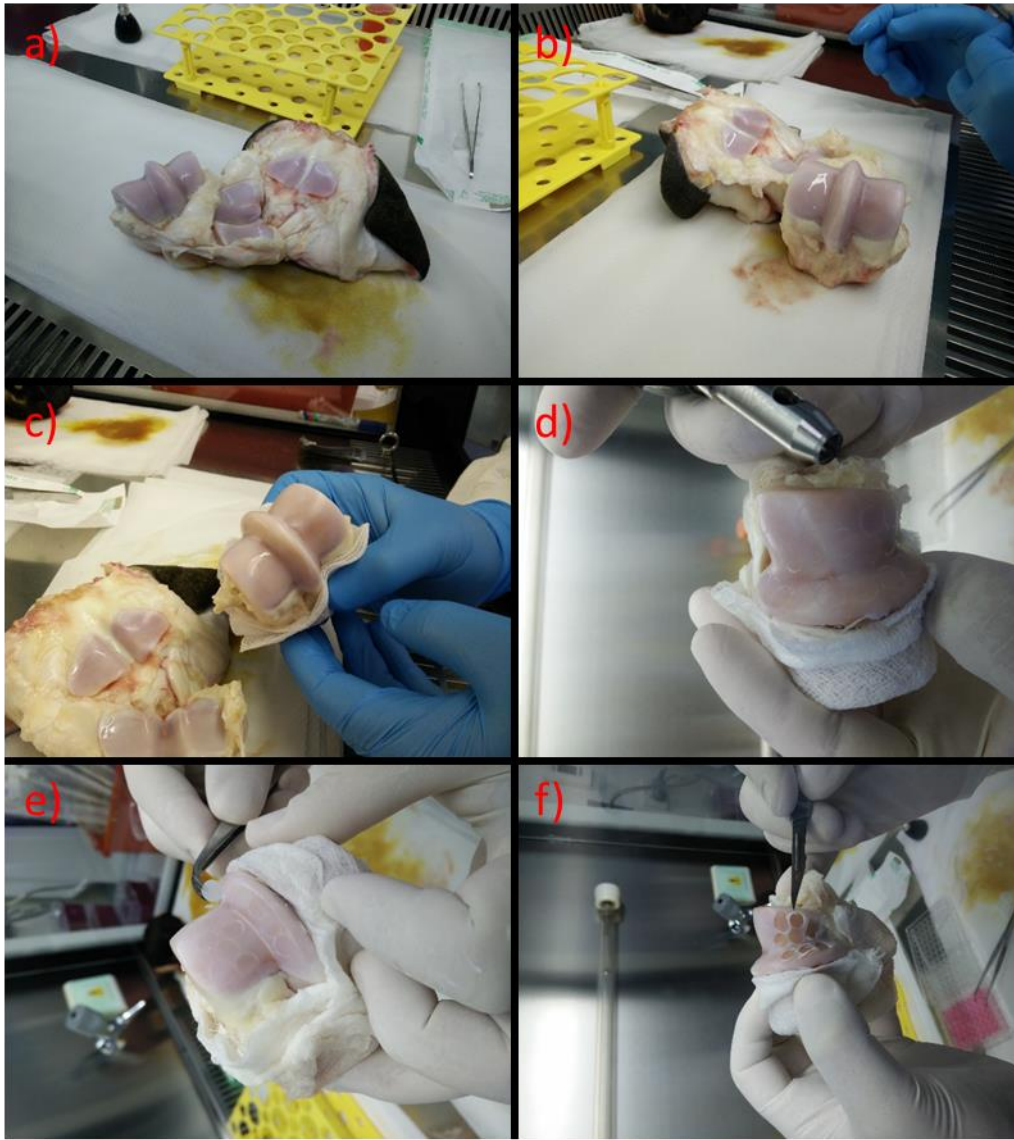**Supplementary figure 4.1 (SF 4.1) Experimental schematic and seeding conditions**

MSCs were cultured until passage 2. After passage all flask were kept in control media for three days. The media was then changed into Control media (flask A), 0.25 mg/mL HA media (flask B), 0.5 mg/mL HA media (flask C), 1 mg/mL HA media (flask D) and 2 mg/ml HA media (flask E). Following HA treatment, cells were then stripped, counted using the standard enumeration technique and then seeded into test wells in a range of media formulations to make up the different conditions. Conditions were always compared back to the control (flask A) which consisted of cells grown in control media and seeded into test wells in control media *(- -)*. Cells grown in flask A were seeded into test wells using HA media *(- +)*. Cells grown in flasks B-E were seeded into test wells using control media *(+ -)* (primed). Cells grown in flasks B-E were seeded into test wells using HA media at the same concentration of HA used to treat *(+ +)*.

4.11.5 MSCs kinetics on equine articular cartilage explants: *ex vivo* cartilage assay

All horses used in this study were due to be sacrificed at a commercial abattoir to be processed and sold as dog food (Kankool Pet Food, Australia). The fetlock joint from mature horses was cut out by sawing 15 cm below and above the joint. The fetlock was then shaved, scrubbed and soaked in iodine bath for a minimum contact time of 5 minutes and then frozen for later use.

4.11.6 Cartilage sectioning

The frozen fetlock joint was thawed at 37 degrees for 1-2 hours until the fetlock joint became flexible. Once thawed, the joint was dis-articulated in a sterile hood (see supplementary figure 2). The third metacarpal was obtained from the fetlock joint and the distal end was soaked in PBS for 5 minutes. The articular cartilage was then perforated using a custom designed hollow cylindrical instrument measuring 6.45 mm in diameter equal to a *standard* 96-well plate. The perforated discs were then sliced off with a scalpel and stored in the correct orientation (Joint side facing up) in a 96-well plate with 200 µL of DMEM and frozen for later use.

Supplementary figure 4.2 (SF 4.2) Articular cartilage disc sectioning protocol

Images depict articular cartilage disc sectioning protocol conducted in a class II sterile safety cabinet. **a)** the fetlock joint was initially dis-articulated and the joint capsule flared open **b)** the third metacarpal is released from the fetlock joint **c)** once the third metacarpal is completely liberated it was wrapped in sterile gauze and the articular joint face was soaked in PBS for 5 minutes **d)** the articular cartilage was then perforated using a custom designed hollow cylindrical instrument which measured 6.45 mm in diameter equal to a standard 96-well plate diameter **e)** perforated discs were sliced off **f)** discs are stored in the correct orientation (joint surface up) in a 96-well plate containing DMEM and frozen for later use.

4.11.7 Cartilage adherence time course of MSCs

MSCs were used from frozen preparations at passage one. The cells were cultured until the second passage before use in this experiment. Prior to seeding cells for the time course, the monolayer was washed in PBS and then stained using CM-DiI (1 µM; life technologies, Australia) membrane dye made in PBS according to the manufacturer's instructions. Cartilage discs were used to plug the bottom of an *ultra-low* adherence 96-well plate in the correct orientation. Cells were then stripped using TrypLE, counted using standard enumeration and then seeded onto the cartilage discs at a density of 5×10^3 cells/disc and the plate then incubated at 37°C and 5% CO_2. At each of the time points (1, 2, 3, 4, 8 & 24 hours), the cartilage disc was washed twice in PBS and then fixed in 4% paraformaldehyde for one hour and then washed in PBS five times. Cartilage discs with attached cells were then permeabilised in 0.1% Triton X-100 for five minutes, washed twice in PBS, blocked in 1% Bovine serum albumin in PBS for 20 minutes before being stained with F-actin specific Alexa Fluor 488-phalloidin (0.165 µM; life technologies, Australia) at room temperature, in the dark for 20 minutes and then washed thrice in PBS. Imaging of the cartilage discs was performed at the Macquarie University Microscopy Unit with the OLYMPUS FLUOVIEW FV1000 IX81 inverted confocal microscope (Olympus, Australia).

4.11.8 HA media viscosity assessment

Falling-ball viscometry was used to determine the viscosity (adapted from Eguchi and Karino, 2008 [356]) of HA media relative to control media. Briefly, a 5 mL serological pipette with a diameter of 5.9 mm was filled with media pre-warmed to 37°C. A bio-silicate sphere, 3 mm in diameter was dropped and the time measured between two defined points with a known distance. A total of five replicates for each media were obtained. The data was expressed as a mean flow rate (millimetres per second) and the stand deviation as error bars.
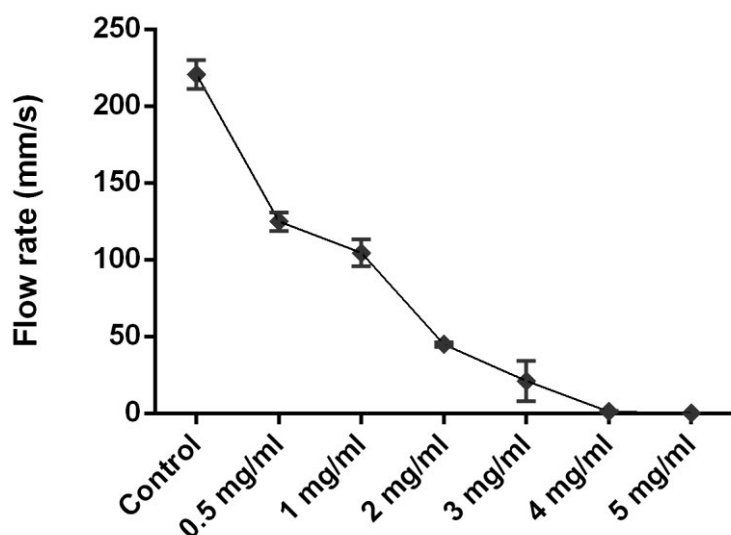
Supplementary figure 4.3 (SF 4.3) falling-ball test to measure viscosity

Falling-ball viscometry was used to determine the viscosity of HA media (0.5-5 mg/ml) relative to control media. A 5 ml serological pipette with a diameter of 5.9 mm was filled with media pre-warmed to 37°C. A bio-silicate sphere, 3 mm in diameter was dropped and time measured between two defined points with a known distance. A total of five replicates for each media were obtained. The data was expressed as a mean flow rate (millimetres per second) and the stand deviation as error bars.

4.11.9 MSC dispersion on cartilage with increasing concentration of HA

MSCs were used from frozen preparations at passage one. The cells were cultured until the second passage before use in this experiment. Cartilage discs were used to plug the bottom of an *ultra-low* adherence 96-well place in the correct orientation. Control media (200 µL) was then added to each well. The media formulations used were control media, 0.5 mg HA media, 1 mg HA media, 2 mg HA media, 3 mg HA media, 4 mg HA media or 5 mg HA media. MSCs were stripped using TrypLE, counted using standard enumeration and then seeded onto the cartilage discs at a density of $5x10^3$ cells/disc. The 96-well plate was then incubated for 24 hours at 37°C and 5% CO_2. After 24 hours the cartilage discs were washed twice in PBS and then fixed in 4% paraformaldehyde for one hour. Cartilage discs were washed in PBS five times and then stained in 1 mL of Hoechst 33342 (2 µg/mL; life technologies, Australia) for five minutes and again washed five times in PBS. Cartilage discs with attached cells were then permeabilised in 0.1% Triton X-100 for five minutes, washed twice in PBS, blocked in 1% Bovine serum albumin in PBS for 20 minutes before being stained with F-actin specific Alexa Fluor 488-phalloidin (0.165 µM; life technologies, Australia) at room temperature, in the dark for 20 minutes and then washed thrice in PBS. Imaging of the cartilage discs was performed at the Macquarie University Microscopy Unit with the OLYMPUS FLUOVIEW FV1000 IX81 inverted confocal microscope (Olympus, Australia).

4.11.10 MSC adherence & proliferation on cartilage

MSCs were used from frozen preparations at passage one. The cells were cultured until the second passage before use in this experiment. The flasks were left in control media for another three days after the seed. On the third day the flasks were treated with either control or 1 mg/mL HA media for another three days. After the cells had been treated for 3 days, the plate containing cartilage discs was thawed. Enough discs were then transferred into *ultra-low* adherence plate in order to obtain 5 technical replicates per condition. The conditions tested in this assay followed the same matrix as shown in supplementary figure 1. These were MSCs grown in control media and seeded in control media *(- -)*, grown in control media and seeded in 1 mg/mL HA culture media *(- +)*, grown in 1 mg/mL HA media and seeded in control media *(+ -)* (primed) and grown in 1 mg/mL HA media and seeded in 1 mg/mL HA media *(+ +)*.

Adherence. MSCs were seeded onto cartilage discs at a density of 5×10^3 cells/disc. After 24 hours the cartilage discs were removed to a new 96-well plate and washed twice with PBS. Fresh culture media (200 µL) was added to the discs followed by 20 µL of CCK-8 reagent and then incubated for four hours. The cartilage discs were then removed from the colour developed well and the absorbance read at 450 nm. Data represents an average of 5 technical replicates and a minimum of 3 biological replicates. *Proliferation.* This experiment was undertaken in the same way as the adherence experiment, however the cells were cultured for 3 days and seeded at a density of 2×10^3 cells/disc.

4.11.11 Secretome analysis

The conditioned media was collected from every flask in this study, centrifuged at 5000 x g for 5 minutes and stored at -80°C. Upon thawing, the samples were filtered through 0.2 mm Nanosep MF Centrifugal Devices with Bio-InertH Membrane (Pall Scientific, USA). Filtrates (50 µL) were analysed using both the Bio-Plex Pro Human Cytokine 27-plex and the Bio-Plex Pro Human Cytokine 21-plex assay (Bio-Rad, USA), according to the manufacturer's instructions. The washing steps were performed using the Bio-Plex Pro II magnetic wash station and the data was acquired using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, USA). The average fluorescence of each cytokine in the conditioned medium samples was calculated from four technical replicates in cultured MSC experiments. To account for biological variation in the secreted cytokines, the data was normalised to fold change in fluorescence of HA treated over control (*n=3*). The fold change was then averaged and graphed with the upper and lower confidence intervals set at 95% as error bars. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment. Any cytokines with a fold change clear of the axis at 1 were reported as significant (determined numerically).
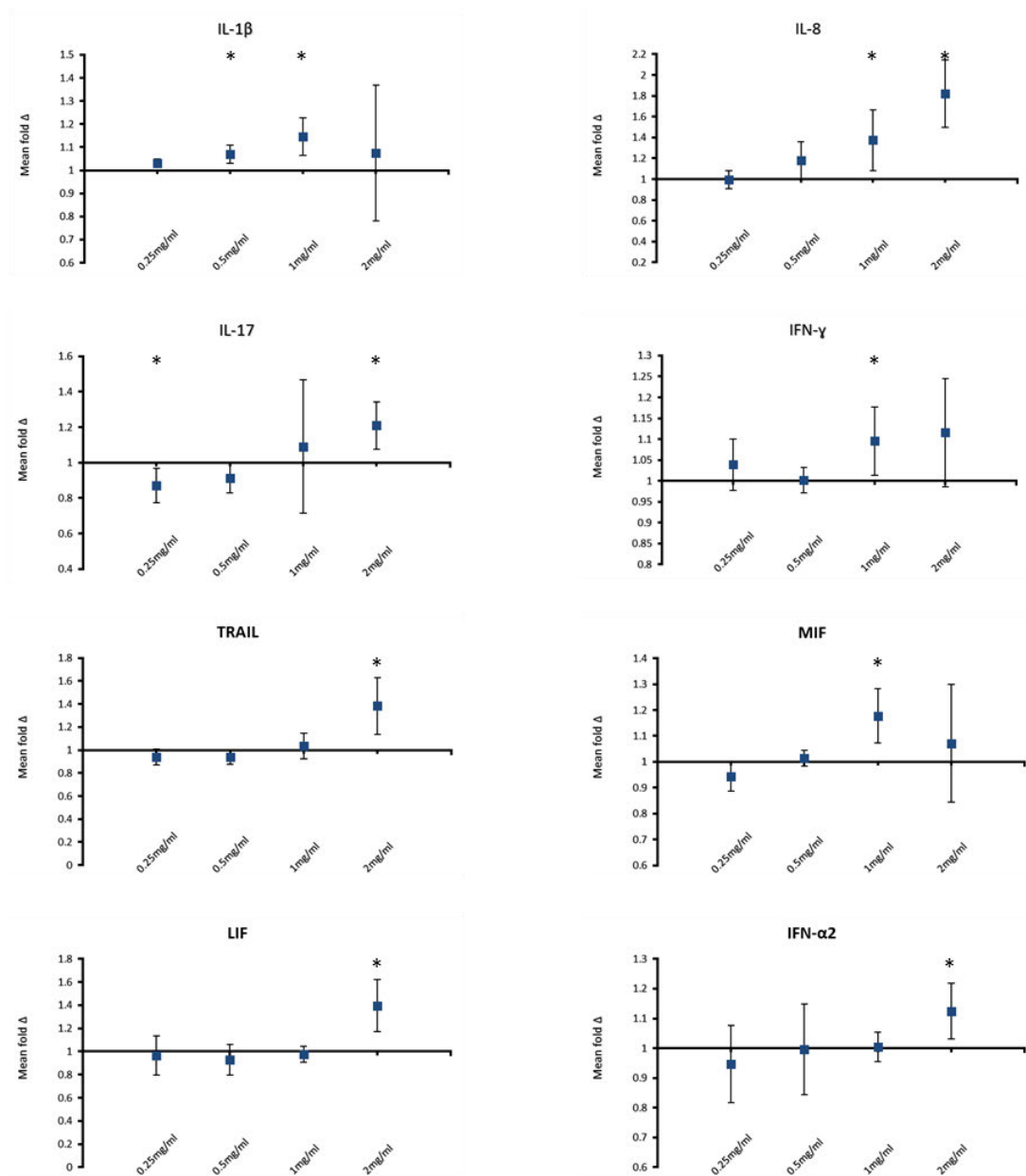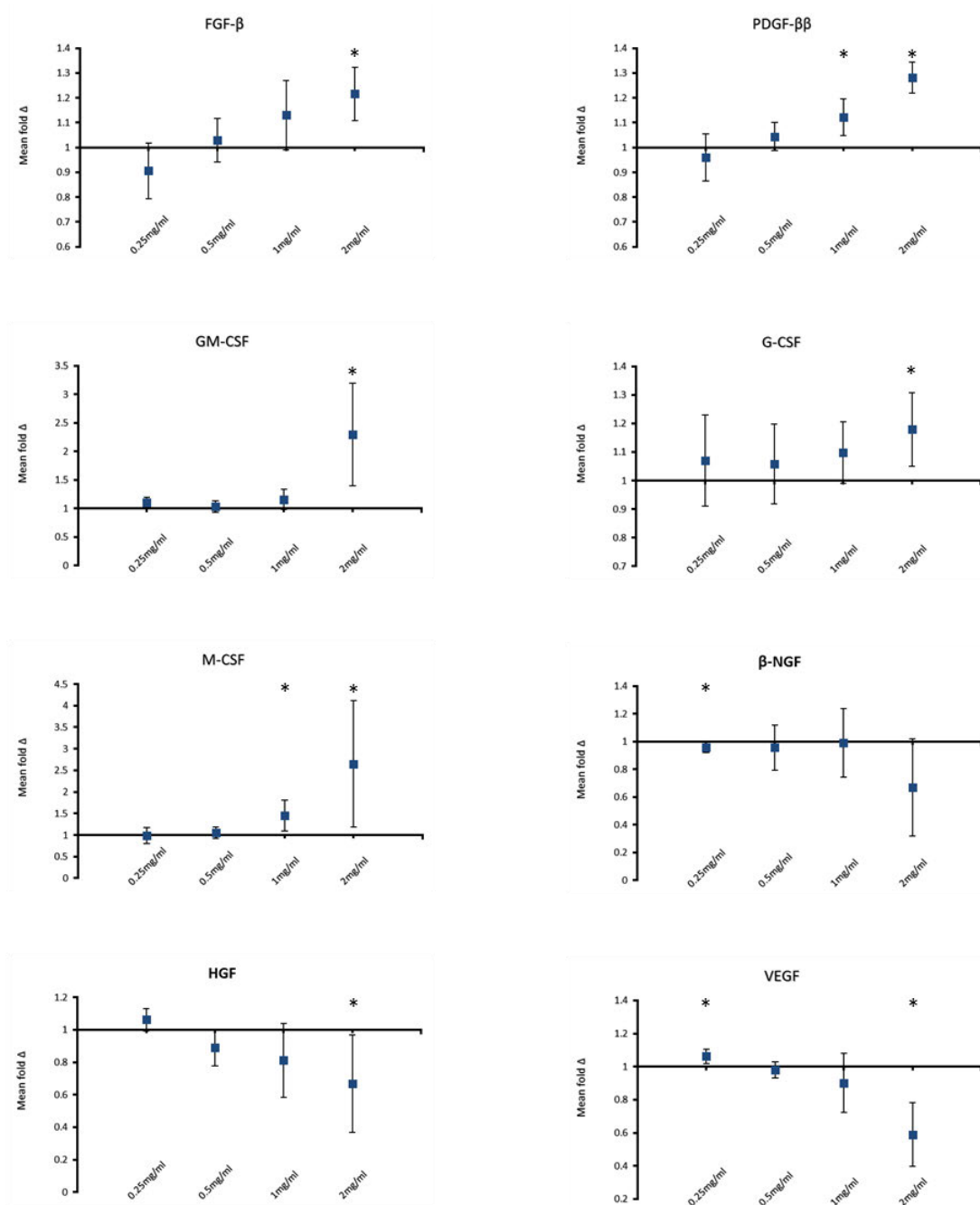
Supplementary figure 4.4 (SF 4.4) fold change of pro-inflammatory cytokine secretion with HA treatment

Conditions were all cultured in either control media or HA media for three days after the media change. Conditions *(x-axis)* were grown in flasks (B-E) HA media, ranging from 0.25 to 2 mg/mL of HA. These were all compared back to the control condition (cells grown control media, flask (A)). Average fold change in fluorescence *(n=3)* ± upper & lower confidence intervals at 95% *(y-axis)*. Cytokines with a fold change less than one indicate a decrease with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment (* cytokines with a fold change clear of the axis at 1 reported as significant). Interleukin-1β (IL-1β), Interleukin-8 (IL-8), Interleukin-17 (IL-17), Interferon-gamma (IFN-ɣ), TNF-related apoptosis inducing ligand (TRAIL), Macrophage migration inhibitory factor (MIF), Leukemia inhibitory factor (LIF) and Interferon alpha-2 (IFN-α2).
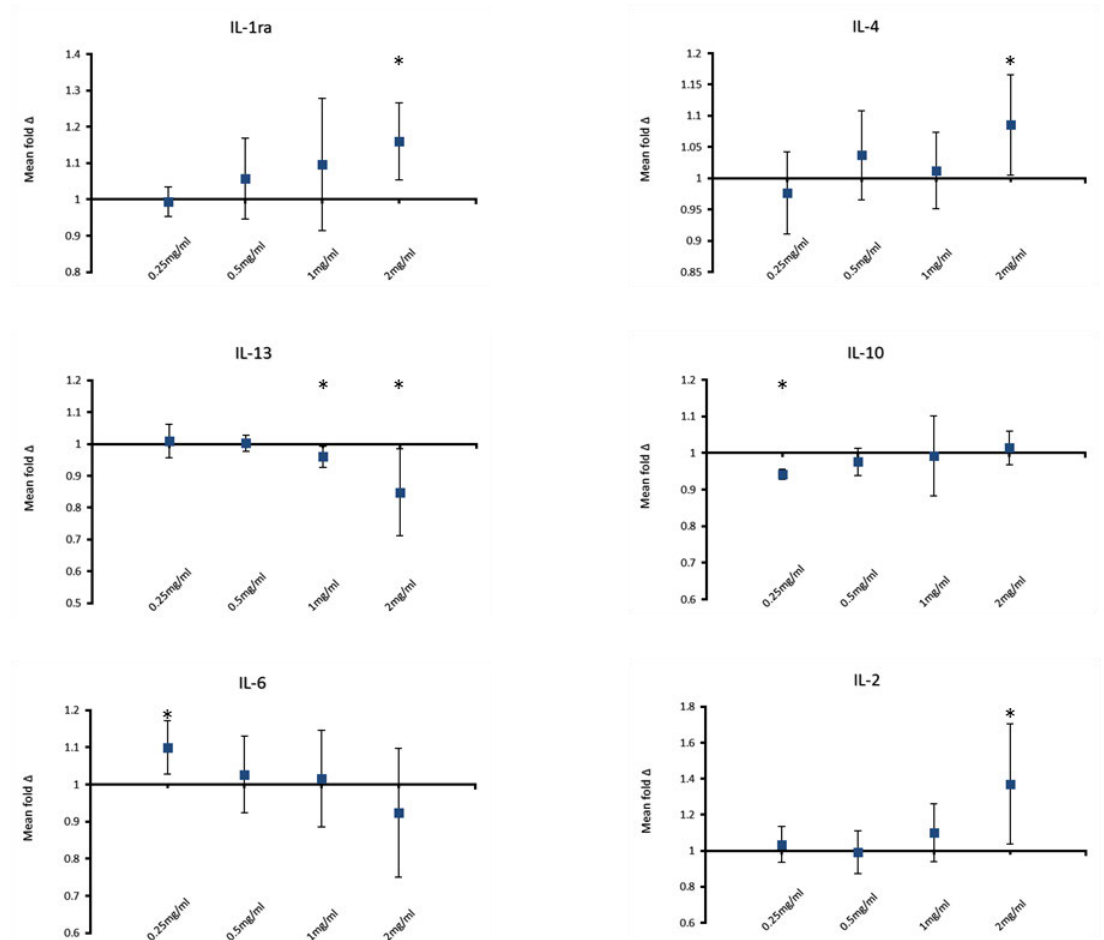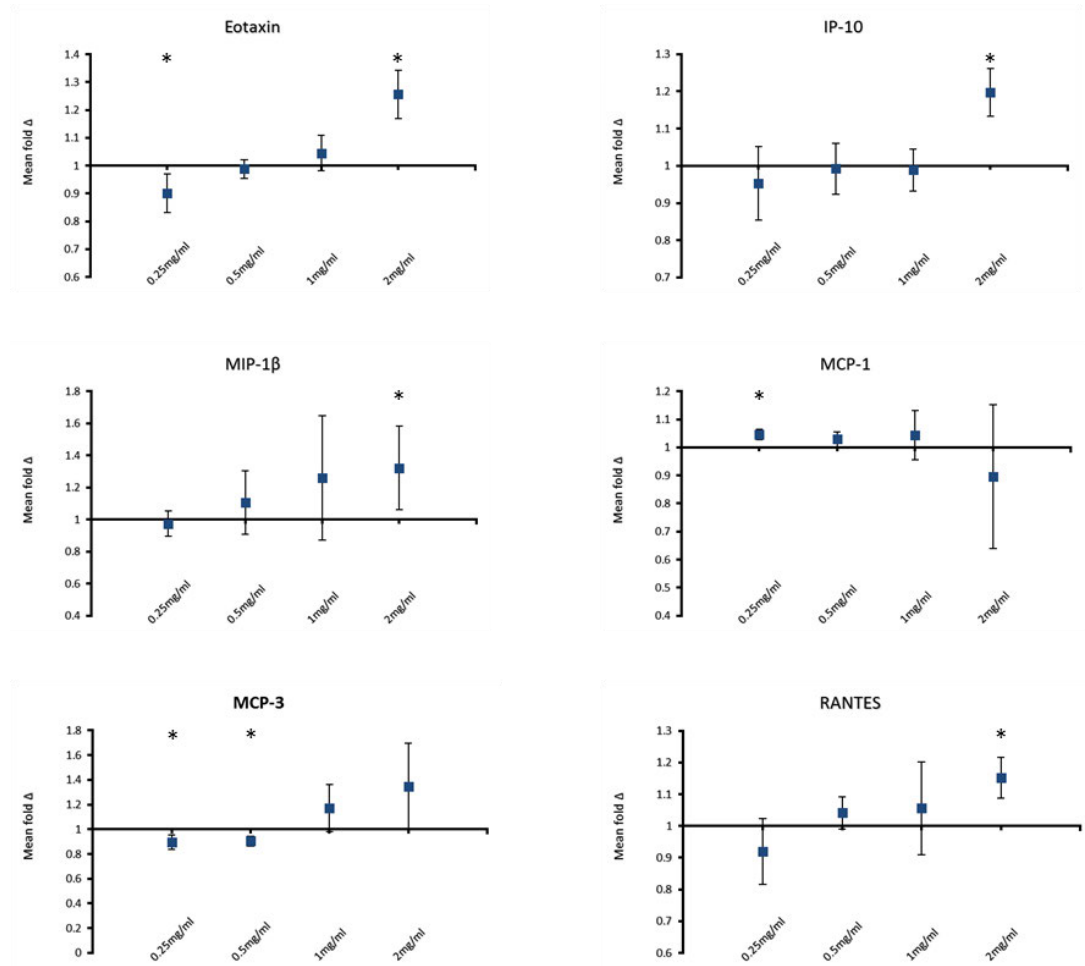
Supplementary figure 4.5 (SF 4.5) fold change of anti-inflammatory and dual role cytokine secretion with HA treatment

Conditions were all cultured in either control media or HA media for three days after the media change. Conditions *(x-axis)* were grown in flasks (B-E) HA media, ranging from 0.25 to 2 mg/mL of HA. These were all compared back to the control condition (cells grown control media, flask (A)). Average fold change in fluorescence *(n=3)* ± upper & lower confidence intervals at 95% *(y-axis)*. Cytokines with a fold change less than one indicate a decrease with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment (* cytokines with a fold change clear of the axis at 1 reported as significant). Interleukin-1 receptor antagonist (IL-1ra), Interleukin-4 (IL-4), Interleukin 13 (IL-13), Interleukin-10 (IL-10), Interleukin-6 (IL-6) and Interleukin-2 (IL-2).

Supplementary figure 4.6 (SF 4.6) fold change of growth factor secretion with HA treatment

Conditions were all cultured in either control media or HA media for three days after the media change. Conditions *(x-axis)* were grown in flasks (B-E) HA media, ranging from 0.25 to 2 mg/mL of HA. These were all compared back to the control condition (cells grown control media, flask (A)). Average fold change in fluorescence *(n=3)* ± upper & lower confidence intervals at 95% *(y-axis)*. Cytokines with a fold change less than one indicate a decrease with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment (* cytokines with a fold change clear of the axis at 1 reported as significant). Fibroblast growth factor-basic (FGF-β), Platelet-derived growth factor-ββ (PDGF-ββ), Granulocyte macrophage colony-stimulating factor (GM-CSF), Granulocyte colony-stimulating factor (G-CSF), Macrophage colony-stimulating factor (M-CSF), Nerve growth factor-beta (β-NGF), Hepatocyte growth factor (HGF) and Vascular endothelial growth factor (VEGF).

Supplementary figure 4.7 (SF 4.7) fold change of chemokine secretion with HA treatment

Conditions were all cultured in either control media or HA media for three days after the media change. Conditions *(x-axis)* were grown in flasks (B-E) HA media, ranging from 0.25 to 2 mg/mL of HA. These were all compared back to the control condition (cells grown control media, flask (A)). Average fold change in fluorescence *(n=3)* ± upper & lower confidence intervals at 95% *(y-axis)*. Cytokines with a fold change less than one indicate a decrease with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment (* cytokines with a fold change clear of the axis at 1 reported as significant). C-C motif chemokine 11 (CCL-11) aka eosinophil chemotactic protein (Eotaxin), C-X-C motif chemokine 10 (CXCL-10) aka interferon gamma-induced protein 10 (IP-10), CC-Chemokine ligand-4 (CCL-4) aka Macrophage inflammatory protein-1 beta (MIP-1β), CC-Chemokine ligand-2 (CCL-2) aka Monocyte chemotactic protein-1 (MCP-1), CC-Chemokine ligand-7 (CCL-7) aka Monocyte chemotactic protein-3 (MCP-3), CC-Chemokine ligand-5 (CCL-5) aka Regulated on Activation Normal T Cell Expressed and Secreted (RANTES).

		Adherence		Proliferation	
		Standard Plate	*High-adherence plate*	*Standard Plate*	*High-adherence plate*
MSCs grown in control & seeded in HA media	*Control*	1368	4627	2293	2373
	0.25 mg	2642	4544	2409	2767
	0.5 mg	2361	5636	2763	2684
	1 mg	2658	4548	***3117***	3039
	2 mg	1746	***2431***	2496	2342
MSCs grown in HA media & seeded in control (primed)	*Control*	1368	4627	2293	2373
	0.25 mg	2967	5092	2506	2930
	0.5 mg	***3990***	3815	***2736***	***3384***
	1 mg	***3410***	3668	***3686***	***3840***
	2 mg	2404	***2681***	***3511***	***2966***
MSCs grown in HA media & seeded HA media	*Control*	1368	4627	2293	2373
	0.25 mg	2952	4830	2911	2997
	0.5 mg	***3797***	3526	***3045***	***3681***
	1 mg	***5931***	2778	***3550***	***3749***
	2 mg	1751	***2119***	***3488***	3868

Supplementary table 4.1 (ST 4.1) MSC growth kinetics summary

Median cell number from all growth kinetics experiments. ***Bold*** numbers indicate a *p-value* < 0.05 as derived from a two-sided t-test using the control as a reference for all the conditions.

Cytokines		Fold Change from control (CI = 95%)				Role in OA
		0.25 mg/mL	**0.5 mg/mL**	**1 mg/mL**	**2 mg/mL**	
Pro-inflammatory	MIF	0.94	1.01	**1.18**	1.07	Induces the release of proinflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-6, IL-8 and MIF levels in synovial fluid correlate to OA severity
	TRAIL	0.94	0.94	1.03	**1.38**	Increased expression in Experimental rat OA-derived cartilage and can induce chondrocyte apoptosis
	LIF	0.96	0.93	0.98	**1.40**	Increased levels in OA-derived synovial fluid
	IL-1β	1.03	**1.07**	**1.15**	1.08	Induces inflammatory reactions and catabolic effects independently as well as in combination with other mediators in OA with respect to the articular cartilage
	IL-8	1.00	1.18	**1.37**	**1.82**	Increased levels in OA-derived synovial fluid and serum
	IL-17	**0.87**	0.91	1.09	**1.21**	Inhibits the synthesis of proteoglycans in OA and upregulates catabolic enzymes which break down cartilage
	IFN-γ	1.04	1.00	**1.10**	1.12	Stimulate cartilage breakdown by production of enzymes via IL-1 β
	IFN-α2	0.95	1.00	1.01	**1.12**	Induces immunosuppressive enzyme; indoleamine-2,3-dioxygenase (IDO)
Anti-inflammatory / Dual role	IL-1ra	0.99	1.06	1.10	**1.16**	Blocks interactions between cell surface receptors and IL-1 inhibiting inflammatory cascades in OA
	IL-4	0.98	1.04	1.01	**1.09**	Can decrease the release of prostaglandin E2 in OA-derived synoviocytes and suppress synthesis of IL-1β and TNF-α in OA tissue

	IL-13	1.01	1.00	**0.96**	**0.85**	Inhibits production of proinflammatory cytokines in immune cells and increases IL-1Ra production. Inhibits IL-1β synthesis in OA-derived synovium
	IL-10	**0.94**	0.98	0.99	1.01	Expressed in chondrocytes and involved in collagen and aggrecan synthesis. Inhibition of catabolic enzymes and apoptosis of chondrocytes
	IL-2	1.04	0.99	1.10	**1.37**	Elevated levels are found in OA synovial fluid and increased levels correlate to increase severity
	IL-6	**1.10**	1.03	1.02	0.92	Catabolises aggrecan via aggrecanase activity. Involved in the synthesis of tissue inhibitor of metalloproteinases known to stop cartilage breakdown
Chemokines	Eotaxin	**0.90**	0.99	1.05	**1.26**	Increased concentrations in OA-derived plasma compared to healthy controls
	IP-10	0.95	0.99	0.99	**1.20**	Plasma and synovial concentrations are inversely associated with knee OA severity
	MIP-1β	0.97	1.11	1.26	**1.32**	Present in significantly higher levels in OA synovial fluid compared to normal synovial fluid
	RANTES	0.92	1.04	1.06	**1.15**	Secreted by IL-1 stimulated osteoarthritic chondrocytes and TNFα stimulated synovial fibroblasts
	MCP-1	**1.05**	1.03	1.04	0.90	Constitutively expressed in osteoarthritic chondrocytes and increased secretion in IL-1 stimulated osteoarthritic chondrocytes
	MCP-3	**0.90**	**0.90**	1.17	1.35	Activates immune cells such as monocytes, T lymphocytes, basophils and eosinophils
Growth Factors	HGF	1.06	0.89	0.81	**0.67**	Promotes osteophyte formation via MCP-1 mediated infiltration of immune cells into OA affected joint
	FGF-β	0.91	1.03	1.13	**1.22**	Potentiates articular cartilage resurfacing and may stimulate expression of MMP-13

β-NGF	**0.96**	0.96	0.99	0.67	Higher expression is found in osteoarthritic derived chondrocyte compared to healthy chondrocytes
GM-CSF	1.11	1.03	1.15	**2.29**	Key mediator in inflammation and arthritic pain
VEGF	**1.06**	0.98	0.90	**0.59**	Expressed in osteoarthritic-derived chondrocytes and shown to increase osteo-chondral angiogenesis in OA patients
G-CSF	1.07	1.06	1.10	**1.18**	Can significantly increase nitrite levels in cartilage when combined with IL-1β stimulated explants
M-CSF	0.98	1.05	**1.45**	**2.65**	Increased gene expression in articular-derived chondrocytes during inflammation
PDGF-ββ	0.96	1.04	**1.12**	**1.28**	OA-derived synovial fibroblasts stimulated with PDGF-ββ decreased total MMP activity

Supplementary Table 4.2 (ST 4.2) Summary of Cytokines across all conditions

A summary of cytokines across all the concentrations of HA treatment. The data is represented as fold change in fluorescence compared to control. A value more than one indicates the HA treatment increased the secretion of that cytokine in that condition. ***Bold*** values represent a significant fold change from control determined numerically using confidence interval set at 95%.

5

Hyaluronan-mediated stem cell secretions in osteoarthritis: A pilot study

Prologue

Our previous work provided insight into the alterations of the MSC secretome when co-cultured with HA. During our investigation of MSC growth kinetics, we began harvesting secretions of MSCs and MSCs cultured in HA. Our discovery of increased attachment to cartilage by priming of MSCs with HA came after this pilot study had begun and therefore primed MSC secretions could not be used in the pilot. Our data pertaining to alterations in the MSC secretome with HA consistently showed increases of IL-1β and MIF. We hypothesised these hallmark OA proteins would detrimentally effect MSC mode of action with respect to osteoarthritic explants. Through the use of functional endpoints on OA cartilage, we disproved our initial hypothesis and showed that HA modulated MSC function to the benefit of outcomes.

The results of this research have been submitted in the form of a peer-reviewed journal article in *Arthritis Research & Therapy, 2016.*

Letter of Submission

ARRT-D-16-00616

Hyaluronan-mediated stem cell secretions in osteoarthritis: A pilot study

Peter Succar; Chris Little; Cindy Shu; Susan Smith; Edmond Breen; Peter Walker; Margaret Smith;

Ben Herbert

Arthritis Research & Therapy

Dear Dr Herbert,

Thank you for submitting your manuscript 'Hyaluronan-mediated stem cell

secretions in osteoarthritis: A pilot study' to Arthritis Research & Therapy.

The submission id is: **ARRT-D-16-00616 -** Please refer to this number in any future

correspondence.

During the review process, you can keep track of the status of your manuscript by

accessing the following website: http://arrt.edmgr.com/

Best wishes,

Editorial Office

Arthritis Research & Therapy

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Contributions to publication statement

The concept of this publication was developed in collaboration with my supervisors Benjamin Herbert. Edmond Breen provided statistical support throughout all projects. Peter Succar performed all of the experimental work, collection of human samples, processing of samples, data analysis and the preparation of the manuscript.

Table 5.1: Author contribution matrix for Publication.

	Peter Succar	Edmond J Breen	Cindy Shu	Susan Smith	Peter Walker	Margaret Smith	Christopher Little	Benjamin Herbert
Experiment Design	●						●	●
Collection of human samples	●							
Histological preparations	●			●				
Technical assistance			●			●		
Provision of Study Materials	●				●		●	●
Data Collection	●							
Data Analysis & Interpretation	●	●						
Manuscript	●		●		●		●	●

Citation

Succar, P., Shu, C., Smith, S., Breen, E. J., Walker, P., Smith, M. M., Little, C. B., Herbert, B. R. Hyaluronan-mediated stem cell secretions in osteoarthritis: A pilot study. Arthritis Research & Therapy (Submitted, 2016)

5.1 Hyaluronan-mediated stem cell secretions in osteoarthritis: A pilot study

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5.2 Disclosures

Benjamin Herbert is an A/Prof Translational Regenerative Medicine at the University of Sydney. He is also a co-founder, shareholder and consultant to Regeneus Ltd. Peter Succar is a PhD candidate and a casual employee at Regeneus Ltd. Edmond J Breen is the Head of Bioinformatics at the Australian Proteome Analysis Facility (APAF) at Macquarie University and a consultant to Regeneus Ltd. Christopher Little reports grants from Fidia Farmaceutici, grants from Galapagos Pharmaceuticals, grants from CSIRO Australia, grants from PolyActiva Pty Ltd, outside the submitted work. Margaret Smith reports grants from Fidia Pharmaceutici, grants from Galapagos Pharmaceuticals, grants from CSIRO Australia, grants from PolyActiva Pty Ltd, outside the submitted work.

5.3 Abstract

Adipose-derived stem cells (MSCs) can regenerate cartilage in osteoarthritis (HA). The mechanism of action has shifted from direct differentiation to secretion-driven. MSC secretions alone can reduce inflammation in vivo. Using Hyaluronan (HA) for delivery of MSC therapy in osteoarthritis is widespread with no consideration of altered MSC function. We previously identified modulation of the MSC secretome by HA. Herein we sought to determine whether HA-modulated MSC conditioned media affected human OA-cartilage and synovial tissue. Secretions were harvested from MSCs grown in media or HA-containing media and used to treat patient-derived cartilage and synovium. Histopathological outcomes were measured semi-quantitatively. Gene expression for key OA-mediators, soluble glycosaminoglycans (GAG), tissue inhibitor of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) were used as quantitative outcome measures. Synovial sub-intimal fibrosis was reduced significantly in all HA-containing groups and not by the MSC-media. MMP7 secretion increased significantly from cartilage and synovium treated with MSC-media. MMP9 secretion increased significantly from cartilage treated with MSC-media. HA modulated the effects of the MSC-secretome in tissue-derived MMP secretion. Inversely, the MSC/HA-media significantly increased the expression of Aggrecanase 1 (ADAMTS4) in cartilage compared to MSC-media. In conclusion, further investigation is required to elucidate whether secreted MMP7 and MMP9 have an anti-inflammatory role in OA and the clinical significance in knee OA of increased ADAMTS4 expression by cartilage resulting from a HA-MSC combination. Future human trials using HA-MSC combination should be required to have a MSC alone group to elucidate the clinical significance of the HA-MSC combination.

5.4 Introduction

Osteoarthritis is a degenerative disease characterised by degradation of cartilage, subchondral and marginal bone remodelling and inflammation of the synovium [353]. Pathological change in these tissues and associated pain leads to reduced mobility, decreased economic contribution because of limited employment capacity and significant contributions to the healthcare burden of developed nations [1]. Conventional treatments for OA are targeted at relieving pain using analgesics and non-steroidal anti-inflammatories [354]. Other non-surgical treatments include intra-articular injection of exogenous preparations of hyaluronan (HA) as a form of viscosupplementation therapy [355]. End stage treatment of OA involves surgical realignment or total knee replacement (TKR) with artificial prostheses. Increasing life expectancy has exacerbated the problem (because TKR has a limited life span) and widened the treatment gap for younger sufferers who are not suitable candidates for TKR [174].

Adipose-derived mesenchymal stem cells (MSCs) have shown promise as a therapeutic for the treatment of knee OA [310,368]. Although MSCs can differentiate into mesodermal lineages to form cartilage, bone and fat, differentiation of implanted cells *in vivo* is rarely documented as a mode of action. Paracrine effects, both anti-inflammatory and trophic, via the secretion of a complex mixture of cytokines is currently a more accepted and well-studied mode of action of MSCs [242,369-372]. The trophic action of cytokines and growth factors act to stimulate and mobilise endogenous cells to repair and regenerate the target tissue.

In the treatment of knee OA, MSCs have shown improvement in function and pain with minimal adverse events [368]. More importantly, MSC treatment can result in a consistent reduction in the size of cartilage defects by the regeneration and neo-formation of hyaline-like cartilage [310]. Supporting the concept that MSC function is

secretion-driven, we previously demonstrated conditioned medium alone, could reduce the clinical arthritis score in a mouse collagen-induced arthritis model [288]. Moreover, the use of minimally processed autologous tissue, including adipose stromal vascular fraction therapy is exempt from regulation as a drug in many countries. This exemption has enabled rapid growth of adipose-derived autologous treatments, particularly for conditions such as knee OA. In some animal trials and human case studies, researchers have combined MSC therapy with other approved OA treatments, such as HA viscosupplementation. Indeed suspending MSCs in HA seems common practice not only in animal models such as guinea pigs [313] and larger animals [291] [290], but also for the treatment of OA in humans [314,315]. This strategy may be hinged on a hypothesis that the physical rheological benefit of HA and the regenerative effect of MSCs would be synergistic.

It is likely that MSC therapy will move to an allogeneic model as various proprietary cell populations proceed through clinical trials. In this context, the manufacture of cell therapeutics for the treatment of OA may involve the use of HA, as it is a core component of the extracellular matrix and endogenously abundant in the knee joint [118,119]. "Off-the-shelf" allogeneic cells will also be simpler for physicians to use, not requiring tissue harvesting and processing, which is likely to drive increased uptake of cell therapy. These factors mean that combination therapies involving MSCs and HA are more likely to occur in the coming years.

Interestingly, interactions between MSCs and HA are rarely discussed in publications where a combination is used. An interaction is likely to be mediated by CD44, the primary receptor for HA, which is abundantly expressed on the surface of MSCs [358]. Work in our lab demonstrated HA (*MW = 2.6MDa non-crosslinked)* can have profound effects on MSC growth kinetics in a dose-dependent manner. Namely, the HA concentration that produced the greatest effects on MSCs, was centred on the physicochemical phenomena of entanglement point (1 mg/mL HA) [373] of high

molecular weight HA. Using a novel cartilage-adhesion assay, we showed MSCs increased attachment to cartilage if they were *primed* with HA, but not in *combination* with HA. Importantly, because MSC mode of action is secretion-driven, understanding the effect of combining MSCs with HA on the MSC secretome will be of clinical value. We previously showed MSCs increased secretion of interleukin 1 beta, macrophage migration inhibitory factor, and fibroblastic growth factor-beta, and decreased secretion of vascular endothelial growth factor when co-cultured with HA (Hylan G-F 20; Synvisc, *MW = 6MDa*) [311]. In the present study we sought to determine whether the previously identified modulation by HA of the MSC secretome, altered the effect of MSC-conditioned medium on OA human cartilage and synovial tissue.

5.5 Materials and Methods

5.5.1 Isolation and treatment of human adipose-derived mesenchymal stem cell populations

This research was approved by the Macquarie University human research ethics committee (Ref #: 5201100385). Human lipoaspirate was obtained from a female patient (with concealed demography) undergoing elective cosmetic liposuction surgery of the abdomen. The lipoaspirate was digested, the stromal vascular fraction isolated and adherent MSCs cultured as previously described [311], and conditioned media harvested as outlined in figure 5.1. Flasks were imaged using an Olympus IMT-2 inverted microscope (Olympus, Australia) with a mounted Scion VisiCapture Firewire camera (Scion Corporation, USA) as depicted in Figure 5.2.

5.5.2 Culture of osteoarthritis-derived explants

Harvest of tissue from patients undergoing unilateral total knee replacement for end stage osteoarthritis was approved by the Macquarie University HREC (Ref #: 5201300753). The lateral femoral condyles and synovium were collected during in sterile PBS from 5 patients (n = 5) (see table 5.1 for patient demography). Within two hours of harvest, articular cartilage was sectioned off the femoral condyles as previously described [373]. Synovium was dissected and stripped of adipose tissue and sectioned into ≈ 5 mm explants. Explants were sectioned so as to obtain five technical replicates from each patient for each of the 5 conditions tested (see table 5.2: Media, HA-media, Synvisc-media, MSC-media, or MSC/HA-media).
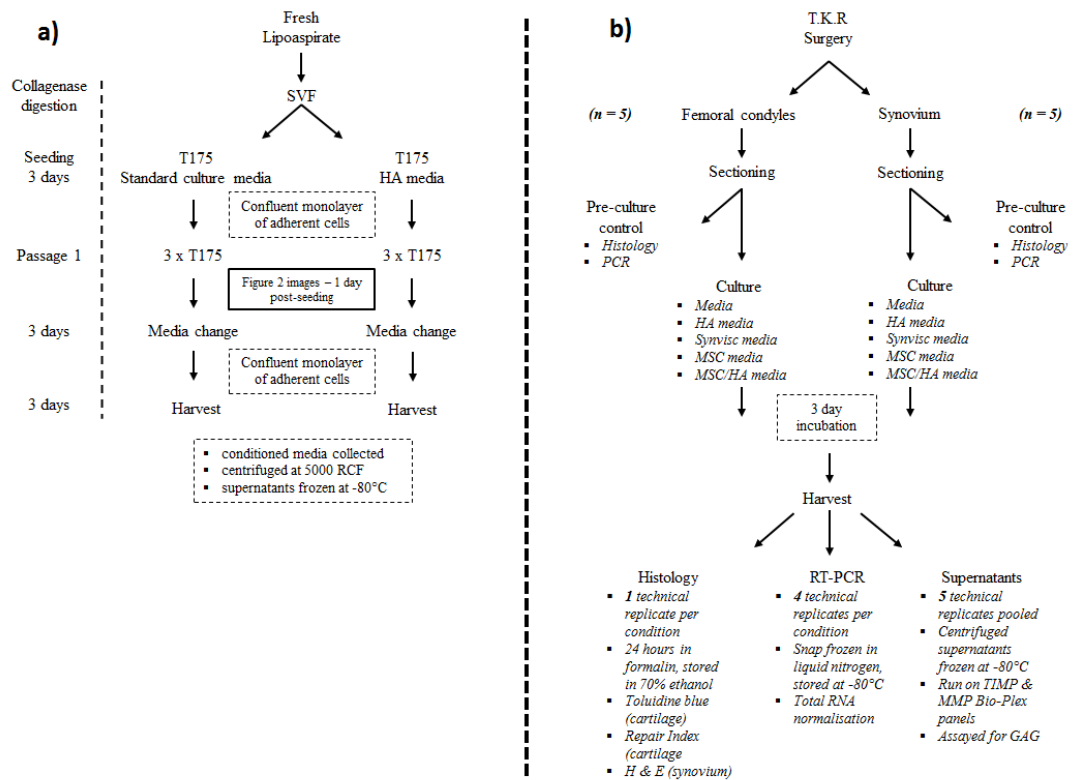
Figure 5.1 Workflow for the derivation of conditioned medium and experimental workflow

a) Human lipoaspirate was obtained from a single female patient undergoing elective cosmetic liposuction surgery of the abdomen. Lipoaspirate was digested with collagenase. The resulting SVF pellet was transferred into two T175cm2 flasks containing either Media HA-media. Flasks were incubated for 72 hours at 37°C with 5% carbon dioxide. At confluence, cells were stripped and passaged at a ratio of 1:3 and termed passage one. Media was changed after three days with either of the two respective formulations. After another three days, the conditioned media was collected, centrifuged at 5000 RCF and supernatants frozen at -80°C. **b)** The lateral femoral condyles and synovium were collected during total knee replacement surgery (n = 5). Cartilage and synovium explants were sectioned so as to obtain five technical replicates for each of the culture conditions and the pre-culture. The pre-culture explant from each of the tissues was snap frozen in liquid nitrogen, another was put into 10% (v/v) neutral buffered formalin for 24 hours and then transferred into 70% ethanol. After three days, conditioned media from five technical replicates were collected, pooled, centrifuged at 5000 RCF and frozen at -80°C for later analysis. An explant (one technical replicate) from each of the treatments was collected into 10% (v/v) neutral buffered formalin for 24 hours, then transferred into 70% ethanol for histological analysis. The remaining four technical replicates for each of the conditions were snap frozen in liquid nitrogen and stored at -80°C for later gene analysis

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Table 5.1 Summary of patient demography used in this study
Average age of patients was 64.4 years with a range of 14 years. Female patients accounted for 80% of the cohort. The average weight in kilograms (kg) was 78.8 kg with a range of 36 kg. All patients were graded by the physician as grade IV and thus candidates for whole knee arthroplasty. (-) data no provided.

Patient ID	Age	Gender	Weight (kg)	Height (cm)	OA grade
ODC – 105	64	female	77	-	IV
ODC – 106	69	male	99	-	IV
ODC – 107	70	female	72	166	IV
ODC – 108	56	female	63	156	IV
ODC – 109	63	female	83	150	IV

Table 5.2 Description of the media and treatment conditions used to treat cartilage and synovium

Media conditions	Description
Media	Standard culture media; Dulbecco's Modified Eagle Medium, 10% foetal bovine serum and 1% Penicillin-Streptomycin
HA-media	Standard culture media with 1 mg/ml HA; 2.6 MDa
Synvisc-media	Standard culture media with 1 mg/ml Synvisc; 6 MDa Hylan G-F 20

MSC conditions	Description
MSC media	50% conditioned media from cells grown in standard culture media and 50% fresh standard culture media
MSC/HA media	50% conditioned media from cells grown in HA media and 50% fresh HA media

5.5.3 Histological analysis

At harvest cartilage and synovial explants were fixed for 24 hours in 10% neutral buffered formalin, processed to paraffin and 4 μm cartilage and synovium sections were cut and stained as previously described [374,375]. Cartilage sections stained with toluidine blue and synovium sections stained with haematoxylin and eosin (H&E), were coded Susan Smith and scored by a single blinded observer (Peter Succar) under the supervision and direction of Professor Christopher Little, an expert and the lab head of the bone and joint research laboratory. The most affected region was scored for each of the parameters using previously published scoring systems for cartilage (n = 5) [374] and synovium sections (n = 4); only sections with a defined synovial edge (see figure 5.4a) were scored [375].

Additionally, a cartilage "repair index" utilizing the percentage of chondrocytes with territorial toluidine blue staining in the peri-cellular region of chondrocytes was used as previously described with some modifications [376]. Briefly, three images per section were generated from distinct regions of interest whilst blinding was maintained. Image acquisition was guided by the rule of having the tangential zone visible in the image using 20 X magnification. Chondrocytes with distinct territorial toluidine blue staining were counted and expressed as a percentage of the total chondrocytes in the tangential zone. The three technical replicates were averaged and following decoding of samples, grouped into respective conditions and compared using non-parametric statistics. Data is presented as mean ± 95% confidence intervals (n = 5 patients/treatment).

5.5.4 Cartilage proteoglycan release in culture

Proteoglycan content of the culture medium was measured as sulphated glycosaminoglycan (GAG) as as previously described [377], with GAG release expressed as μg/μL of culture medium rather than μg/mg as cartilage explants were all uniform size in equal volume of media. All conditions were compared back to the Media condition and data is presented as mean ± 95% confidence intervals (n = 5 patients/treatment).

5.5.5 RNA extraction, reverse transcription and real time PCR

RNA extraction, reverse transcription and real time PCR were performed using validated primers for ACAN, ADAMTS4, ADAMTS5, MMP1, MMP2, MMP3, MMP9, MMP13, COL1A1, COL2A1, COL10A1, TIMP1, TIMP2, TIMP3, WISP1, TNF, IL6, TGFB2, CD44, SOX9 as previously described [378] but normalised to total (0.5 μg) RNA. Concentration was normalised using a log transformation and presented as mean ± 95% confidence intervals (n = 3 patients/treatment).

5.5.6 Secretome analysis

The conditioned media was collected from every well in this study. All technical replicates were pooled and centrifuged at 5000 x g for 5 minutes and supernatants stored at -80°C. Upon thawing, aliquots (50 μL) were analysed using Bio-Plex Pro™ Assays for human matrix metalloproteinases (MMPs; MMP 1, MMP 2, MMP 3, MMP 7, MMP 8, MMP 9, MMP 10, MMP 12 and MMP 13) and tissue inhibitors of matrix metalloproteinases (TIMPs; TIMP 1, TIMP 2, TIMP 3 and TIMP 4) (Bio-Rad, USA), according to the manufacturer's instructions. The washing steps were

performed using the Bio-Plex Pro II magnetic wash station and the data was acquired

using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, USA).

Fluorescence was normalised using a log transformation and presented as mean ±

95% confidence intervals (n = 5 patients/treatment).

5.5.7 Statistical analysis

Two sided Mann-Whitney tests were used on data where normal distribution could

not be confirmed using GraphPad Prism; version 6.04. Statistical significance was set

at the 0.05 level. Other, statistical analysis used to test for significant differences (at

the 0.05 level) between Pre-culture, Media (3) and MSC conditions (2) was done

using R (version 3.1.0) via RStudio (Version 0.98.507). Raw data was log2

transformed to help with assumptions of normality. A linear mixed-effects model

using Ime4 [379] was used to test for treatment and outcome effects plus the

significance of the interactions between these effects. A random effect conditional on

patient was used to account for patient to patient differences. The visualization of

regression results was done using visreg [380] and the significance of interactions terms

in the linear mixed-effects models were obtained using the testInteractions procedure

of De Rosario-Martinez [381]. The *P*-values were multiple test corrected according to

the multiple test correction procedure (FDR) of Benjamini and Hochberg [382].

5.6 Results

5.6.1 Exogenous hyaluronan cleared peri cellular matrix *in vitro*

Adherent adipose-derived mesenchymal stem cell (MSC) populations cultured in standard culture media showed distinct peri cellular halo matrices at 10 X magnification (figure 5.2a) and upon closer observation at 20 X magnification (figure 5.2b) the distinct peri cellular halo matrices created an exclusion zone surrounding cells bodies. This phenomenon was not observed in HA-media cultures (figure 5.2c & d).
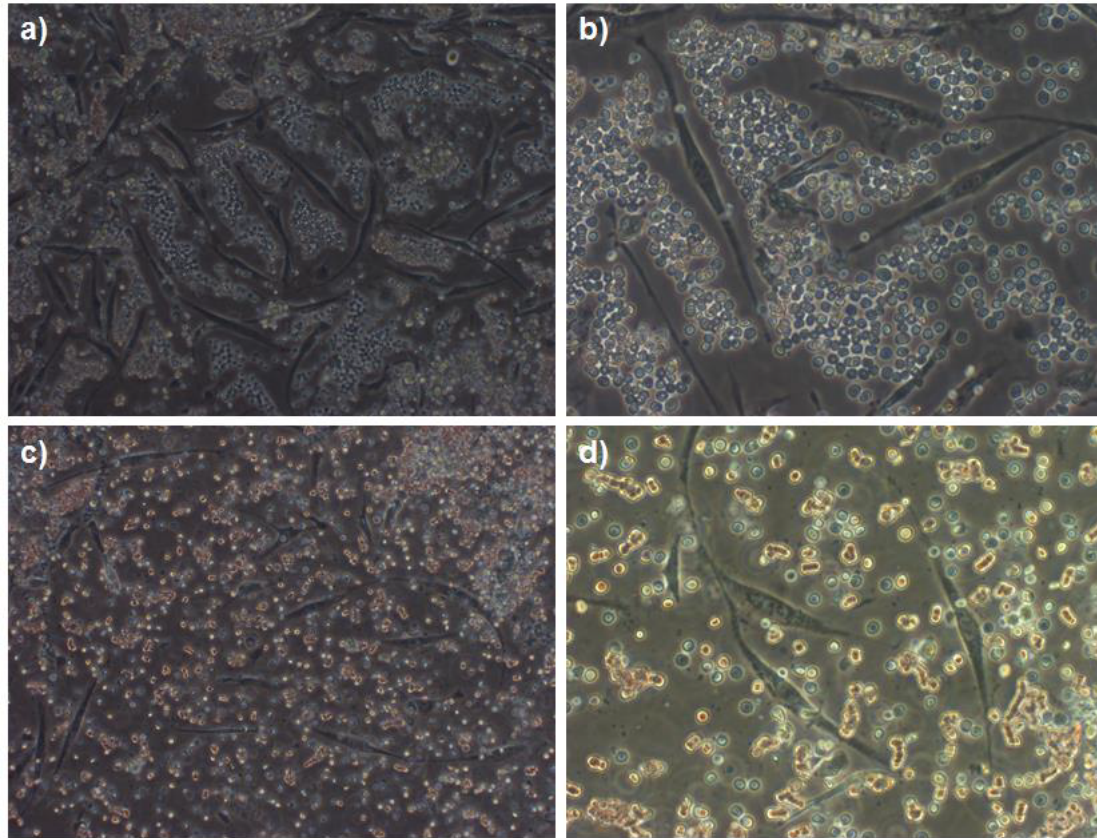
Figure 5.2 Representative images of deteriorated peri-cellular halo matrix in primary cultures containing Hyaluronan

The stromal vascular fraction (SVF) was obtained following enzymatic digestion of lipoaspirate and then seeded into flasks containing either standard culture media or HA media (standard culture media; 1 mg/ml HA). Flasks were incubated for three days at 37°C with 5% carbon dioxide. Flasks were visualised and imaged periodically during this time using an Olympus IMT-2 inverted microscope (Olympus, Australia) with a mounted Scion VisiCapture Firewire camera (Scion Corporation, USA). a) adherent cell population cultured in standard culture media showing distinct peri cellular halo matrices at 10 X magnification b) adherent cell population cultured in standard culture media showing distinct peri cellular halo matrices created a red blood cell exclusion zone surrounding cells bodies at 20 X magnification c) adherent cell population cultured in HA media depicting the absence of peri cellular halo matrices at 10 X magnification d) adherent cell population cultured in HA media depicting the absence of peri cellular halo matrices and a lack of red blood cell exclusion zone surrounding cells bodies at 20 X magnification

5.6.2 HA-media increases territorial secretion of proteoglycans by chondrocytes and retention of glycosaminoglycans in cartilage explants

Representative microscopic images (figure 5.3a-e) depict the parameters used in the scoring system and the spectrum of change observed. There was no change in the cumulative histopathological score (figure 5.3f) or the individual histological parameters (data not shown). A feature of histopathology in human OA cartilage, is loss of inter-territorial and peri-cellular/territorial toluidine blue staining (figure 5.3d and e). In the short time-frame of the cultures, any positive effect of the different culture media would likely be in reduced degradation, increased synthesis and retention of proteoglycan by chondrocytes. We therefore examined the percentage of chondrocytes with positive peri-cellular toluidine blue ("cartilage repair index") and release of GAG into the medium in the different culture conditions. There was no difference in the % of chondrocytes with peri-cellular proteoglycan in control culture media compared with pre-culture tissue (figure 5.3g). The HA-media increased the repair index compared with Media (P = 0.007) and MSC/HA-media (P = 0.007). These differences in peri-cellular proteoglycan were not reflected in total GAG release from the cartilage matrix (figure 5.3h), where compared with Media alone, HA-media and Synvisc-media significantly decreased release of GAG (P = 0.039 and 0.031 respectively).
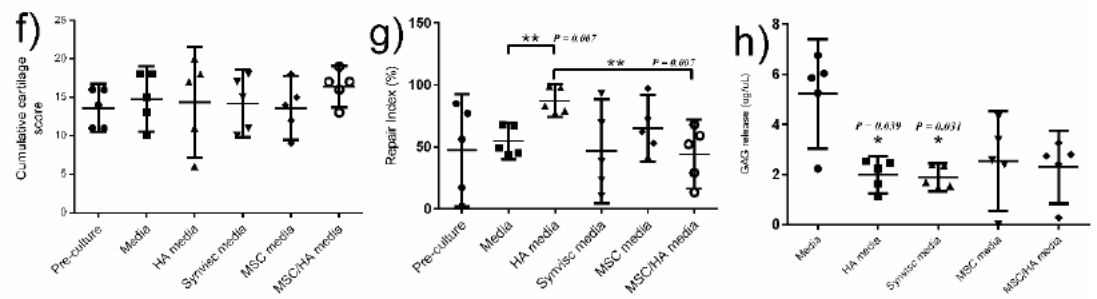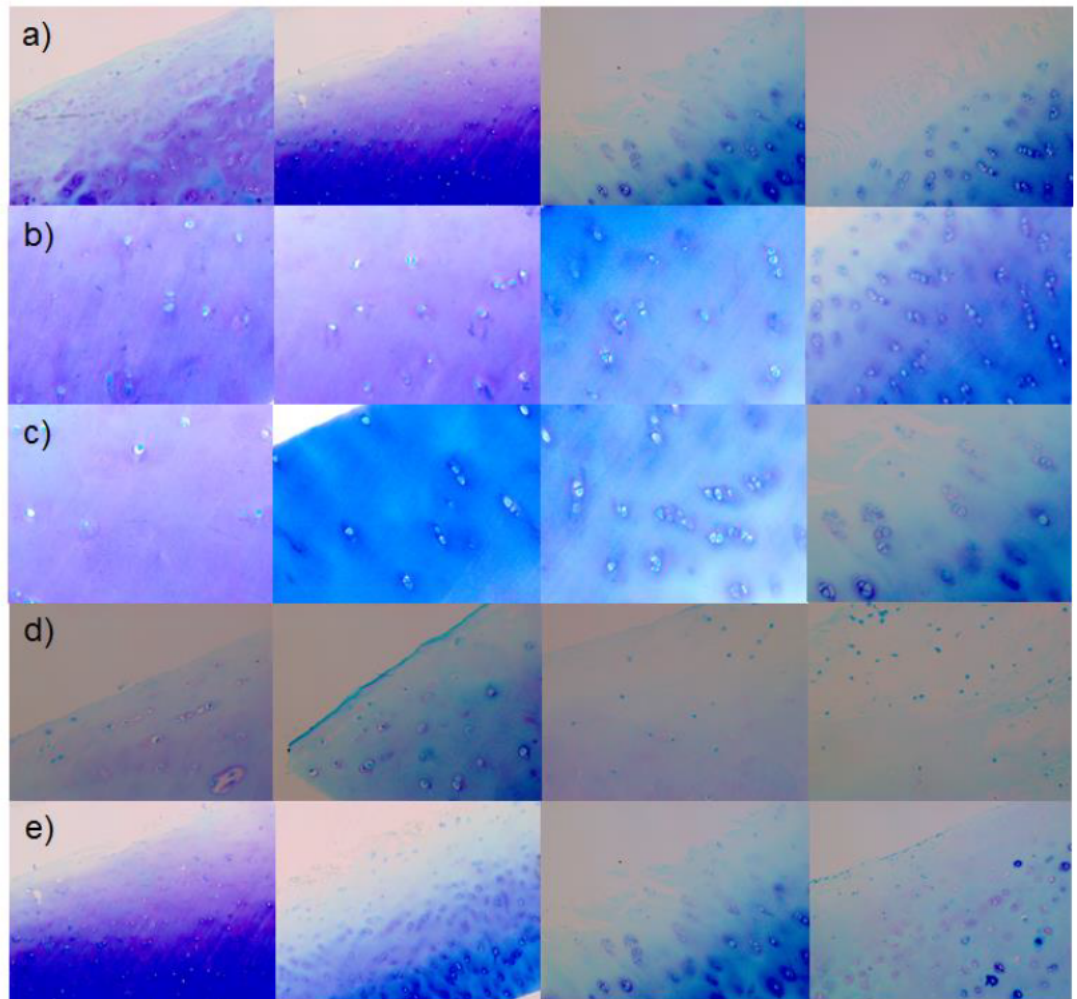
Figure 5.3 Histological changes in pathology of cartilage explants using semi-quantitative scoring methods and relative quantitation of soluble glycosaminoglycans

Following processing to paraffin blocks by standard histological methods, 4 μm cartilage sections were cut and stained with toluidine blue. All sections were coded and scored by a single observer blinded to the conditions. Cartilage sections were scored using a modified scoring system for histopathological assessment (see Table 1). a) to e) representative images of cartilage explants stained with toluidine blue used for scoring pathology. Images depict worsening pathology from left to right for each of the rows. a) Structure b) cellularity c) chondrocyte cloning d) territorial toluidine blue staining e) inter-territorial blue staining f) cumulative cartilage score (mean ± 95% CI) (y axis) comparing all the conditions (x axis) used in this study (n =5) g) conditions (x axis) compared using a repair index derived from a total of three images per section of chondrocytes containing territorial toluidine blue staining expressed as a percentage (mean ± 95% CI) (y axis) of total count (n = 5) h) Conditioned medium analysed using metachromatic dye 1,9-dimethylmethylene blue (DMMB) to determine proteoglycan sulphated glycosaminoglycan (GAG) content (mean ± 95% CI) (y axis) of the culture medium across the different conditions (x axis) (n = 5). All conditions were compared back to standard culture media condition using non-parametric statistics (*$P < 0.05$, ** $P < 0.01$)

5.6.3 HA-based culture conditions reduce synovial sub-intimal fibrosis

Representative microscopic images in figure 5.4a-d depict the parameters used in the scoring system and the spectrum of pathology observed. No significant changes were observed in the synovium cumulative histopathological score (figure 5.4e). Evaluation of the individual parameters showed all HA-containing media (HA-, Synvisc- and MSC/HA-media significantly reduced sub-intimal fibrosis ($P = 0.047$, 0.007 and 0.047 respectively; figure 5.4f) compared to pre-culture.
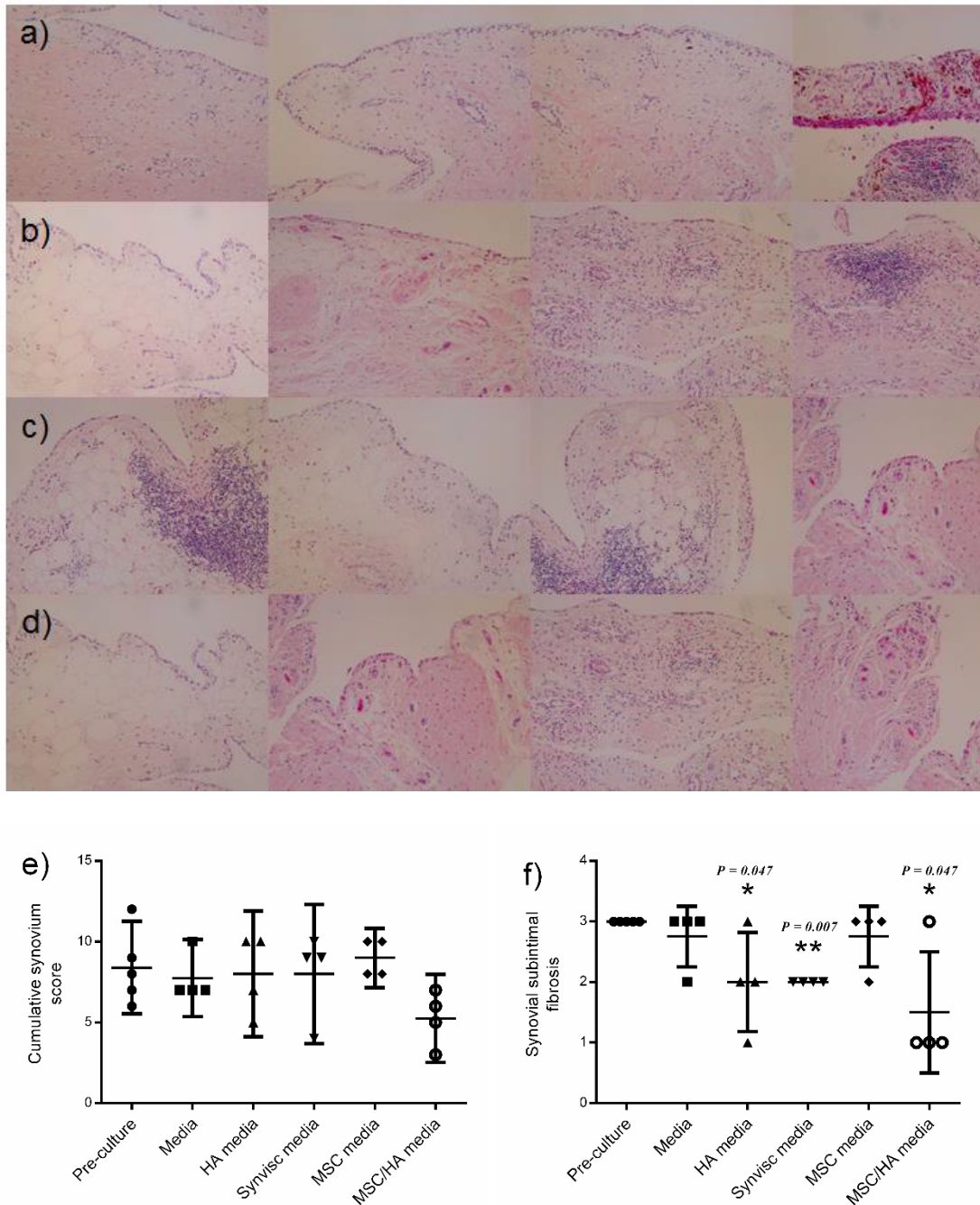
Figure 5.4 Histological changes in pathology of synovial explants using semi-quantitative scoring methods

Following processing to paraffin blocks by standard histological methods, 4 μm synovium sections were cut and stained with haematoxylin and eosin (H&E). All sections were coded and scored by a single observer blinded to the conditions. Synovium sections were scored using a modified scoring system for histopathological assessment. a) to d) representative images of synovium explants stained with H&E used for scoring pathology. Images depict worsening pathology from left to right for each of the rows. a) intimal hyperplasia b) lymphocytic infiltration c) sub-intimal fibrosis d) vascularity e) cumulative synovium score and f) synovial sub-intimal fibrosis score; both (mean $\pm$ 95% CI) (y axis) comparing all the conditions (x axis) used in this study (n = 4) (*$P < 0.05$, ** $P < 0.01$).

5.6.4 Alterations in gene expression of key OA-mediator between treatment groups in cartilage tissue

Interactions were tested between Pre-culture, Media-conditions and MSC-conditions within each of the genes. No significant differences in any gene expression were observed for synovial explants (data not shown). However in cartilage, Aggrecanase 1 (ADAMTS4) expression increased significantly with MSC/HA-media compared to MSC-media ($P = 0.011$; figure 5.5a). No other genes measured in cartilage changed significantly.
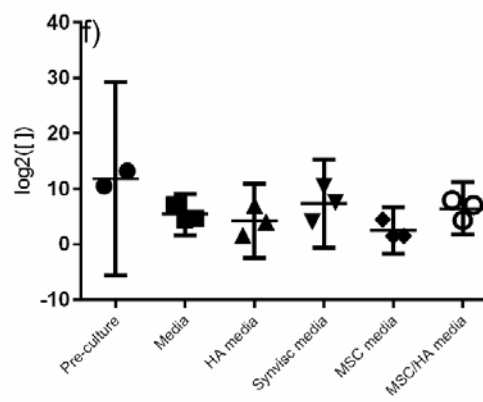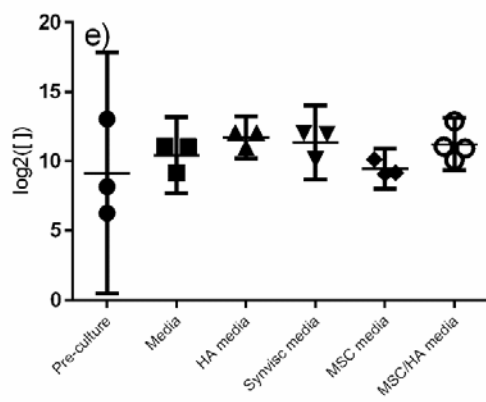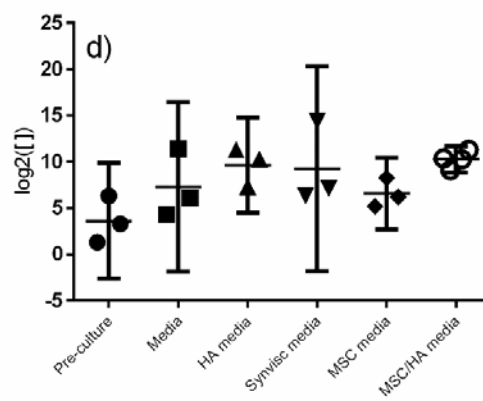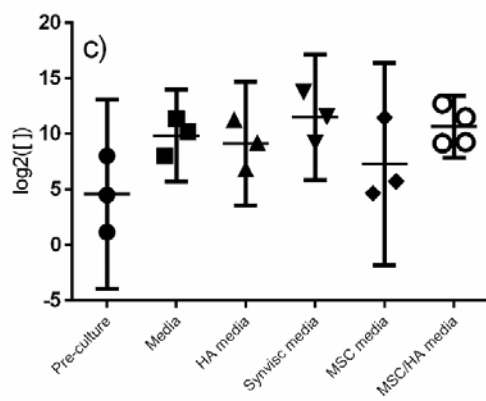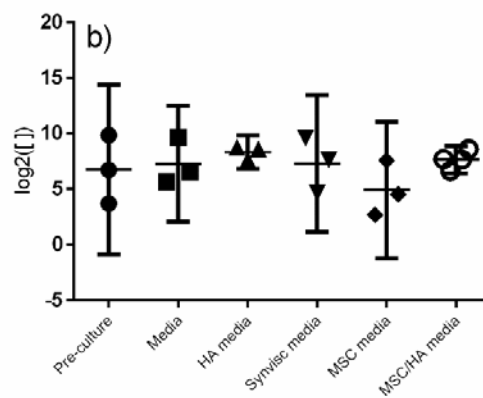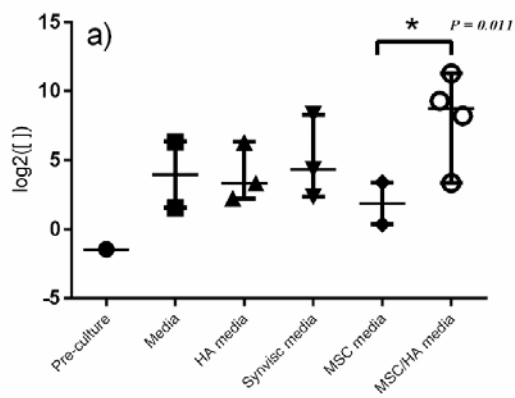
Figure 5.5 Gene expression changes in cartilage explants following three days of culture in each of the conditions

Cartilage explants were either cultured for three days in five conditions, snap frozen in liquid nitrogen and stored at -80°C or collected prior to culture in the same way (Pre-culture condition). Following RNA extraction and reverse transcription, real time PCR was performed using validated human specific primers. Only samples containing sufficient total RNA (0.5μg) were analysed and results were corrected to total RNA to avoid bias in regulatory house-keeping gene fluctuations found in mechanically loaded tissue. Concentration data was log transformed (y axis) and presented as mean ± 95% confidence intervals for each of the conditions (x axis). A linear mixed-effects model using lme4 was used for statistical comparisons between each condition and between the two treatment groups with FDR correction (n = 3) (*$P < 0.05$). a) Aggrecanase 1 (ADAMTS4) b) Aggrecanase 2 (ADAMTS5) c) Matrix Metalloproteinase 3 (MMP3) d) Matrix Metalloproteinase 13 (MMP13) e) Aggrecan (ACAN) f) Tissue Inhibitor of Metalloproteinases 2 (TIMP 2).

5.6.5 MSC-secretome altered release of TIMP and MMP from cartilage & synovium

Cartilage explants cultured in MSC-media released more TIMP3 compared to MSC/HA-media ($P = 0.017$) and Synvisc-media ($P = 0.011$; figure 5.6a). MMP9 release from cartilage increased significantly in MSC-media compared to Media ($P = 0.005$), HA-media ($P = 0.008$) and Synvisc-media ($P = 0.008$; figure 5.6e). MMP12 release from cartilage increased significantly in MSC-media compared to Synvisc-media ($P = 0.041$; figure 5.6f). MSC-media increased MMP7 compared with Synvisc-media *($P < 0.001$)*, MSC/HA-media ($P = 0.001$) and HA-media ($P = 0.008$). Likewise synovium release of MMP7 increased significantly with MSC-media compared to both MSC/HA-media ($P = 0.004$), HA-media ($P = 0.006$) and Synvisc-media ($P = 0.050$) (figure 5.6c-d). The remaining TIMPs and MMPs were detectable in the supernatants but no significant changes between conditions were observed.
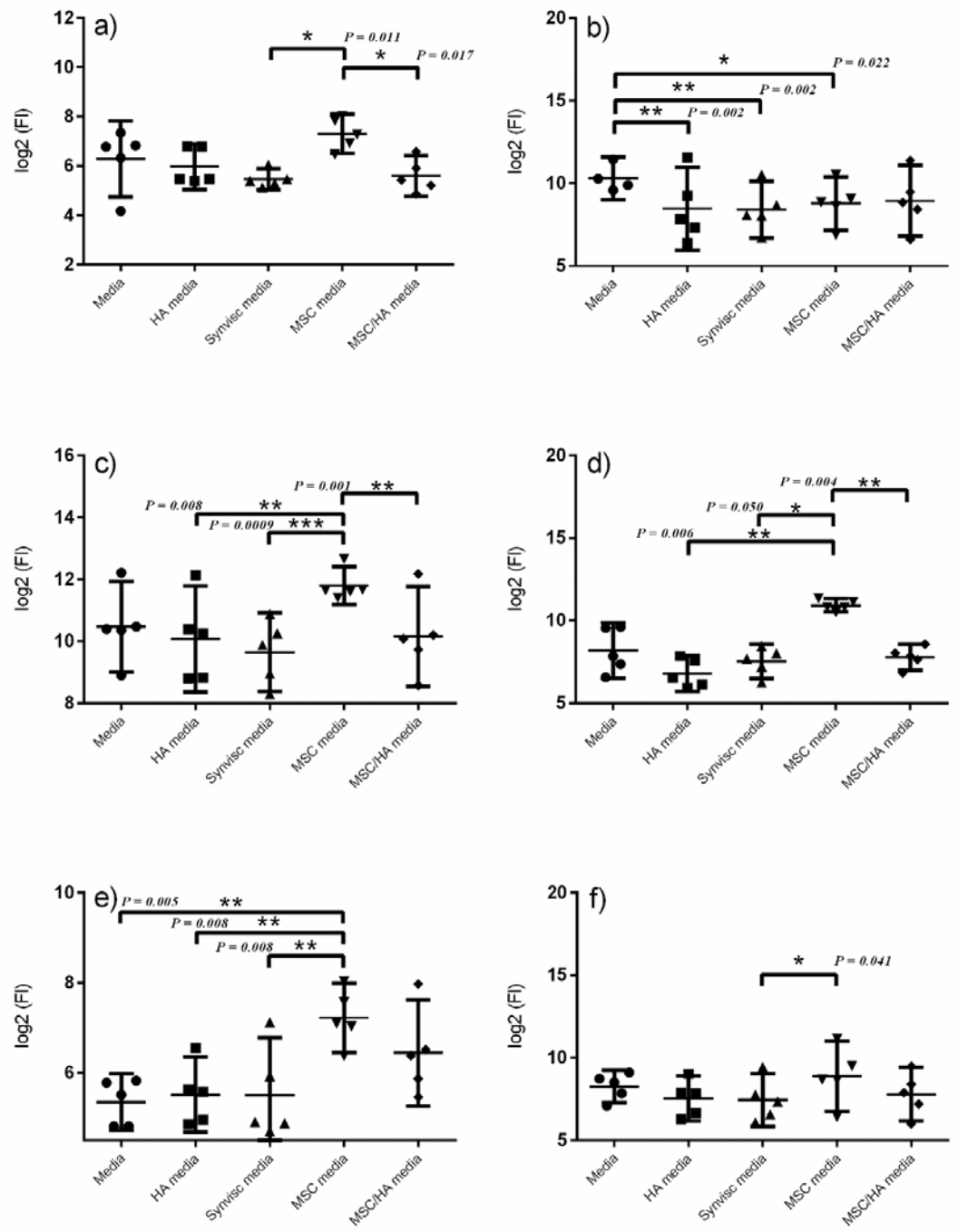
Figure 5.6 Changes in TIMP and MMP secretion by cartilage and synovium explants after three days of culture in each of the conditions

Cartilage and synovium explants were cultured for three days and the conditioned media collected from every well. All technical replicates were pooled. Samples were analysed using Bio-Plex Pro™ Assays for human matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs). Fluorescence data was log transformed (y axis) and the mean ± 95% CI presented for each of the conditions (x axis). A linear mixed-effects model using lme4 was used for statistical comparisons between each condition and between the two treatment groups with FDR correction (n = 5) (* $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$). a) TIMP 3 (cartilage culture) b) TIMP 4 (synovium culture) c) MMP 7 (cartilage culture) d) MMP 7 (synovium culture) e) MMP 9 (cartilage culture) and f) MMP 12 (cartilage culture).

5.7 Discussion

Investigations in our lab have demonstrated MSC secretions in filter-sterilized conditioned media alone, could reduce the clinical arthritis score in a mouse collagen-induced arthritis model [288]. Hence anything that might alter the secretome would be expected to affect the therapeutic efficacy of MSCs *in vivo.* Monitoring of the MSC secretome in the HA-MSC combination showed an increase in interleukin 1 beta, a catabolic cytokine and key mediator in OA pathophysiology [311]. Further work into the HA-MSC combination revealed the HA concentration that produced the greatest effects on MSCs, was centred on the physio-chemical phenomena of entanglement point (1 mg/mL HA) [373]. Our novel cartilage-adherence assay showed MSCs increased attachment to cartilage if they were primed with HA, but not in combination with HA. Attempts to translate the results on fresh patient-derived OA cartilage were unsuccessful, due to limitations in the signal to noise ratio produced from live cartilage. Therefore to minimise noise in the data, fresh OA-derived material was treated with just the conditioned media from either MSCs or MSCs cultured in the presence of 1mg/mL HA. In this way, finite amounts of secretions could be added to the tissue, which allowed the independent assessment of the HA-MSC combination on patient-derived OA tissue.

In primary culture of MSCs, adhered cells sometimes produce a peri-cellular halo which excludes red blood cells and other stromal vascular fraction components. Peri-cellular halos are not unique to MSCs and have been observed in many cell types [134-137, 139]. Studies have determined HA is the core component of the peri-cellular halo, with the HA chains tethered to the cell by binding to specific HA-receptors i.e. CD44 [140, 141, 142], which is abundantly expressed on the surface of MSCs [311]. Our study showed the addition of exogenous HA dissolved or possibly stopped the formation of the peri-cellular halo in MSC cultures. The function of the peri-cellular halo is

unclear, however it may promote cell proliferation by facilitating rounding during mitosis within a hydrated peri cellular zone. Therefore it may be the addition of exogenous HA created global hydration and therefore dispersed the cell-specific hydrated zones via competitive binding to CD44.

Media derived from cartilage or synovial explant culture contains proteins from both the tissue and the initial added conditioned media (MSC- and MSC/HA-media). TIMP3 is a member of an inhibitor family of proteins responsible for preserving the cartilage-matrix via the inhibition of catabolic enzymes. The level of TIMP3 in cartilage culture-media increased significantly in the MSC-media compared with MSC/HA- and Synvisc-media. This may indicate HA modulation of the TIMP3 release, however there was no difference between HA-media, which suggests another mechanism may be responsible. Whilst TIMP3 can inhibit the activity of all MMPs, it more selectively inhibits *ADAMTS4* and *ADAMTS5* [383]. The affinity towards the aggrecanases rather than the MMPs suggests inhibition of these molecules may be the primary role of TIMP3 [384].

MMPs are enzymes that can degrade all components of extracellular matrix and are primary drivers of tissue remodelling. MMP9 increased significantly compared to Media, HA- and Synvisc-media. MMP9 has minimal expression in normal adult chondrocyte and is greatly increased in osteoarthritic-chondrocytes suggesting an involvement in cartilage destruction [385]. Indeed it is only secreted from osteoarthritic-cartilage and not healthy-cartilage [386]. However, MSC/HA-media did not increase which suggests HA is modulating the MSC-secretome or the cartilage-tissue.

Surprisingly, in the conditioned medium from both cartilage and synovium explants, MMP7 release increased significantly in MSC-media compared with MSC/HA-, HA- and Synvisc-media. Previous investigations showed MMP7 to be overexpressed in OA-derived cartilage compared to control and that cytokine stimulated chondrocytes enhanced expression of MMP7 [387], [388]. Others have shown similar results with

protein, mRNA and serum expression levels of MMP7 significantly higher in an OA compared to control [389]. In a study exploring immunolocalisation of MMPs in rheumatoid arthritis (RA) and patient-derived OA synovia, MMP7 was observed only in the RA and never in the OA synovium [390]. In an investigation of synovial fluid from 43 patients suffering from osteoarthritis MMP7 was not detected [391]. A study of the synovial membranes from human patients indicated MMP7 can be detected in both healthy, OA pathology and interestingly in pyogenic arthritic tissue samples using immunohistochemistry [392]. MMP7 is the smallest of the matrix metalloproteinases and may have a role in antimicrobial defence because of its broad substrate specificity. In a study of healthy and pathological lung tissue where MMP7 was found to be constitutively expressed by lung epithelial cells, alveolar type II pneumocytes secreted high amount of MMP7 in response to PMA stimulation [393]. To the best of our knowledge, this is the first report of MMP7 secretion for cartilage and synovial tissue.

No structural changes were observed in the histopathological analysis of cartilage. However, upon closer examination of territorial toluidine blue staining surrounding chondrocytes, we found HA-media significantly increased the repair index i.e. more chondrocytes in these cultures secreted and or retained proteoglycan (as measured by toluidine blue staining) in the peri-cellular zone. Proteoglycans make up 10-15% of the wet weight in the extracellular matrix of articular cartilage [20], with aggrecan, biglycan, decorin, and fibromodulin the most predominant, and having distinct functions in the tissue. Aggrecan is the principal large proteoglycan of cartilage and it propensity to aggregate with hyaluronan into much larger complexes is essential for the mechanical properties of cartilage [21]. Thus if chondrocytes treated with HA-media secrete or retain more aggrecan, this would be expected to better protect them from excessive mechanical loading. The small leucine rich proteoglycans (SLRPs) such as biglycan, decorin and fibromodulin can affect collagen fibrillogenesis, and

their presence on the surface of collagen fibrils impedes cleavage by collagenases [394].

Experimental-arthritis models have shown that during the hypertrophic phase of attempted cartilage repair there is increased expression of these SLRPs [395]. Future studies will aim to characterise the proteoglycan in the peri-cellular space or cartilage-cultured in the different media and how the change might be expected to impact OA-patients.

Unlike the distinct differences between media in peri-cellular proteoglycan, overall GAG-release from the cartilage matrix was reasonably uniformly decreased by HA- and Synvisc-media but not significantly by MSC- and MSC/HA-media. It is likely media-GAG is largely that lost from the inter-territorial matrix in OA-cartilage, the reduction in different media therefore reflecting inhibition of accelerated aggrecanolysis in OA and consistent with previously reported effects of HA [164]. The effect of MSC-and MSC/HA-media on GAG-release while still inhibitory was less consistent than either HA-preparation, which may reflect secretion of both anti- and pro-catabolic effects by MSCs [23]. Loss of GAG from OA-cartilage in culture may also arise from failure to incorporate or stabilise newly synthesised proteoglycan into damaged matrix. In this scenario reduced GAG-release in response to HA or MSC-media would be indicative of inhibition of proteoglycan synthesis. The lack change in ACAN mRNA suggests synthesis of this proteoglycan may not be affected, but future studies examining expression of other proteoglycans and/or radiolabelling would provide insight on how the balance of synthesis and degradation might be differentially modulated by the different conditioned media.

Cumulative histopathological scoring of synovium showed no significant changes for any of the conditions tested. However, upon further sub-analysis of the individual parameters used for scoring, all HA-containing groups, including MSC/HA-media showed a decrease in synovial sub-intimal fibrosis. This was consistent with prior investigation of HA in which Cake *et al.,* found HA could decrease sub-intimal

fibrosis in an ovine-model of experimental osteoarthritis [375]. Thus, we have shown for the first time HA but not MSC-media can reduce synovial sub-intimal fibrosis in human osteoarthritic-tissue in vitro and unlike the GAG retention data in cartilage, the effect of HA was not negatively impacted by the MSC-secretome.

In this study we measured the expression of major matrix constituents, key enzymes implicated in cartilage degradation, inhibitors of catabolic enzymes, chondrogenic transcription factors and drivers of disease. No significant changes were observed in the synovial gene expression potentially in part due to excessive variability in the synovium data that may reflect the focal nature of synovial change in OA and/or unlike cartilage the greater variability in tissue explant harvest. Cartilage sectioning techniques used in this study utilised a hollow cylindrical punch that provides uniform explants as previously described [373]. Cartilage gene expression revealed *ADAMTS4* (Aggrecanase-1) increased significantly in the MSC/HA-media compared to MSC-media. *ADAMTS4* is a proteinase responsible for the cleavage of aggrecan and heavily implicated in early events of cartilage remodelling observed in knee OA [396] and as such its activity is considered a hallmark of cartilage degradation in knee OA. The expression of *ADAMTS4* can be induced by cytokines which play a catabolic role in OA pathogenesis such as interleukin 1β [397] and tumor necrosis factor-α [398]. Our preceding investigations have shown primary cultures of MSCs can secrete increased amounts of interleukin 1β, when combined with HA [311]. Further work with cultured-MSCs treated with 1mg/ml hyaluronan for 3 days (the same protocol used in this study prior to collection of conditioned media), showed interleukin 1β increased significantly compared to control whereas tumor necrosis factor-α did not [373], implicating the former in increased *ADAMTS4* in the MSC/HA-media. Future investigations should competitively block the interleukin 1β in vitro to test this, nevertheless the increased expression of *ADAMTS4* warrants further investigation for the use of a MSC-HA combination in the treatment of knee OA.

5.8 Conclusion

Adipose-derived mesenchymal stem cells (MSCs) have shown promise as a therapeutic for the treatment of knee OA. MSC therapies are rapidly evolving from a three-step liposuction-digestion-injection methodology to a simplified and commercially viable off-the-shelf therapeutic. This study is based on the use of a single donor and therefore inferences from the data are limited. However this was an exploratory pilot study attempting to mimic the direction of current commercial preparations which are dependent on a single, batch controlled donor verified by a series of quality control tests.

MSCs act by secreting a complex mixture of cytokines and these secretions alone can reduce clinical arthritis scores. To elucidate the effects of combining MSCs with HA for the treatment of knee OA, our study functionally tested MSC-media and MSC/HA-media on OA-derived tissues. To the best of our knowledge, this is the first report to show HA-containing media and MSC/HA media can decrease human synovial sub-intimal fibrosis in vitro. This is also the first report of increased MMP7 secretion for OA-cartilage and synovial tissue cultured with MSC-media. In contrast, MMP7 secretion decreased in all HA-containing media. ADAMTS4 gene expression increased significantly in cartilage treated with MSC/HA-media compared with MSC-media. Based on our prior secretion profiling of MSC/HA-media, the increase in ADAMTS4 was perhaps initiated by the IL-1β proinflammatory cytokine present in MSC/HA-media. Significantly higher levels of soluble TIMP3 were detected in MSC-media treated cartilage, which was modulated by MSC/HA-media. In conclusion, further investigation is required to elucidate whether secreted MMP7 has an anti-inflammatory role in OA and the clinical significance in knee OA of increased ADAMTS4 expression by cartilage resulting from a HA-MSC combination.

5.9 Acknowledgments

5.10 References

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5.11 Supplementary materials

5.11.1 RNA extraction, reverse transcription and real time PCR

RNA extraction, reverse transcription and real time polymerase chain reaction (PCR) were performed as previously described with minor changes [378]. Vials containing a stainless steel bearing were pre-cooled in liquid nitrogen and then loaded with each individual explant. Each frozen explant underwent a powdering cycle of 1800 rpm for 30 seconds in the dismembrator (B Braun Mikro Dismembrator S, United Kingdom), followed by the addition of 1 ml of TRIzol® and incubated at room temperature for 45 minutes. Total RNA was purified using Qiagen RNeasy minikits with an on-column DNase digestion (Qiagen, Australia). RNA quality was assessed by Nanodrop®, MultiNA microchip electrophoresis. A uniform amount of RNA (0.5 μg) was reverse transcribed using a Qiagen Omniscript kit with added random decapentamers and RNase inhibitor (Bioline, Australia)). Real time PCR was performed using validated human specific primers for *ACAN, ADAMTS4, ADAMTS5, MMP1, MMP2, MMP3, MMP9, MMP13, COL1A1, COL2A1, COL10A1, TIMP1, TIMP2, TIMP3, WISP1, TNF, IL6, TGFB2, CD44, SOX9* (see supplementary table 3) and internal standards (random mix of sample cDNA) using a Rotor-gene 6000 (Qiagen) as previously described [399]. The Rotor-gene 6000 Series software (version 1.7) generated relative fluorescent units (RFU) for sample cycle thresholds based on calculated, optimized, baseline- and slope-corrected thresholds generated by tissue-matched cDNA standards. Mechanically loaded tissues typically display fluctuations in regulatory house-keeping genes [400] and thus to avoid bias, results were corrected for total RNA as previously recommended [401]. Concentration data was normalised using a log transformation and presented as median ± standard deviation (N = 3; three biological replicates).

Supplementary table 5.1 (ST 5.1) Real time PCR primers to human genes used in this study
* in cartilage only; ** in synovium only

Protein name	Gene name	Accession #	Sequence 5' to 3'	T °C	Product (bp)
Aggrecan	*ACAN*	NM_001135	F – TCA CCA TCC CCT GCT ATT TCA TC R – TCT CCT TGG ACA CAC GGC TC	56	105
Aggrecanase 1	*ADAMTS4*	AF148213	F – AGA CAC AGG CAG GGA GAG ACA AAG R – GGA GAA AAC TTA GTC CTT GGG CTT G	57	110
Aggrecanase 2	*ADAMTS5*	NM_007038	F – AAC TCC CAG GAC AGA CCT ACG ATG R – GCA GAT TCT CCC CTT TCC ACA AG	59	191
Matrix Metalloproteinase 1	*MMP1*	NM_002421.2	F – TGG ACC TGG AGG AAA TCT TGC R – TCA CAC GCT TTT GGG GTT TG	55	312
Matrix Metalloproteinase 2	*MMP2*	NM_004530	F – TGA CGG AAA GAT GTG GTG TG R – CTC CTG AAT GCC CTT GAT GT	57	223
Matrix Metalloproteinase 3	*MMP3*	NM_002422.3	F – GGC AAG ACA GCA AGG CAT AGA GAC R – CGT CAC CTC CAA TCC AAG GAA C	55	280
Matrix Metalloproteinase 9	*MMP9*	NM_004994	F – CAG AGA TGC GTG GAG AGT R – AAT AGG TGA TGT TGT GGT	54	198
Matrix Metalloproteinase 13	*MMP13*	NM_002427.2	F – AAA ACG CCA GAC AAA TGT GAC C R – GCA TCA ATA CGG TTG GGA AGT TC	55	173
Collagen type I **	*COL1A1*	BC036531	F – ACA GGG CGA CAG AGG CAT AAA G R – AAC AGG ACC AGC ATC ACC AGT G	60	229
Collagen type II*	*COL2A1*	NM_033150.2	F – CAG TTC GGA CTT TTC TCC CCT C R – AGT TTC CTG CCT CTG CCT TGA C	55	129
Collagen type X	*COL10A1*	NM000493	F – CCC TCT TGT TAG TGC CAA CC R – AGA TTC AGT CCT TGG GTC A	55	155
Tissue Inhibitor of Metalloproteinases 1	*TIMP1*	NM_003254.2	F – GAC ACC AGA AGT CAA CCA GAC C R – GGT AGT GAT GTG CAA GAG TCC A	55	220
Tissue Inhibitor of Metalloproteinases 2	*TIMP2*	BC071586	F – GCG GTC AGT GAG AAG GAA GTG G R – CTT GCA CTC GCA GCC CAT CTG	58	321
Tissue Inhibitor of Metalloproteinases 3	*TIMP3*	NM_000362.4	F – TGC CCT TCT CCT CCA ATA CA R – CTT CCT TCC CTC CCT CAC TC	55	197
WNT1 Inducible Signaling Pathway Protein 1	*WISP1*	NM_003882	F-TGTCCTTCCAGTGTCCTGATGG R-CAGCAAAGATGTCATTGGGATTCC	60	105
Tumor Necrosis Factor Alpha	*TNF*	NM_000594.2	F – CCA ATC CCT TTA TTA CCC CCT CC R – TGG TTG CCA GCA CTT CAC TGT G	56	171
Interleukin 6	*IL6*	BC015511	F – GAA GAT TCC AAA GAT GTA GCC GC R – GAA GGT TCA GGT TGT TTT CTG CC	55	178
Transforming Growth Factor, Beta 2	*TGFB2*	NM_003238	F – GCA GAA CCC AAA AGC CAG AGT G R – ATG TGG AGG TGC CAT CAA TAC C	54	314
Cluster of Differentiation 44	*CD44*	AY101193.1	F – AAA GGA GCA GCA CTT CAG GA R – TGT GTC TTG GTC TCT GGT AGC	55	128
SRY (Sex Determining Region Y)-Box 9	*SOX9*	NM_00346.3	F – AAA CGG TGC TGC TGG GAA AC R – CTC CTT GCT TGC CTT TTA CCT C	55	103

5.11.2 Synovium and Cartilage processing

Fixation: Fix in 10% (v/v) neutral buffered formalin for 24 hours. (May now be stored in 70% (v/v) ethanol)

Decalcification: Decalcify in 10% (v/v) formic acid (v/v) 5% formalin for 24 hours. Cartilage decalcified in 70ml specimen containers with agitation.

Specimens stored in 70% (v/v) ethanol until ready to process.

Processing;

1. 70% (v/v) ethanol 1 hour

2. 75% (v/v) ethanol 1 hour

3. 85% (v/v) ethanol 1 hour

4. 95% (v/v) ethanol 1 hour

5. 100% (v/v) ethanol 1 hour

6. 100% (v/v) ethanol 1 hour

7. 100% (v/v) ethanol 1 hour

8. Chloroform 2 hours

9. Chloroform 2 hours

10. Chloroform 2 hours

11. Wax (Paraplast Plain) 2 hours with vacuum

12. Wax (Paraplast Plain) 2 hours with vacuum

13. Wax (Paraplast Plain) 2 hours with vacuum

14. Wax (Paraplast Plain) 6 hours with vacuum

15. Embed in wax (Paraplast plain)

Note: The above protocol is for the processing of cartilage. The processing of synovium was similar, however step 14 is not included.

5.11.3 Haematoxylin and Eosin Technique

This protocol uses Mayer's Haematoxylin which is a progressive stain containing an aluminium mordant that complexes to the tissue to produce a clear blue staining of nuclei under alkaline conditions (Scott's Blue solution).

The counterstain is Eosin, and acidic dye (negatively charged) the enables positively charged groups within the proteins of the tissue to unite with the ionised eosin dye molecules. This results in a contrasting pink staining of the tissue around the blue nuclei.

Tissues start this procedure surrounded by paraffin on a glass slide. The aim of this procedure is to **rehydrate** the tissues to water and so remove the paraffin, stain the tissue and then **dehydrate** to xylene before mounting.

Procedure:

Place slides to be stained in a black slide holder all facing the same way.

Xylene 1 (paraffin) = 3 min. Immerse and initially agitate

Xylene 2 (paraffin) = 3 min. Immerse and agitate.

100% Ethanol 1 = 1 min. Immerse and agitate.

100% Ethanol = 1 min. Immerse and agitate.

95% Ethanol = 1min. Immerse and agitate.

70% Ethanol = 1 min Immerse and agitate.

Running tap water ~ 1 min. Immerse and agitate.

Mayer's Haematoxylin = 5 min. Immerse and agitate.

Running tap water. Rinse until runs clear.

Scott's blueing solution = 1 min. Immerse and agitate.

Running tap water = 1 min.

 Check the slides microscopically for the correct ratio of staining.

Eosin solution = 5 min. Immerse and agitate.

Wash briefly in running tap water to remove excess eosin.

The next steps are dehydration in ethanol, clearing in xylene and mounting in a permanent mountant.

100% Ethanol. Immerse and agitate.

100% Ethanol. Immerse and agitate.

100% Ethanol. Immerse and agitate.

100% Ethanol. Immerse and agitate.

Xylene. Immerse and agitate.

Xylene. Immerse and agitate.

Xylene. Immerse and agitate.

Mount slide in a resinous mountant (Eukitt, Sigma)

Supplementary table 5.2 (ST 5.2) The synovial histopathological scoring system used in this study

PARAMETERS	Score & Description
INTIMAL HYPERPLASIA	0 normal (1-2 layers)
	1 mild-moderate, focal thickening (3-4 layers)
	2 mild-moderate, diffuse thickening
	3 moderate-marked, diffuse thickening (>5 layers)
LYMPHOCYTIC / PLASMACYTIC INFILTRATION	0 none
	1 mild-moderate, focal infiltrate
	2 mild-moderate, diffuse infiltrate
	3 moderate-marked, diffuse infiltrate
SUBINTIMAL / PERIVASCULAR FIBROSIS	0 none
	1 slight fibrosis
	2 moderate fibrosis
	3 marked fibrosis
VASCULARITY	0 normal
	1 slight increase in vascular elements
	2 moderate increase in vascular elements
	3 marked increase in vascular elements

5.11.4 Toluidine blue staining technique

Procedure:

Place slides to be stained in a black slide holder all facing the same way.

Xylene 1 (paraffin) = 3 min. Immerse and initially agitate

Xylene 2 (paraffin) = 3 min. Immerse and agitate.

100% Ethanol 1 = 1 min. Immerse and agitate.

100% Ethanol = 1 min. Immerse and agitate.

95% Ethanol = 1min. Immerse and agitate.

70% Ethanol = 15 min Immersion and then thoroughly drain.

Stain in 0.04% Toluidine Blue O (C.I. 52040) in 0.1M sodium acetate buffer pH 4.0

for 10 minutes.

Rinse quickly in running tap water.

Stain in 0.1%Fast Green FCF (C.I. 42053) for 2 minutes.

Rinse quickly in running tap water.

Dehydrate in 3 changes of isopropyl alcohol then 3 changes of xylene.

Mount in a resinous mountant (Eukitt, Sigma)

Supplementary table 5.3 (ST 5.3) Modified grading method used to derive histopathological scoring of articular cartilage (adapted from Cake et al.)

PARAMETERS	Score & Description
STRUCTURE	0 Normal
	1 Slight surface irregularities
	2 Moderate surface irregularities
	3 Severe surface irregularities
	4 Clefts/fissures into transitional zone (one-third depth)
	5 Clefts/fissures into radial zone (two-thirds depth)
	6 Clefts/fissures into calcified zone (full depth)
	7 Fibrillation and/or erosion to transitional zone (one-third depth)
	8 Fibrillation and/or erosion to radial zone (two-thirds depth)
	9 Fibrillation and/or erosion to calcified zone (full depth)
	10 Fibrillation and/or erosion to subchondral bone
CELLULARITY	0 Normal
	1 Increase or slight decrease
	2 Moderate decrease
	3 Severe decrease
	4 No cells present
CHONDROCYTE CLONING	0 Normal
	1 Several doublets
	2 Many doublets
	3 Doublets and triplets
	4 Multiple cell nests
TERRITORIAL TOLUIDINE BLUE STAINING	0 Normal
	1 Increase or slight decrease
	2 Moderate decrease
	3 Severe decrease
	4 No staining
INTER-TERRITORIAL TOLUIDINE BLUE STAINING	0 Normal
	1 Decrease staining to tangential zone (one-third depth)
	2 Decrease staining to transitional zone (two-thirds depth)
	3 Decrease staining to radial zone (full depth)
	4 Loss of staining to tangential zone (one-third depth)
	5 Loss of staining to transitional zone (two-thirds depth)
	6 Loss of staining to radial zone (full depth)

6

Conclusions & Future Directions

6.1 Conclusion

There is currently a treatment gap in the therapies available for younger sufferers of knee OA. Current therapies provide short to medium term symptomatic relief, but cannot halt disease progression and thus patients who are not candidates for total knee replacement, generate economic and healthcare burdens for governments around the world. End stage knee OA inevitably results in the need for prosthetic interventions, which also have a limited lifespan and often require revision surgery. MSC therapy for the treatment of knee OA is emerging as an alternative biological therapy, which may significantly improve pain, function, slow disease progression, regenerate articular and meniscal cartilage, reduce inflammation and modulate the immune system. Although most of the studies performed have not been controlled and the evidence is not strong enough that this therapy is effective in treating the underlying pathology of osteoarthritis or can regenerate the damaged tissue.

Investigators have combined MSC therapy with HA for the treatment of knee OA and have shown there are minimal adverse events associated with the combination. However, all studies thus far have been uncontrolled and no comparison on the efficacy is made between MSC therapy with or without HA for the treatment of knee OA.

The initial investigation aimed to assess the feasibility of current clinically relevant and available preparations of MSC therapy in combination with HA. The investigation aimed to initially determine the effects on differentiation and immunophenotypic expression of regular MSC surface markers. The data showed MSCs maintained their multipotent state when co-cultured with HA and did not differentiate unless chemically induced to do so. Furthermore there were no

substantial changes observed in typical immunophenotypic expression on the surface of MSCs.

Following the preservation of multipotency of MSCs, it was imperative to explore the effects of HA on the MSC secretome, since the mode of action of MSCs is secretion driven. An understanding of the effects HA can have on the MSC secretome could be of clinical value for future investigations involving MSC therapy and HA. The cytokine analysis captured alterations in the MSC secretome at the protein level and showed the cytokine profile was substantially affected depending on the type of preparation. This was most prominent in the preparations containing mixed cell populations rather than the culture purified MSCs.

Although results from this work demonstrated HA can have profound effects on the MSC secretome in a range of clinical preparations, the investigation was purely *in vitro.* Thus the artificial nature of the experiment did not allow any clinically meaningful conclusions about the *in vivo* activity to be drawn.

Culture expanded MSC therapy for the treatment of knee OA is advantageous compared to other preparations and is likely to be the approach of future investigations. However if HA was to be used in combination with MSC therapy, practical considerations needed to be made for the concentration which would be used. Additionally, the likely move of MSC therapy towards an off-the-shelf style therapeutic raised questions about how MSCs would be combined with HA. That is, would they be co-administered separately? Would MSCs be cultured in HA and then administered independently? Or cultured with HA and administered together. We therefore sought to test the effect of a range of HA concentrations on MSC growth-kinetics *in vitro* in a range of different ways to re-capitulate our speculations on the future use of a MSC-HA combination.

The current HA treatment concentration of 10 mg/mL, was not feasible due to constraints of viscosity. The exploration of HA concentrations found that 1 mg/mL, which incidentally coincided with the HA entanglement point, was the optimum concentration to achieve maximum adherence and proliferation of MSCs. To then better understand what effects HA would have on MSC binding to cartilage *in vivo*, a quantitative explant model was developed to validate the previous findings of MSC adherence and proliferation to articular cartilage in culture. Surprisingly, it was revealed only the primed 1 mg/mL concentration of HA showed the greatest adherence and proliferation on cartilage and not in the presence of HA. This suggested that HA may act to inhibit MSC adherence to articular cartilage.

Moreover the cartilage explant model was used to qualitatively show for the first time that HA viscosity acts as a constraint on the dispersion of MSCs on cartilage. Higher concentrations of HA limited MSC adherence to cartilage to singular colonies, whereas lower concentrations allowed for full surface coverage. Although the clinical significance of these results remain unknown, the data can be well utilized for physical dispersion studies of cells in the practical sense depending on the future applications. The use of the cartilage explant model in a time course for the first time has also shown the time needed for MSCs to adhere to cartilage. The model could not take into account actual biological forces MSCs would be subjected to in the knee joint, however the data suggested patients undergoing MSC therapy would benefit greatly from a period of unloading to allow MSCs to thoroughly adhere to cartilage.

Upon discovering a biological significance for the physicochemical phenomena in HA known as the HA entanglement point, assessment of the MSC cytokine profile revealed striking dose-dependent increases of two major pro-inflammatory proteins involved in the pathogenesis of OA, MIF and IL-1β. As the mode of action of MSCs

is secretion driven, functionally testing how the secretions resulting from MSCs cultured in HA would fair compared to unaltered MSC secretions was imperative. To elucidate the effects of the HA/MSC secretome, we translated the previously refined cartilage explant model to include live, biologically active, OA-derived cartilage and synovium from patients undergoing total knee replacements.

The investigation of osteoarthritic synovium revealed for the first time that HA can decrease human synovial sub-intimal fibrosis, which was consistent with previous findings in animal models of OA. More importantly, secretions from MSCs cultured in HA showed the same decrease as HA media, whereas MSC secretions alone did not. MSC secretions but not MSC secretions derived from HA culture, increased the levels of MMP7 released by cartilage. Similarly, other catabolic enzymes, MMP9 and MM12, showed increased levels from cartilage when treated with MSC secretions but not from MSC secretions derived from HA culture. Thus our data suggests that HA plays an important modulatory role on mode of action of MSCs.

In conclusion, the investigation sought to elucidate the effects of combining MSCs with HA. Our growth kinetics data showed HA can have profound dose-dependent effects on MSC adherence and proliferation, especially with respect to cartilage. Although early data showed increased secretion of pro-inflammatory cytokines by MSCs cultured in HA, our functional assessments of human osteoarthritic cartilage and synovium demonstrated HA culture modulated negative effects produce by the MSC secretome. Thus we have shown through exploration of the MSC secretome that MSCs cultured in HA would have a synergistic effect in MSC therapy for the treatment of knee OA.

6.2 Technical considerations and future directions

The assessment of MSC growth kinetics when cultured in the presence of HA showed the concentration of HA can have profound dose dependent effects on adherence and proliferation, especially with respect to cartilage. In these investigations, viscosity of the media as a result of increasing HA concentration limited our ability to determine the effects of clinically relevant preparations of HA, which are typically manufactured at 10 mg/mL. Our assessment of viscosity using the falling-ball test showed an inordinate increase in viscosity beyond the entanglement point of 1 mg/mL. Moreover, our physical dispersion experiment highlighted how the cells would clump into a single colony at 5 mg/mL.

The measurement of adherence and proliferation relied on the metabolic activity of adhered MSCs. Although adherence of MSCs is inherently constrained by viscosity, proliferation measures could be determined using different types of culture methods. For example, the use of suspension culture or pellet culture (non-adherent culture) would curb the constraint of viscosity in that the cells would not need to adhere before proliferating. This would negate the use of CCK-8, which is metabolically dependent and reliant on a consistent, accurate and reproducible standard curve to extrapolate cell number. Our experience with seeding standard curves with such culture methods, indicated these alternatives were inaccurate and inconsistent when seeding standard curves and thus were avoided.

Future investigations measuring proliferation of MSCs using non-adherent culture methods, would benefit greatly from the use of flow cytometric proliferation assays. One such assay is the 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay. BrdU is incorporated in place of

thymidine into the newly synthesized DNA of proliferating cells. The use of a fluorescent antibody is then used to capture the incorporated molecule and the amount of absorbance is a proportional measure of cell number.

The novel cartilage explant model allowed for quantitation of adhered MSCs on the surface of cartilage. The processing steps used to derive the cartilage discs ensured low metabolic activity of articular chondrocytes within the cartilage. The CCK-8 assay was therefore appropriate to measure the adhered cells, because the explants with no MSCs seeded onto to them could be used as a blank and thus the absorbance from the test explants produced sufficient signal. Attempts to translate the cartilage explant model to live human OA-derived articular cartilage was not successful. Chondrocyte metabolic activity in the blank samples saturated the absorbance and therefore no signal could be achieved in the test explants. In order to curb the excessive signal produce using CCK-8, future investigations could employ semi-quantitative image analysis methods. By pre-labelling MSCs with CM-DiI, explants could be fixed in paraformaldehyde at the endpoint. Fluorescent microscopy could then be used to generate images of the surface to identify the MSCs on cartilage. The images would then be processed through image analysis software, such as Imaris, in order to obtain comparable MSCs numbers seeded from different concentrations HA.

The effect of MSC and HA/MSC secretions on osteoarthritic cartilage showed HA modulated the negative effects produced by MSC secretions alone. However the pilot study of only five patients could not account for the excessive biological variability between patients and thus most of the gene expression data was inconclusive. Due to PhD time constraints the investigation was limited to only 5 patients. To curb the biological variability in the explants, future investigations would benefit from conducting a power analysis this data in order to determine how many more patients are required to achieve substantial signal differentiation. Moreover, the methods used

for cartilage sectioning had previously been standardized whereas the sectioning of synovium was not. It was assumed that although we utilized approximate sectioning techniques for synovium, the presence of 5 technical replicates within each biological sample would enable signal production and differentiation between treatments. However the excessive variability proved to be the principal constraint in obtaining discernable gene expression data between conditions. To limit the variability in synovium analysis, future investigations would benefit from standardizing the explants by weight.

This study relied on the quantification of MSC growth kinetics, characterization of the MSC secretome and the effect of secretions on osteoarthritic explants. Although MSC mode of action is secretion driven, only a snapshot of the secretome was captured and used which could not account for the dynamic biological function that MSCs would continuously produce *in vivo*. Future research needs to ultimately be conducted in the knees of OA patients to ascertain the effects of HA on MSC function.

We propose that future investigations could translate our experimental methodologies into the patient population with end stage OA and that are scheduled for a total knee replacement. In the period leading up to the surgery, the recruited patients could be injected with fluorescent iron oxide labelled MSCs, suspended in HA at 1, 3 or 7 days prior to the day of surgery. In this way, any untoward adverse events can be monitored in patients, bloods can be taken for biomarker analysis, synovial fluid (collected prior to total knee replacement) can be assessed for HA content and cytokine changes, cartilage and synovium explants (collected post total knee replacement) could be analysed using fluorescent microscopy to determine MSC dispersion in the joint and finally a gene expression analysis of the cartilage and synovium explants could be conducted for OA drivers of disease. This approach

would holistically address the effect of combining MSCs and HA for the treatment of

knee OA in a clinical setting.

7

Appendices

7.1 Appendix of Publications

Research Article

Alterations in the Secretome of Clinically Relevant Preparations of Adipose-Derived Mesenchymal Stem Cells Cocultured with Hyaluronan

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Osteoarthritis (OA) can be a debilitating degenerative disease and is the most common form of arthritic disease. There is a general consensus that current nonsurgical therapies are insufficient for younger OA sufferers who are not candidates for knee arthroplasties. Adipose-derived mesenchymal stem cells (MSCs) therapy for the treatment of OA can slow disease progression and lead to neocartilage formation. The mechanism of action is secretion driven. Current clinical preparations from adipose tissue for the treatment of OA include autologous stromal vascular fraction (SVF), SVF plus mature adipocytes, and culture-purified MSCs. Herein we have combined these human adipose-derived preparations with Hyaluronan (Hylan G-F 20: Synvisc) *in vitro* and measured alterations in cytokine profile. SVF plus mature adipocytes showed the greatest decreased in the proinflammatory cytokines IL-1β, IFN-γ, and VEGF. MCP-1 and MIP-1α decreased substantially in the SVF preparations but not the purified MSCs. The purified MSC preparation was the only one to show increase in MIF. Overall the SVF plus mature adipocytes preparation may be most suited of all the preparations for combination with HA for the treatment of OA, based on the alterations of heavily implicated cytokines in OA disease progression. This will require further validation using *in vivo* models.

1. Introduction

Osteoarthritis (OA) can be a debilitating degenerative disease and is the most common form of arthritic disease. Onset is usually in the third and fourth decade of life and has a gradual worsening prognosis over time.

OA is most prevalent in weight bearing joints; the highest incidence of the disease occurs in the knee. It is classified as idiopathic and or secondary. Primary clinical intervention currently involves limited systemic pharmacotherapy such as analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs). Controlling mechanical overload through weight loss and supporting braces can be used autonomously or in combination with physical therapy. Viscosupplementation can also be used to treat OA. This aims to replace lost synovial fluid in an OA knee with Hyaluronan (HA) to reduce pain and increase mobility by lubricating the joint. HA is an endogenous polysaccharide found in all tissues and body fluids of vertebrates. HA is especially abundant in loose connective tissue and is a major component of cartilage [1] and the synovium [2]. Aside from its rheological properties, high molecular weight HA can decrease apoptosis, oxidative stress, and necrosis [3].

Arthroscopic surgical interventions are used for the debridement of mechanical cartilage tears, lesions, and defects, as well as cartilage resurfacing or microfracture to stimulate fibrocartilaginous repair. However, due to inherent degenerative factors of OA and the limited capacity of

conventional therapies to halt disease progression, arthroplasties (artificial joint replacement surgery) are commonly performed for a large proportion of OA patients. The useful lifetime of an artificial knee joint is between 10 and 20 years. Average time for revision surgery has been reported at 35 months and is most commonly caused by infection, instability, and stiffness [4]. For these reasons arthroplasties are often not recommended for patients under 60. There is a general consensus that current nonsurgical therapies are insufficient for younger OA sufferers who are not candidates for knee arthroplasties [5]. Although current therapies may provide temporary pain relief and increased mobility, there is a pressing need for treatments that slow or stop the degradation of cartilage.

The use of alternative biological therapies, such as mesenchymal stem cells (MSCs), for the treatment of OA is increasing as a result of a gap in healthcare options for middle age patients [5, 27]. MSCs can differentiate into mesodermal lineages making them an attractive source for cell based therapies in musculoskeletal conditions such as OA [28]. However, differentiation is not the primary mode of action and *in vitro* animal models and human data have demonstrated that a complex set of secretions drive the anti-inflammatory and regenerative effects that have been observed [29]. Thus, the secretome of MSCs has become of particular interest and multiplex cytokine analyses are used to assess cell populations and the "cytokine profile" is predictive of *in vivo* effect [30]. Proinflammatory cytokines such as Interleukin-1β (IL-1β) play a pivotal role in the pathogenesis of OA. IL-1β can dramatically increase the expression of matrix metalloproteinases, which contribute to cartilage degradation in OA. Chondrocytes stimulated with IL-1β increase secretion of Nitric oxide and Prostaglandin E2, which are key mediators essential to inflammation in OA [31].

The role of inflammation in the pathophysiology of OA has shifted from originally being considered a by-product of the disease to the current understanding as a key driver of disease progression [32]. MSCs have an anti-inflammatory effect and are able to stimulate cartilage proliferation via paracrine signaling [33]. As such, regenerative therapies using MSCs are emerging as a suitable treatment for degenerative musculoskeletal defects.

Surgeons have experimented with possible combination therapies; most notably Lee et al. showed in a porcine model using histopathological observation that injection of bone marrow-derived MSCs suspended in Hyaluronan (Hylan G-F 20: Synvisc) could home to the site of injury, adhere, proliferate, and lead to neocartilage formation [34]. One limitation of this study as concluded by the authors was that regeneration may have been limited by a dose-dependent response to the number of MSCs injected.

One of the first MSCs sources to be isolated came from bone marrow, however as shown by Kern et al. in a comparative analysis between adipose and bone marrow-derived, these MSCs may be inferior to adipose-derived MSCs, in terms of clinical feasibility. It was shown that adipose tissue had the highest concentration of MSCs and a greater proliferative capacity when compared to bone marrow-derived MSCs [35]. The above components combined with minimal

invasiveness spearhead adipose tissue as a favorable source of MSCs in emerging regenerative therapies.

Hyaluronan (HA) shows great potential as an adjunct to adipose-derived MSC regenerative therapy for the treatment of OA not only for its ability to provide rheological cushioning but also for the mechanistic biochemical relationship it forms with MSCs. The relationship with high molecular weight HA and MSCs is founded in CD44, the primary HA receptor, and abundantly expressed on the surface of MSCs. It has been proposed that the cross-talk between CD44 and HA may promote rapid resolution of immune responses [36] and thus enhance the anti-inflammatory effect of MSCs in OA.

In this study we sought to assess the feasibility of a range of preparations in which adipose-derived mesenchymal stem cell therapy could be administered in combination with HA by assessing the secreted soluble protein fraction produced by MSCs that is the secretome. We employed multiplex analyses of 48 cytokines involved in OA to assess the suitability of conditions. These conditions include autologous therapies such as the stromal vascular fraction (SVF), the SVF coinjected with autologous mature adipocytes, and cultured allogeneic preparations such as culture purified adipose-derived MSCs. As adipose-derived MSC therapy moves closer to mainstream medicine, secretome data will be a valuable assessment tool to inform animal studies and human clinical trials in years to come.

2. Materials and Methods

2.1. Isolation of Human Adipose-Derived Mesenchymal Stem Cell Populations. This research was approved by the Macquarie University human research ethics committee (Reference number 5201100385). Human lipoaspirate was obtained from patients undergoing elective cosmetic liposuction surgery. The lipoaspirate was digested as previously described [37]. Briefly, 200 mL of fresh lipoaspirate was mixed and enzymatically digested in prewarmed (37°C) saline containing 0.5 mg/mL collagenase (Lomb Scientific, USA). The lipoaspirate was then incubated in a 37°C water bath for 30 minutes and mixed periodically to circumvent layer separation. The digested lipoaspirate was then passed through an 800 μm mesh to exclude undigested tissue clumps. Finally, the suspension was centrifuged at 1500 ×g for 5 minutes to obtain the pelleted stromal vascular fraction cells (SVF) and floating adipocytes. At this point, the SVF was either used for experimentation or propagated to obtain an adherent purified adipose-derived mesenchymal stem cell (MSC) population.

2.2. Propagation of Adherent Adipose-Derived Mesenchymal Stem Cells. To obtain a population of adherent MSCs, the SVF pellet was transferred into three T175 cm^2 flasks containing standard culture media which consisted of Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, USA) supplemented with 10% foetal bovine serum (FBS; Bovogen, Australia) and 1% penicillin-Streptomycin solution (Invitrogen, USA). Flasks were incubated for 72 hours at 37°C with 5% carbon dioxide. To prevent iron toxicity to adherent MSCs, the flasks were washed using DMEM with no additives to

wash away nonadherent cells and the media replaced with fresh standard culture media. Once the monolayer of MSCs reached 80% confluency, cells were passaged with enzymatic digestion using TrypLE express (Invitrogen, USA) and then resuspended in standard culture media. Cells from each flask were then transferred to three new T175 cm^2 flasks now termed passage 1. Media changes were performed every 72 hours.

Standardized enumeration of the SVF, dependent on nucleated cell clusters, was achieved with Trucount tubes (Becton Dickinson, USA) containing sheath fluid (isoflow; Becman Coulter, USA) and nucleic acid dyes; propidium iodide (10 μg/mL; Sigma, USA), Syto11 (1 μM; Invitrogen, USA), and a defined bead number. The combination of a charged (propidium iodide (Pi)) and a cell permeate (Syto11) dye allows for discriminant cell population gating, which are based on the principal of Pi membrane exclusion and thus viability can be calculated from defined bead populations run as standards. Samples were all run on FACSCalibur flow cytometer (Becton Dickinson, USA).

2.3. SVF Seeded with Hyaluronan Treatment. Following on from the digestion of the fresh lipoaspirate, the pelleted SVF was resuspended in standard culture media. Viable SVF cells were all seeded at two million nucleated cells per T175 cm^2 flask with or without the addition of adipocytes. Flasks were always seeded in either two conditions: the control, which consisted of standard culture media, or treatment, which consisted of HA media (standard culture media; hyaluronan (6% (v/v))). In each condition, three subset arms were tested; SVF and mature adipocytes, SVF alone, and mature adipocytes. Adipocytes were used at 2.5 mL (suspended in saline) and flasks were normalized to 20 mL total containing 17.5 mL media and 2.5 mL saline equivalent (with or without adipocytes). Flasks were incubated for 72 hours at 37°C with 5% carbon dioxide.

2.4. Purified Adherent-MSC Hyaluronan Treatment

2.4.1. Seeding Experiment. Once the flasks reached 80% confluency at passage 2, cells were stripped off the plastic and enumerated. Viable MSCs were then seeded at 1.1 million cells per T175 cm^2 flask. Total media contained within the flasks was standardized to 20 mL. Flasks were seeded with standard culture media or HA media for treatment flasks. This method approximates the intra-articular coinjection of HA and MSCs. After 24 hours of incubation, conditioned media was collected from all flasks. Cells were then stripped off the plastic and a portion was used for immunophenotypic characterization.

2.4.2. Media Change Experiment. Once the flasks reached 80% confluency at passage 2, cells were stripped off the plastic and enumerated. Cells were seeded at 1.1 million cells per T175 cm^2 and incubated in standard culture conditions. On the third day the media was changed with either standard culture media in control flasks or HA media for treatment flasks. After three days, conditioned media was collected from

each of the flasks. Cells were then stripped off the plastic and a portion was used for immunophenotypic characterization.

2.5. Morphology and Differentiation

2.5.1. Morphology. Prior to harvesting the purified adherent-MSCs were imaged under a Zeiss Primo Vert inverted light microscope (Zeiss, Australia) using an eye-piece attached, Canon EOS 5D Mark II Digital SLR camera (Canon, Australia).

2.5.2. Differentiation. Adipogenic and osteogenic differentiation media formulations were used as previously described [38] with slight modification. In brief, cells received media changes twice weekly for 28 days. Upon termination of the differentiations, monolayers were washed in PBS and fixed in 4% paraformaldehyde for 1 hour. To confirm the multipotency of our MSCs, differentiated adipocytes and osteocytes were challenged with Oil Red O (Sigma, Australia) and Alizarin Red (Sigma, Australia), respectively.

The adipogenic differentiation was washed with milliQ water, incubated with 60% isopropanol, stained using three parts filtered Oil Red O stock (0.3% w/v) with two parts milliQ water for five minutes at room temperature, and washed with tap water. The osteogenic differentiation was washed with milliQ, stained with 2% Alizarin Red S solution for two minutes at room temperature and washed three times with milliQ water.

All differentiations were visualized using an Olympus IMT-2 inverted microscope (Olympus, Australia) and imaged with a mounted Scion VisiCapture Firewire camera (Scion Corporation, USA).

2.6. Immunophenotypic Characterization. MSC characterization can be achieved through adherence to cell culture flasks, differentiation, and immunophenotype. A portion of the endpoint cells collected was used for MSC immunophenotypic characterization. Cells from either control or treated flasks were diluted in their respective media (standard culture media or HA media) and centrifuged at 2000×g for 5 minutes. The cells were washed in PBS and resuspended in PBS with 2% FBS. The cells were stained with the following antibodies, which were all sourced from BD Biosciences: CD34 (number 550619), CD45 (number 557059), CD73 (number 550257), CD90 (number 555596), CD105 (number 560839), and IgG1$_K$ isotype control (number 551436). Cell were incubated with each of the antibodies for 45 minutes and then washed with ice cold PBS, centrifuged at 2000×g for 5 minutes and resuspended in 1x FACS Lysing Solution (Becton Dickinson, USA). The cells were all stained with phycoerythrin (PE) conjugated antibodies and thus were resuspended in Syto11 (1 μM) and isoflow to achieve contrast fluorescence. Stained and unstained control cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, USA). CD44 has long been recognized as an established receptor for HA [39]; therefore in addition to the immunophenotypic MSC characterization above, CD44 (number 550989) was also tested using the same protocol.
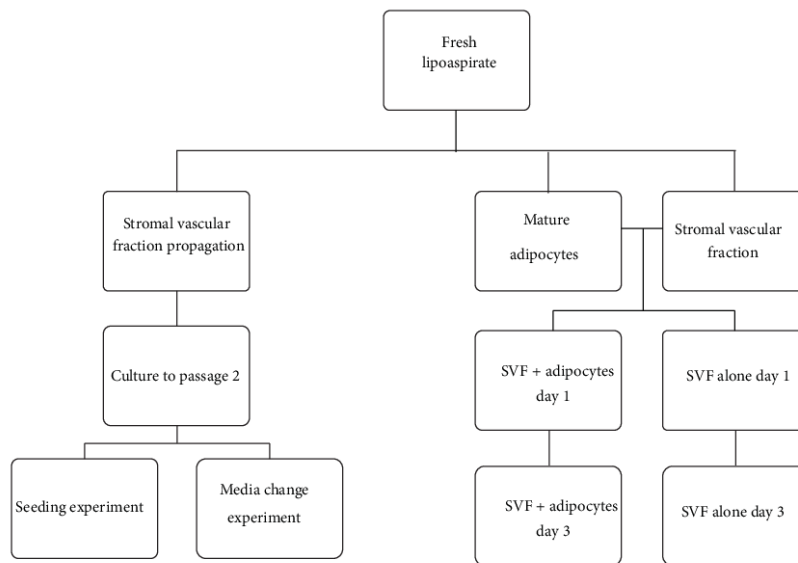
FIGURE 1

2.7. Secretome Analysis. The conditioned media was collected from every flask in this study, centrifuged at 5000 ×g for 5 minutes, and stored at −80°C. Upon thawing, the samples were filtered through 0.2 mm Nanosep MF Centrifugal Devices with Bio-InertH Membrane (Pall Scientific, USA). Filtrates (50 μL) were analyzed using either the Bio-Plex Pro Human Cytokine 27-plex or the Bio-Plex Pro Human Cytokine 21-plex assay (Bio-Rad, USA), according to the manufacturer's instructions. The washing steps were performed using the Bio-Plex Pro II magnetic wash station and the data was acquired using the Bio-Plex 200 system with version 5.0 software (Bio-Rad, USA). The average concentration of each cytokine in the conditioned medium samples was calculated from four technical replicates in cultured MSC experiments. To account for biological variation in the concentration of secreted cytokines and, in the stromal vascular fraction, the variable concentration of MSCs, the data was normalized to fold change of concentration (pg/mL) of HA treated over control ($n = 3$). The fold change was then averaged and graphed with the upper and lower confidence intervals set at 95% as error bars. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment, a fold change greater than one indicates an increase in the cytokine with HA treatment. Any cytokines with a fold change clear of the axis at 1 were reported as significant (determined numerically).

2.8. Experimental Schematic. See Figure 1.

3. Results

3.1. Multipotent MSC Validation. Previously published data from our lab demonstrated methods used within this study to both isolate and culture/propagate the SVF and obtain adherent-MSC populations [37, 38]. These were in accordance with the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy statement [40]. Herein we have supplemented media used to grow MSCs with HA media in an attempt to quantify the effects on the secretome.

Cultured MSCs were tested for immunophenotypic expression with and without HA media treatment. The MSC profile was maintained for the duration of the study. Monolayers from both the control and HA treated MSCs presented with equivalent differentiation potential. Figure 2 shows no detectable difference in morphology and differentiation potential between MSCs cultured in standard culture media and HA media. The differentiated MSCs were tested with either Alizarin Red S, which stains positive for calcium deposits confirming osteogenic potential, or Oil Red O, which stains positive for lipid accumulation confirming adipogenic potential. The consistency in the take-up of both stains used to test differentiated cell monolayers indicates the differentiation potential is maintained.

In addition to differentiation potential, immunophenotypic expression on the control and HA treated MSCs is depicted in Figure 3. The presence of CD73, CD90, and CD105 and the absence of hematopoietic markers CD34 and
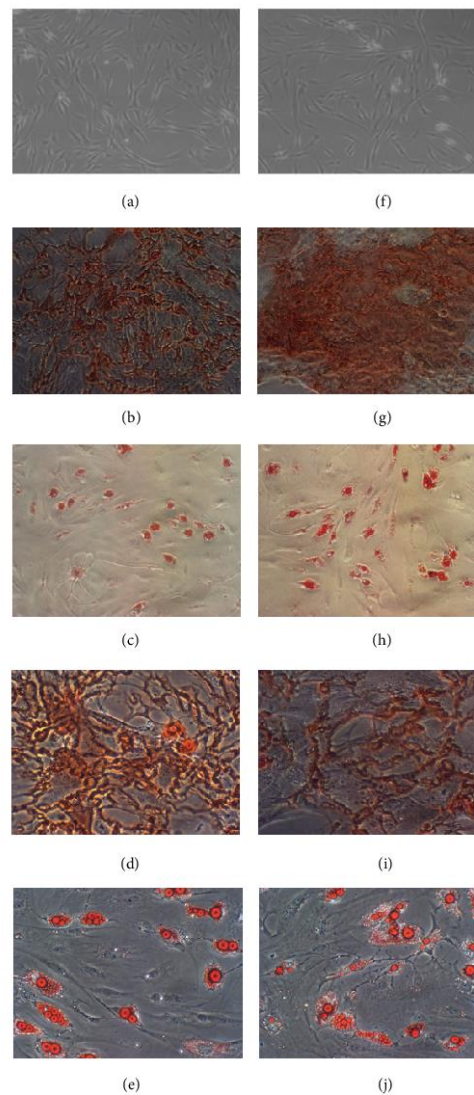
FIGURE 2: Morphology and differentiation potential of control and treated MSCs. MSCs cultured in either standard culture media or HA media. No detectable differences were seen in the morphology of control (a) MSCs and HA treated (f) monolayers. Osteogenic differentiation of control MSCs (b) and of the HA treated (g) at 10x magnification. Adipogenic differentiation of control (c) and of the HA treated (h) at 10x magnification. At 20x magnification no detectable differences in Alizarin Red S staining were observed in the osteogenic differentiation of control MSCs (d) and HA treated (i). At 20x magnification no detectable differences in Oil Red O staining were observed in the adipogenic differentiation of control MSCs (e) and HA treated (j).

(a)   (b)   (c)

(d)   (e)   (f)

FIGURE 3: Immunophenotypic expression of control and HA treated MSCs. Immunophenotypic expression of MSCs cultured in either standard culture media or HA media. Each of the histograms depicts the IgG iso-type control (black dashed lines), control media (red dashed lines), and HA media (blue dashed lines). Maintenance of MSC profile was confirmed with the lack expression in the haematopoietic markers CD34 (a), CD45 (b), and positive expression of CD73 (c), CD90 (d), and CD105 (e) in both the control and HA treated MSCs. CD44 (f), the major receptor for HA, showed no major increases in immunophenotypic expression.

CD45 indicated the presence of replicative and self-renewal capacity of MSCs of both the control and the HA media treated. CD44 is a major receptor for HA; as such it was also tested. The number of CD44$^+$ cells in the control and the HA media treatment showed no major quantifiable difference in the tested replicates.

3.2. Alterations of Secretion Profile in HA Media Treatment. In this study we sought to profile changes in the secretome to ascertain the effects of combining HA with MSCs. Changes in the secretions profile can be an indication of positive or negative effect in the context of OA; that is, increase in the anti-inflammatory cytokines may be considered positive or an increase in proinflammatory cytokines may be negative. All the cytokines were represented as a percentage of the control, that is, fold change (fold Δ). The average fold change of three biological replicates for each of the cytokines was plotted as an average ± the upper and lower confidence

intervals at 95%. The horizontal axis was set at one (i.e., no change), and any point with error bars that cleared one was considered a significant difference from the control (determined numerically; see Table 1 for numerical summary of cytokine data).

3.3. Stromal Vascular Fraction. The stromal vascular fraction (SVF) alone prepared from fresh lipoaspirate via enzymatic digestion is currently used in autologous therapy for the treatment of OA. When the SVF alone was combined with HA, proinflammatory cytokine IL-1α (Figure 4), increased 21% on the first day and significantly on the third day of culture by 34%. IL-12 decreased significantly on the first day by 33% (Figure 4) but later recovered on the third day of culture with a 15% decrease. IL-17 decreased significantly by 30% (Figure 4) when combined with HA on the first day of culture but on the third day increased by 17%.

Table 1: Summary of cytokines across all conditions.

Cytokines	Fold change from control (CI = 95%)						Role in OA
	SVF day 1	SVF day 3	SVF + adipo day 1	SVF + adipo day 3	MSC seeding	MSC media	
Proinflammatory							
MIF	0.94	0.93	0.89	0.99	**1.49**	0.79	Induce the release of proinflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-6, and IL-8 [6] and MIF levels in synovial fluid correlate to OA severity [7]
TNF-β	1.00	**1.26**	1.03	1.28	**1.52**	1.18	TNF-beta stimulates cartilage matrix breakdown [8]
IL-1α	1.21	**1.35**	0.98	1.26	1.34	N.D.	Highly expressed in OA-derived human articular cartilage [9]
IL-1β	1.03	*1.12*	**0.59**	0.89	1.00	0.94	Induce inflammatory reactions and catabolic effects independently as well as in combination with other mediators in OA with respect to the articular cartilage [10]
IL-12	**0.67**	0.86	**0.49**	0.78	1.00	**0.90**	Expressed by infiltrating macrophages and synovial cells in OA [11]
IL-17	**0.70**	1.17	0.83	0.90	0.96	*0.09*	Inhibit the synthesis of proteoglycans in OA and upregulate catabolic enzymes which break down cartilage [10]
IFN-γ	0.96	0.86	0.89	**0.80**	0.99	0.93	Stimulate cartilage breakdown by production of enzymes via IL-1β [12]
Anti-inflammatory/dual role							
IL-10	0.90	0.85	**0.40**	**0.61**	1.05	**0.86**	Expressed in chondrocytes and involved in collagen and aggrecan synthesis. Inhibition of catabolic enzymes and apoptosis of chondrocytes [10]
IL-2	0.81	2.35	**0.69**	**0.74**	1.23	1.35	Elevated levels are found in OA synovial fluid and increased levels correlate to increase severity [13] Catabolism aggrecan via aggrecanase activity [14]
IL-6	0.93	0.84	0.47	0.94	1.11	**0.84**	Involved in the synthesis of tissue inhibitor of metalloproteinases known to stop cartilage breakdown [15]

Table 1: Continued.

Cytokines	Fold change from control (CI = 95%)						Role in OA
	SVF day 1	SVF day 3	SVF + adipo day 1	SVF + adipo day 3	MSC seeding	MSC media	
Chemokines							
GROα	1.02	0.89	0.79	0.87	**0.94**	**1.12**	Constitutively secreted by chondrocytes and secretion of this chemotactic protein is increased in stimulated osteoarthritic chondrocytes and synovial fibroblasts [16]
MIP-1β	0.92	0.83	0.86	**1.17**	1.16	1.14	Present in significantly higher levels in OA synovial fluid compared to normal synovial fluid [17]
RANTES	0.78	0.99	0.94	1.31	1.09	**0.67**	Secreted by IL-1 stimulated osteoarthritic chondrocytes and TNF-α stimulated synovial fibroblasts [16]
MCP-1	0.79	**0.65**	**0.75**	0.90	0.95	**0.91**	Constitutively expressed in osteoarthritic chondrocytes and increased secretion in IL-1 stimulated osteoarthritic chondrocytes [16]
MCP-3	0.83	1.07	**0.82**	0.95	0.97	N.D.	Activate immune cells such as monocytes, T lymphocytes, basophils, and eosinophils [18]
MIP-1α	0.83	**0.67**	0.82	0.90	1.04	0.95	Expressed by osteoarthritic derived chondrocytes and production is enhanced by TNF-α stimulated chondrocytes [19]
Growth factors							
HGF	0.69	**0.83**	0.83	1.56	1.42	0.95	Promote osteophyte formation via MCP-1 mediated infiltration of immune cells into OA affected joint [20]
FGF-β	0.89	1.21	**0.79**	0.98	**1.26**	0.84	Potentiate articular cartilage resurfacing and may stimulate expression of MMP-13 [21, 22]
β-NGF	1.02	0.92	**0.73**	0.86	**0.78**	0.86	Higher expression is found in osteoarthritic derived chondrocyte compared to healthy chondrocytes [23]
GM-CSF	0.84	**1.82**	0.76	0.91	1.38	N.D.	Key mediator in inflammation and arthritic pain [24]
VEGF	0.71	0.82	**0.51**	0.71	1.00	**0.84**	Expressed in osteoarthritic-derived chondrocytes and shown to increase osteochondral angiogenesis in OA patients [25]
G-CSF	0.97	0.96	**0.51**	0.80	1.06	0.84	Can significantly increase nitrite levels in cartilage when combined with IL-1β explants [26]

A summary of cytokines across all the conditions treated with HA. The data is represented as fold change in concentration compared to control. A value more than one indicates the HA treatment increased the secretion of that cytokine in that condition. Bold values represent a significant fold change from control determined numerically using confidence interval set at 95%. Italic values were only detected in one of the three biological replicates for that condition.
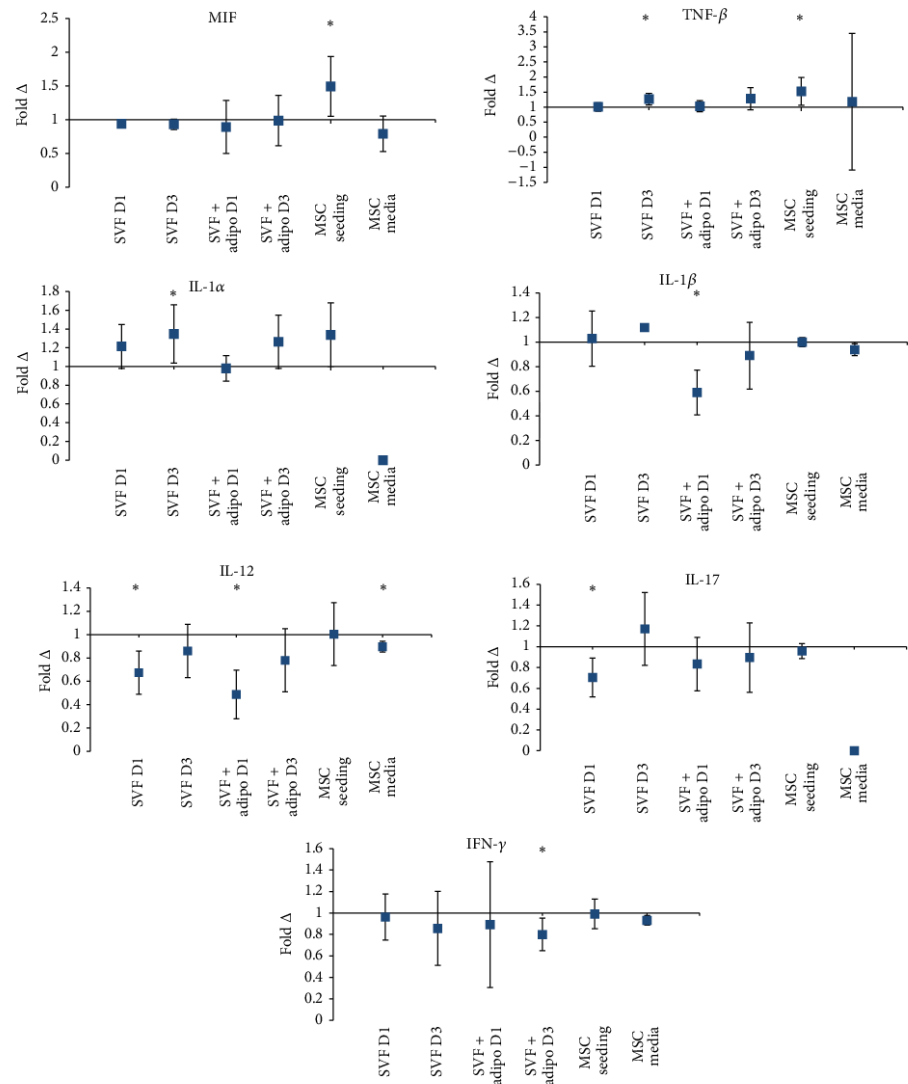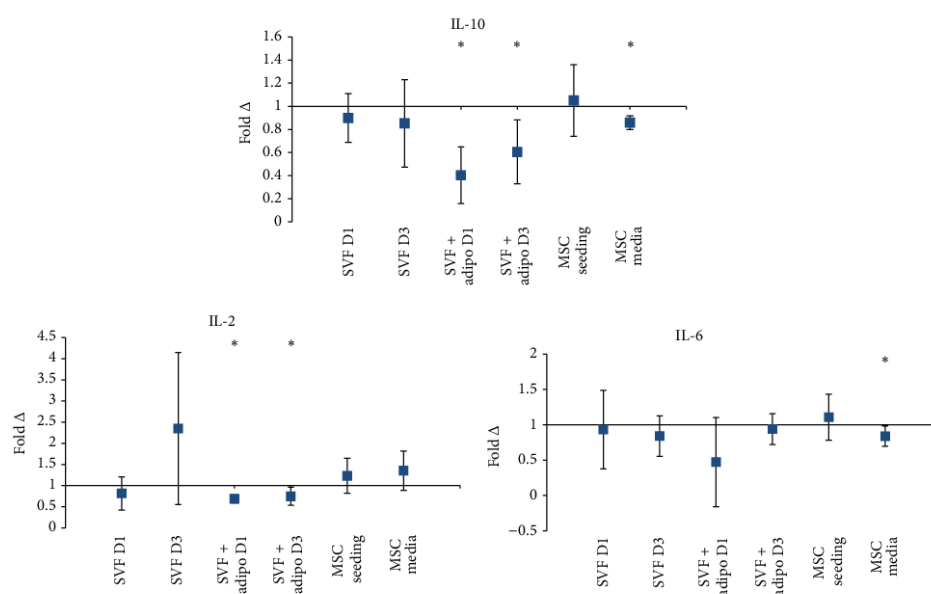
FIGURE 4: Fold change in concentration of proinflammatory cytokines detected across experiments Average fold change in concentration ($n = 3$) ± upper and lower confidence intervals at 95% (y-axis). Conditions (x-axis): stromal vascular fraction on day 1 (SVF D1), stromal vascular fraction on day 3 (SVF D3), stromal vascular fraction plus mature adipocytes on day 1 (SVF + adipo D1), stromal vascular fraction plus mature adipocytes on day 3 (SVF + adipo D3), seeding experiment with purified MSCs (MSC seeding), and media change experiment with purified MSCs (MSC media). Conditions were all cultured in either standard culture media or HA media. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment; a fold change greater than one indicates an increase in the cytokine with HA treatment. Macrophage migration inhibitory factor (MIF), tumor necrosis factor-beta (TNF-β; lymphotoxin-alpha), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-12 (IL-12), interleukin-17 (IL-17), and interferon-gamma (IFN-γ). IL-1α was not detected in the MSC media condition. IL-17 was only detected in one biological replicate in the MSC media condition. IL-1β was only detected in one biological replicate in the SVF condition on day 3.

FIGURE 5: Fold change in concentration of anti-inflammatory and dual role cytokines detected across experiments. Average fold change in concentration ($n = 3$) ± upper and lower confidence intervals at 95% (y-axis). Conditions (x-axis): stromal vascular fraction on day 1 (SVF D1), stromal vascular fraction on day 3 (SVF D3), stromal vascular fraction plus mature adipocytes on day 1 (SVF + adipo D1), stromal vascular fraction plus mature adipocytes on day 3 (SVF + adipo D3), seeding experiment with purified MSCs (MSC seeding), and media change experiment with purified MSCs (MSC media). Conditions were all cultured in either standard culture media or HA media. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment, and a fold change greater than one indicates an increase in the cytokine with HA treatment. Interleukin-10 (IL-10), interleukin-2 (IL-2), and interleukin-6 (IL-6).

FGF-β is one of six detected growth factors across the range of conditions tested. On the third day of culture, the SVF increased FGF-β secretion by 20% on the third day (Figure 6). Also on the third day of culture the SVF decreased secretion of GM-CSF significantly by 81% (Figure 6). VEGF also decreased on the first and third day culture by 21% and 19%, respectively, but not significantly (Figure 6).

Chemokines are chemotactic cytokines responsible for homing and immune cell recruitment. RANTES in the first day of culture in the SVF decreased by 23% but not significantly (Figure 7). The SVF on day one decreased secretion of MCP-1 by 21% and then significantly on the third day by 36% (Figure 7). MIP-1α decreased by 17% on the first day of SVF culture and then significantly on the third day of culture by 33% (Figure 7).

3.4. Stromal Vascular Fraction Plus Adipocytes. Aside from the stromal vascular fraction (SVF), another by-product of the enzymatic digestion of adipose tissue is mature adipocytes. A combination of the SVF and adipocytes can be used to treat knee OA. This therapy is available commercially for use in the autologous setting and has been used in a placebo control trial. In this study, SVF plus adipocytes were combined with HA and in the first day IL-β decreased significantly by 41% but then later recovered on the third day to just a 11% decrease (Figure 4). Also another very significant decrease was observed on the first day of culture, and the SVF plus mature adipocytes decreased the secretion of IL-12 by 52% when combined with HA, and this later recovered on the third day to just a 22% decrease (Figure 4).

Anti-inflammatory cytokine IL-10 decreased significantly on the first (60%) and on the third day (40%) of the SVF plus adipocytes combined with HA culture (Figure 5). The SVF plus adipocytes combined with HA also decreased secretion of IL-2 significantly on the first day by 31.5% and again on the third day significantly by 26% (Figure 5).

Growth factor secretions were also measured from the SVF plus adipocytes when combined with HA. β-FGF on the first day of culture decreased significantly by 21% but later recovered by the third day culture to no change from the control (Figure 6). β-NGF decreased significantly on the first day of culture by 27.5% but on the third day of culture was not significant and only decreased by 14% (Figure 6). VEGF secretion by the SVF plus adipocyte preparation when combined with HA decreased significantly by 50% and 29% on the first and third day of coculture, respectively (Figure 6). G-CSF also decreased significantly on the first day by 50% but
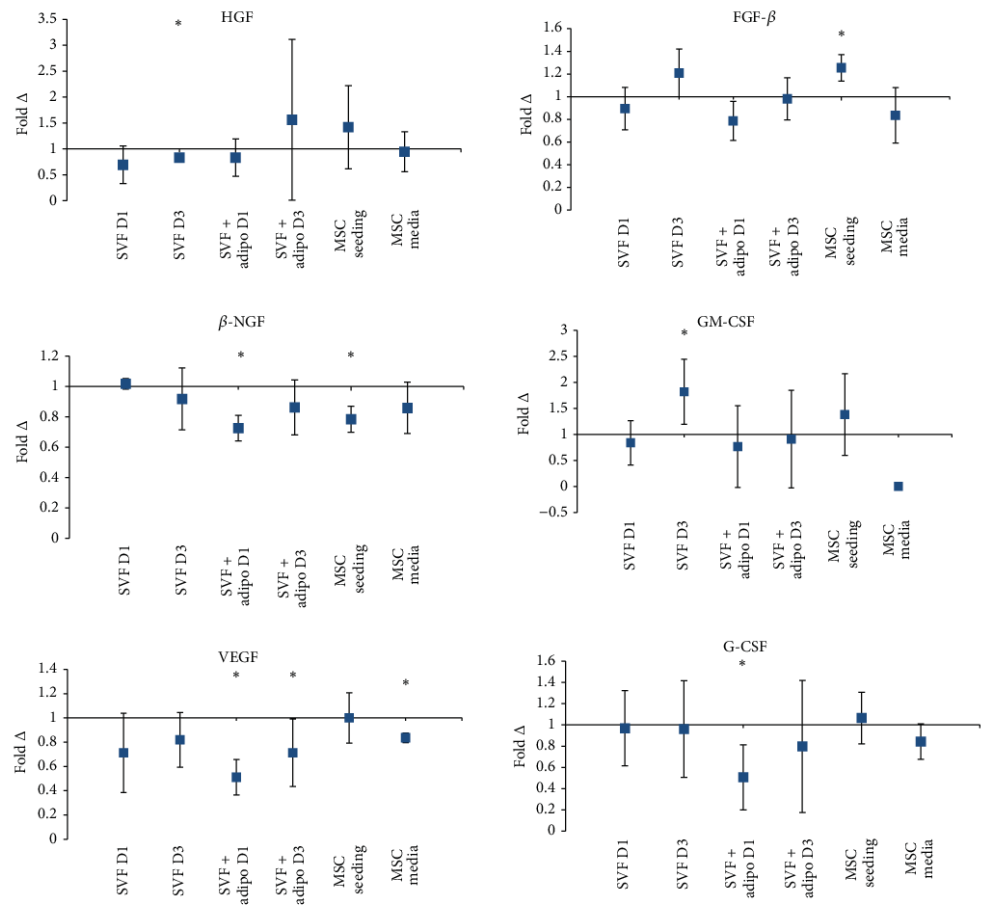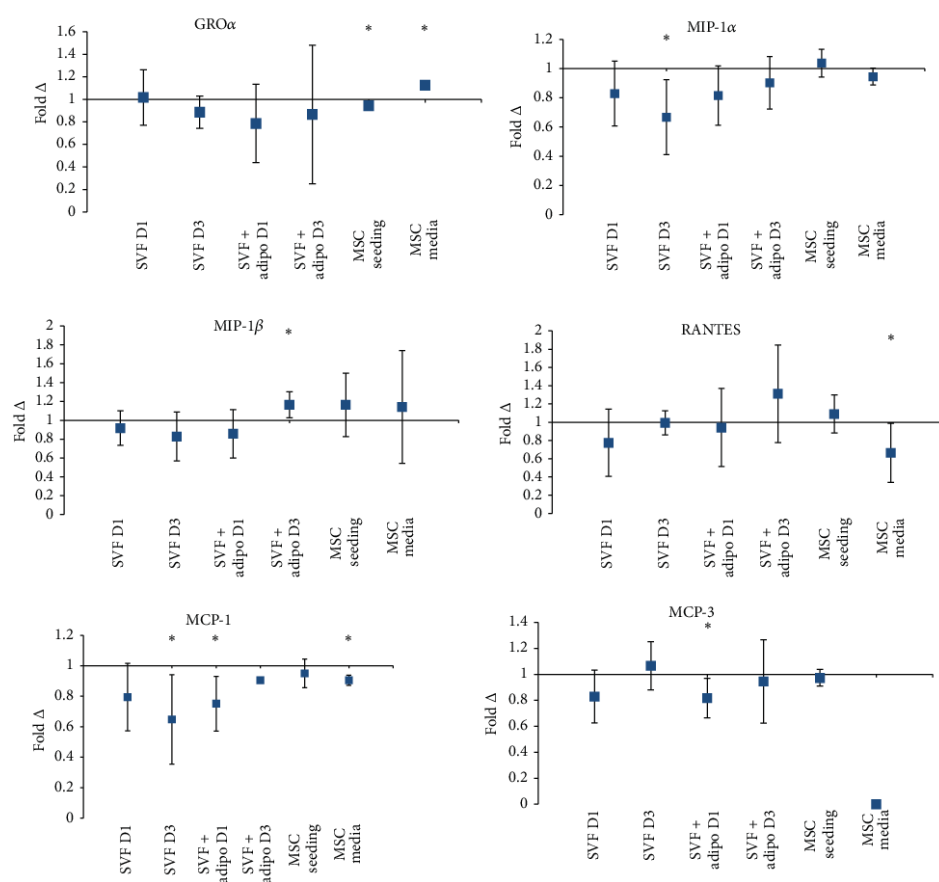
FIGURE 6: Fold change in concentration of growth factors detected across the experiments. Average fold change in concentration ($n = 3$) ± upper and lower confidence intervals at 95% (y-axis). Conditions (x-axis): stromal vascular fraction on day 1 (SVF D1), stromal vascular fraction on day 3 (SVF D3), stromal vascular fraction plus mature adipocytes on day 1 (SVF + adipo D1), stromal vascular fraction plus mature adipocytes on day 3 (SVF + adipo D3), seeding experiment with purified MSCs (MSC seeding), and media change experiment with purified MSCs (MSC media). Conditions were all cultured in either standard culture media or HA media. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment, and a fold change greater than one indicates an increase in the cytokine with HA treatment. Hepatocyte growth factor (HGF), fibroblast growth factor-basic (FGF-β), nerve growth factor-beta (β-NGF), granulocyte macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), and granulocyte colony-stimulating factor (G-CSF). GM-CSF was not detected across all biological replicates in the MSC media condition.

then later recovered on the third day to decrease secretion not significantly by 20% (Figure 6).

MIP-1β is one of six detectable chemokines across all the preparations. Secretion of MIP-1β by the SVF plus adipocytes when combined with HA decreased on the first day but on the third day increased significantly by 16.5% (Figure 7). MCP-1 decreased significantly on the first day when combined with HA by 25% on the third day and recovered to just a 10%

decrease, and it was however only detected in one of three biological replicates (Figure 7). Finally MCP-3 decreased significantly by 18% on the first day when combined with HA but on the third day recovered with no observable change (Figure 7).

3.5. Culture Purified MSCs. The final preparation tested in this study and a likely future therapeutic in MSC therapy

FIGURE 7: Fold change in concentration of chemokines detected across the experiments. Average fold change in concentration ($n = 3$) ± upper and lower confidence intervals at 95% (y-axis). Conditions (x-axis): stromal vascular fraction on day 1 (SVF D1), stromal vascular fraction on day 3 (SVF D3), stromal vascular fraction plus mature adipocytes on day 1 (SVF + adipo D1), stromal vascular fraction plus mature adipocytes on day 3 (SVF + adipo D3), seeding experiment with purified MSCs (MSC seeding), and media change experiment with purified MSCs (MSC media). Conditions were all cultured in either standard culture media or HA media. Cytokines with a fold change less than one indicate a decrease in concentration with HA media treatment, and a fold change greater than one indicates an increase in the cytokine with HA treatment. Chemokine ligand-1 (CXCL-1) aka growth-regulated peptide alpha (GROα), CC-Chemokine ligand-4 (CCL-4) aka macrophage inflammatory protein-1 beta (MIP-1β), CC-Chemokine ligand-5 (CCL-5) aka regulated on activation normal T cell expressed and secreted (RANTES), CC-chemokine ligand-2 (CCL-2) aka monocyte chemotactic protein-1 (MCP-1), CC-chemokine ligand-7 (CCL-7) aka monocyte chemotactic protein-3 (MCP-3), and CC-chemokine ligand-3 (CCL-3) aka macrophage inflammatory protein-1-alpha (MIP-1α). MCP-3 was not detected across all biological replicates in the MSC media condition. MCP-1 was only detected in one of three biological replicates in the SVF + adipo D3 condition.

is the culture purified and expanded MSC population. In the MSC seeding experiment the combination with HA saw a significant increase in a major proinflammatory cytokine MIF by 49% but then a nonsignificant decrease after 3 days in the MSC media experiment of 21% (Figure 4). TNF-β secretion by purified MSCs in the MSC seeding experiment increased significantly by 52% when combined with HA but

on the third day recovered to a 17% increase (Figure 4). IL-1α increased in the MSC seeding experiment by 34% but not significantly (Figure 4). There was no detectable IL-1α in the MSC media experiment.

IL-6 is considered a dual role cytokine and in the MSC seeding experiment increased by 10.5% but then decreased significantly in the MSC media experiment by 16% (Figure 5).

Culture purified MSCs in combination with HA also secrete appreciable amounts of growth factors. FGF-β increased significantly in the MSC seeding experiment by 25.5% but decreased in the MSC media by 16.5% (Figure 6). In the MSC seeding experiment β-NGF decreased significantly by 21.5% but in the MSC media experiment decreased nonsignificantly by 14% (Figure 6).

Chemokine secretion by culture purified MSCs in combination with HA was also measured. GROα decreased significantly in the MSC seeding experiment by 6% but inversely significantly increased in the MSC media experiment by 12% (Figure 7). RANTES secretion did not change in the MSC seeding experiment; however in the MSC media experiment it decreased by 33.5% when combined with HA (Figure 7). MCP-1 showed no appreciable change in secretion in the MSC seeding experiment but decreased significantly by 9.5% in the MSC media experiment when combined with HA (Figure 7).

4. Discussion

Biological therapeutics such as MSC therapy are gaining acceptance for the treatment of inflammatory conditions, including musculoskeletal ailments such as OA.

It is likely that MSC therapy will be administered with viscosupplementation such as HA, which has extensive evidence for therapeutic efficacy in OA. In this study we used a number of adipose MSC preparations that are currently in commercial use or undergoing clinical trial for the treatment of OA. We cocultured the different cell preparations with HA to investigate the effects on the secretome. We found that MSCs maintained differentiation potential when cocultured with HA. These results are consistent with [34] although enhanced adipogenic potential was observed in the HA treated MSCs (data not shown). Immunophenotypic expression was characteristic of multipotent mesenchymal stem cells following coculture, and additionally, no observable changes could be observed in the expression of the hyaluronan receptor CD44.

Changes in the cytokine profile may be an indication of therapeutic efficacy reflective of combining HA and MSCs. We therefore profiled proinflammatory cytokines in an attempt to measure the effects of HA on MSCs either in stromal vascular fraction (SVF), SVF plus mature adipocytes, and culture MSC populations. Significant reductions in a well-established proinflammatory cytokine and interferon gamma in the SVF plus adipocyte preparation (Figure 4) suggest that this is a favorable preparation, although the fold change of all other preparations trended downward also. Interleukin-12 also trended downward for all preparations and significantly for the SVF, SVF plus adipocytes, and the MSC media. Interleukin-12 is expressed in the OA milieu [41] by infiltrating immune cells such as mature dendritic cells [42]; however MSCs are known to inhibit the maturation of dendritic cells [43] and thus a combination with HA for this cytokine may be desirable. The MSC seeding preparation was intended to model co-intra-articular injection of purified MSCs with HA. The significant increase in macrophage migration inhibitory factor (MIF) (Figure 4) may contraindicate the combination of purified MSCs with HA. Animal models have shown a correlation with reduced severity of

OA and depletion of MIF [44]. MIF acts in local tissue to increase neutrophil and macrophage migration to regions of inflammation. Recent investigations have shown that TNF-β (lymphotoxin-alpha) initiates an inflammatory response in human chondrocytes as a consequence of increase NF-κB signaling [45]. Figure 4 shows that there is both a significant increase of TNF-β in both the SVF alone and MSC seeding preparations, although the SVF plus adipocytes increased but not significantly, further supporting the SVF plus adipocytes as an ideal preparation. The secretion of interleukin-1 alpha (IL-1α) trended upward for most preparations, however only significantly for the SVF alone preparation. Interleukin-1 family of cytokines have long been implicated in degradation of the knee joint cartilage in OA [9], pending *in vivo* trials, and this increase may amplify endogenous interleukin-1 secretion in the OA joint much to the detriment of MSC therapy. Interleukin-1 beta (IL-1β) is heavily implicated in cartilage catabolism [46]. Collagen-induced arthritis models in mice have shown that HA alone can reduce IL-1β mRNA expression in articular cartilage [47]. Here we have shown minimal changes in purified MSC secretion of IL-1β when combined with HA; however when the SVF plus mature adipocytes were exposed to HA there was a significant 41% (Figure 4) decrease in secretion of IL-1b as opposed to an increase in secretion when the SVF alone was combined with HA. This may contraindicate the use of the SVF alone in combination with HA but encourage the addition of mature adipocytes.

Anti-inflammatory cytokines and growth factors play a crucial role in the regenerative potential of MSCs. As such, a decrease in anti-inflammatory cytokines as a result of the combination with HA may not be desirable in the context of trophic efficacy. Interleukin-10 decreased significantly in both SVF plus adipocytes and cultured MSC media preparation (Figure 5). Interleukin-10 secretion by MSCs has been proposed to be a cytokine involved in the inhibition of immune cell, Th17, and differentiation [48]. Therefore a downward trend in the preparation when combined with HA, as seen in Figure 5, may not be desirable for MSC therapy as it may hinder the immunomodulatory capacity. Basic fibroblast growth factor (FGF-β) increased significantly in the cultured MSC seeding preparation (Figure 6) which could suggest that increased proliferative capacity of MSCs as FGF-β is a potent mitogen. It may also play a synergistic role in regenerating cartilage in OA with MSCs. Animal models of cartilage defects have shown that recombinant human FGF-β alone potentiates articular cartilage resurfacing after just 6 weeks [21]. However other studies suggest that endogenous FGF-β stimulates the expression of matrix metalloproteinase-13 [22], an enzyme which degrades type II collagen present in articular cartilage. Granulocyte macrophage colony-stimulating factor (GM-CSF) significantly increased in the SVF alone preparation (Figure 6). This change may negatively affect MSC therapy for the treatment of OA as GM-CSF is a key mediator in inflammation and arthritic pain [24]. Animal studies of experimental osteoarthritis using knockout mice showed that the development of OA was strictly dependent on GM-CSF [49]. Nerve growth factor-beta (β-NGF) showed a downward trend in most preparations and significantly in the SVF plus

adipocytes and the cultured MSC seeding preparations (Figure 6). β-NGF in human osteoarthritic-derived chondrocytes shows increased mRNA expression when compared to chondrocytes from healthy donors [23] suggesting that the combination of HA with MSCs might be beneficial with decreased overall levels of β-NGF. Vascular endothelial growth factor (VEGF) is expressed in osteoarthritic-derived chondrocytes and shown to increase osteochondral angiogenesis in OA patients [25]. Animal models have shown that intra-articular injection of recombinant VEGF causes cartilage degradation as in OA [50]. VEGF showed a downward trend across all preparations and decreased significantly in the SVF plus adipocyte and cultured MSC media preparations when combined with HA (Figure 6). Colony stimulating factors are pivotal in influencing cartilage metabolism; a combination of IL-1β and granulocyte colony stimulating factor (G-CSF) significantly increased nitrite levels by cartilage explants [26]. In the SVF plus mature adipocytes preparation G-CSF secretion decreased 50% when combined with HA, the other preparation showed no appreciable changes. This again reiterates the SVF plus mature adipocyte preparation as an ideal combination with HA.

Chemokines are chemoattractant cytokines that play a pivotal role in regulating migration and infiltration of immune cell populations. In a study of OA-derived chondrocytes it was found that monocyte chemotactic protein-1 (MCP-1) induced matrix metalloproteinase-3 expression and inhibited proteoglycan synthesis [19]. MCP-1 in OA-derived subchondral bone marrow stromal cells also showed constitutive expression [51]. MCP-1 secretion decreased across all preparations and significantly for the SVF alone and SVF plus mature adipocytes by 35% and 25%, respectively. This suggests that a combination of these preparations with HA may synergistically work to reduce immune cell recruitment. Macrophage inflammatory protein-1-alpha (MIP-1α) showed increased expression in OA-derived bone marrow stromal cells when challenged with IL-1β [51]. Therefore modulating levels of MIP-1α is desirable for the treatment of OA. Secretion of this chemokine decreased across both the SVF alone and SVF plus adipocyte preparation but not appreciably in the purified MSC preparations.

5. Conclusion

In this study we investigated a range of adipose-derived mesenchymal stem cell preparations, some of which are currently used for autologous therapy and some of which will become important for future off-the-shelf allogeneic preparations. As the mode of action of MSCs is driven by the secretion of immunomodulatory and trophic factors, we assessed changes in the secretion of cytokines in the conditioned media when cells were cocultured with HA. When cocultured with HA, SVF plus mature adipocytes showed the greatest decrease in the proinflammatory cytokines IL-1β, IFN-γ, and the growth factor VEGF, which has been identified as a negative influence in OA. Two chemokines, MCP-1, and MIP-1α decreased substantially in the HA SVF preparations, with and without adipocytes, but not the purified MSCs. Both the SVF alone and purified MSC populations also had small but significant

increases in TNF-β when cocultured with HA. There was an increase in TNF-β in the HA coculture of SVF and adipocytes, but this was not significant.

The purified MSCs cocultured with HA were the only preparation to show increased MIF, a major proinflammatory cytokine, which is correlated with OA severity. The increased concentration was observed at the initial stage of cell seeding and MIF was below the control concentration by the media change at day three. It is unknown what effect a transient spike of MIF may have on OA pathology and this would be ideally tested in an animal model.

As shown in previous *in vitro* and *in vivo* studies from our group, the mixed population of cells in adipose SVF plus adipocytes produces a distinct and therapeutically superior cytokine profile. In this study the SVF plus mature adipocytes preparation appears to be most suited of all the preparations for combination with HA.

Disclosures

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Priming Adipose-Derived Mesenchymal Stem Cells with Hyaluronan Alters Growth Kinetics and Increases Attachment to Articular Cartilage

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Background. Biological therapeutics such as adipose-derived mesenchymal stem cell (MSC) therapy are gaining acceptance for knee-osteoarthritis (OA) treatment. Reports of OA-patients show reductions in cartilage defects and regeneration of hyaline-like-cartilage with MSC-therapy. Suspending MSCs in hyaluronan commonly occurs in animals and humans, usually without supporting data. *Objective*. To elucidate the effects of different concentrations of hyaluronan on MSC growth kinetics. *Methods*. Using a range of hyaluronan concentrations, we measured MSC adherence and proliferation on culture plastic surfaces and a novel cartilage-adhesion assay. We employed time-course and dispersion imaging to assess MSC binding to cartilage. Cytokine profiling was also conducted on the MSC-secretome. *Results*. Hyaluronan had dose-dependent effects on growth kinetics of MSCs at concentrations of entanglement point (1 mg/mL). At higher concentrations, viscosity effects outweighed benefits of additional hyaluronan. The cartilage-adhesion assay highlighted for the first time that hyaluronan-primed MSCs increased cell attachment to cartilage whilst the presence of hyaluronan did not. Our time-course suggested patients undergoing MSC-therapy for OA could benefit from joint-immobilisation for up to 8 hours. Hyaluronan also greatly affected dispersion of MSCs on cartilage. *Conclusion*. Our results should be considered in future trials with MSC-therapy using hyaluronan as a vehicle, for the treatment of OA.

1. Introduction

Osteoarthritis is a degenerative disease characterised by degradation of cartilage and inflammation of the synovium [1]. Arthritic-degeneration and associated pain lead to reduced mobility, decreased economic contribution, and a significant healthcare burden of developed nations [2]. Conventional treatments of OA for middle-age sufferers are targeted at relieving pain using analgesics and nonsteroidal anti-inflammatories [3]. Others include intra-articular injection of exogenous preparations of hyaluronan (HA) as a viscosupplementation therapy [4]. End stage treatment of OA involves surgical realignment or total knee replacement (TKR) with artificial prostheses. Increasing lifespan has exacerbated the problem and widened the treatment gap for middle aged sufferers who are not suitable candidates for TKR [5].

Recently, adipose-derived mesenchymal stem cells (MSCs) have shown promise as an OA therapeutic. Numerous *in vitro* studies have demonstrated that MSCs can differentiate into mesodermal cell types that form cartilage, bone, and fat. However, differentiation of implanted cells *in vivo* is a rarely documented mode of action. Perhaps differentiated allogeneic cells are not immune privileged and would be removed by the host immune system.

Paracrine effects, both anti-inflammatory and trophic, via the secretion of a complex mixture of cytokines are currently a more accepted and well-studied mode of action [6]. The trophic action of cytokines acts to stimulate and mobilise endogenous MSCs to repair and regenerate tissue.

MSCs can improve function and pain in patients suffering from knee OA with minimal adverse events. Patients show a consistent reduction in the size of cartilage defects by the regeneration and neoformation of hyaline-like cartilage [7]. Manufacture of cell therapeutics for OA may involve the use of HA, particularly as a vehicle. The rationale for this is that HA is a core component of the extracellular matrix, endogenously abundant in the knee joint [8, 9] and approved for the treatment of knee OA.

Suspending MSCs in HA is common practice in rodents [10] and larger animals [11, 12] and for the treatment of OA in humans [13, 14]. MSCs and HA are safe and are used independently in humans; however there are few studies which explore the effect of HA on MSC growth kinetics, function, and tissue binding, especially with respect to cartilage.

Therefore our study sought to address alterations of MSC-HA growth kinetics and whether changes would persist in HA primed cells both on plastic and on articular cartilage. We employed a novel cartilage disc assay in a time series to determine adherence time and assessed the physical dispersion of MSCs over cartilage shedding light on the biological relevance of the physiochemical phenomena of HA entanglement point. As the mode of action of MSCs is secretion-driven, the cytokine profile was measured to assess clinical feasibility of the MSC-HA combination.

2. Materials and Methods

2.1. MSC Growth Kinetics

Adherence. This experiment was undertaken on *standard* 96-well plates and CellBind 96-well plates (*high*-adherence; Corning, Australia) to test the effect of HA on different binding chemistries. Cells were seeded into 96-well plates at 5×10^3 cells/well using a combination of media preparations (see Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/9364213). A standard curve was seeded ranging from 1×10^3 to 16×10^3 cells/well. After 24 hours all wells were washed in PBS and $100 \mu L$ of control media was added. A further $10 \mu L$ of Cell Counting Kit-8 (water soluble tetrazolium salt, WST-8; CCK-8) reagent was added and following a four-hour incubation at $37°C$, the absorbance measured at 450 nm (5 technical replicates; $n = 3$). Cell number was extrapolated from the standard curve using a trend line with a polynomial of two. Combinations of the media preparations $(- +)$, $(+ -)$, and $(+ +)$ were compared back to cells grown in control media and seeded in control media $(- -)$ using a Mann-Whitney test (nonparametric test).

Proliferation. This experiment was similar to the *adherence* protocol with minor differences. Cells were seeded into 96-well plates at 2×10^3 cells/well (5 technical replicates; $n = 3$).

The standard curve was seeded on the second day, 24 hours before the 3-day endpoint.

2.2. MSCs Kinetics on Equine Articular Cartilage Explants: Ex Vivo Cartilage Assay.
All horses used in this study were due to be sacrificed at a commercial abattoir to be processed and sold as dog food (Kankool Pet Food, Australia). The fetlock joint from mature horses was cut out by sawing 15 cm below and above the joint. The fetlock was then shaved, scrubbed, and soaked in iodine bath for a minimum contact time of 5 minutes and then frozen for later use.

2.3. Cartilage Sectioning.
The frozen fetlock joint was thawed at 37 degrees for 1-2 hours until the fetlock joint became flexible. Once thawed, the joint was disarticulated in a sterile hood (see Supplementary Figure 2). The third metacarpal was obtained from the fetlock joint and the distal end was soaked in PBS for 5 minutes. The articular cartilage was then perforated using a custom designed hollow cylindrical instrument measuring 6.45 mm in diameter equal to a *standard* 96-well plate. The perforated discs were then sliced off with a scalpel and stored in the correct orientation (joint side facing up) in a 96-well plate with $200 \mu L$ of DMEM and frozen for later use.

2.4. Cartilage Adherence Time Course of MSCs.
Cartilage discs were used to plug *ultra-low* adherence 96-well plates. Passage 2 cells were seeded onto the cartilage discs at a density of 5×10^3 cells/disc and incubated at $37°C/5\%$ CO_2. At each time point (1, 2, 3, 4, 8, and 24 hours), cartilage discs were washed twice in PBS, fixed, and washed again. Cartilage discs were stained (see Supplementary Methods) and imaged using confocal microscopy.

2.5. HA Media Viscosity Assessment.
Falling-ball viscometry was used to determine the viscosity (adapted from Eguchi and Karino, 2008 [15]) of HA media relative to control. Briefly, a 5 mL serological pipette was filled with prewarmed media. A biosilicate sphere was dropped and the time measured between two defined points. The data was expressed as a mean (5 technical replicates) flow-rate (millimetres/second) ± standard deviation.

2.6. MSC Dispersion on Cartilage with Increasing Concentration of HA.
The media formulations used were control media and 0.5 mg/mL, 1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL, and 5 mg/mL HA media. MSCs were seeded onto the cartilage discs at a density of 5×10^3 cells/disc. Following a 24-hour incubation, cartilage discs were washed twice in PBS, fixed, and washed again. Cartilage discs were stained (see Supplementary Methods) and imaged using confocal microscopy.

2.7. MSC Adherence and Proliferation on Cartilage.
MSCs were treated with either control or 1 mg/mL HA media for three days. *Ultra-low* adherence 96-well plates were plugged with cartilage discs. The conditions tested included MSCs grown in control and seeded in control $(- -)$, grown in control and seeded in 1 mg/mL HA media $(- +)$, grown in

1 mg/mL HA media and seeded in control (+ −) (primed), and grown in 1 mg/mL HA media and seeded in 1 mg/mL HA media (+ +).

Adherence. MSCs were seeded onto cartilage discs at a density of 5×10^3 cells/disc. After 24 hours cartilage discs were removed to a new 96-well plate and washed twice with PBS. Fresh culture media (200 μL) were added to discs followed by 20 μL of CCK-8 and incubated for four hours at 37°C. Colour-developed wells were read at an absorbance of 450 nm (5 technical replicates; $n = 3$).

Proliferation. This experiment was undertaken in the same way as the *adherence* experiment; however the cells were cultured for 3 days and seeded at a density of 2×10^3 cells/disc.

2.8. Secretome Analysis. Conditioned media were collected from every flask in this study ($n = 3$), centrifuged at 5000 ×g for 5 minutes, and stored at −80°C. Filtrates (50 μL) were analysed using both the Bio-Plex Pro-Human Cytokine 27-Plex and Bio-Plex Pro-Human Cytokine 21-Plex assay (Bio-Rad, USA), according to the manufacturer's instructions (see Supplementary Methods).

3. Results

3.1. MSCs Grown in Control Media and Seeded in HA Media. HA concentrations were compared to control, that is, (− −) versus (− +). In *standard* 96-well plates, the highest cell proliferation was observed for 1 mg/mL HA ($p < 0.07$). In *high*-adherence 96-well plates the 2 mg/mL HA decreased adherence by 45% ($p < 0.05$) (Figure 1).

3.2. MSCs Grown in HA Media and Seeded in Control Media (Priming). MSCs grown in HA media (B–E) were compared to control (− −) versus (+ −). In *standard* 96-well plates, HA primed conditions showed peaked adherence for 0.5 mg/mL and 1 mg/mL HA ($p < 0.05$). Inversely, in *high*-adherence 96-well plates, adherence decreased with increasing concentration of HA and significantly for 2 mg/mL HA ($p < 0.05$). However in *high*-adherence 96-well plates, proliferative effects of HA priming recovered with 0.5, 1, and 2 mg/mL HA significantly ($p < 0.05, <0.001,$ and <0.01, resp.) increased (Figure 2).

3.3. MSCs Grown in HA Media and Seeded in HA Media. MSCs grown in HA media (B–E) were compared to control (− −) versus (+ +). In *standard* 96-well plates, adherence showed a dose-dependent trend with a peak at 1 mg/mL HA ($p < 0.001$) and decreased in 2 mg/mL HA. Similarly proliferation with 0.5, 1, and 2 mg/mL HA increased but peaked at 1 mg/mL HA ($p < 0.05, <0.01,$ and <0.01, resp.). Inversely in *high*-adherence 96-well plates, adherence decreased with increasing concentration of HA and significantly for 2 mg/mL HA ($p < 0.01$). However proliferation again increased with 0.5 and 1 mg/mL HA ($p < 0.01, <0.001,$ resp.) (Figure 3).

3.4. Time Course of MSC Grown on Cartilage. Ultra-low adherence 96-well plates were plugged with cartilage discs and MSCs seeded in control media to assess adherence times from one to 24 hours (Figure 4). At the one-hour time point (a), only a handful of cells adhered to cartilage. At higher magnification (g) MSCs appeared to be spherical with extensions clearly visible. From three to four hours (c-d), MSCs covered the surface with overlapping flat cell bodies. Overlapping between cells (e) was greatly reduced by eight hours and cell began to display (h) spindle morphology. At 24 hours (f) MSCs adhered to cartilage and dissipating membrane stain indicated possible doubling; (i) typical fibroblastic-like MSC morphology and organisation can be observed.

3.5. MSC Dispersion on Cartilage with Increasing Concentration of HA and Viscosity of Media Formulations. Ultra-low adherence 96-well plates were plugged with cartilage discs and MSCs were seeded, suspended in either control or HA media (0.5–5 mg/mL HA). At lower concentrations of HA, MSCs uniformly dispersed across the cartilage surface. As concentration increased to 3 mg/mL dispersion was maintained but with reduction in adhered cells. Singular colonies were observed at highest concentrations 4 mg/mL and 5 mg/mL HA (Figure 5).

The flow-rate of the sphere in the falling-ball test was measured to show viscosity of HA media formulations relative to control (see Supplementary Figure 3). The control displayed the highest flow-rate, which decreased with increasing concentrations of HA. The flow-rate for 0.5 mg/mL and 1 mg/mL HA was 57% and 51% of the control, respectively. Flow-rate of 2 mg/mL HA dropped to 20% of control and then a plateau was observed for higher concentrations.

3.6. MSC Adherence and Proliferation on Cartilage. The 1 mg/mL HA formulation was tested for all conditions (Figure 6). *Ultra-low* adherence 96-well plates were plugged with cartilage discs and cells seeded at either 5×10^3 cells (*adherence*) or 2×10^3 cells (*proliferation*) per well. In the adherence experiment, a peak was observed in the primed (+ −) condition ($p < 0.05$). A similar peak was observed for the primed condition in the proliferation experiment ($p = 0.1$).

3.7. HA Media Alter the Cytokine Secretion Profile of MSCs. MSCs were treated for three days in either control or HA media formulations. Changes in concentration were observed in 28 of the 48 measured cytokines (see SF 4–7 and Supplementary Table 2). MSCs treated with 1 mg/mL HA increased secretion for Interleukin-1β (IL-1β) and macrophage migration inhibitory factor (MIF) (Figure 7). Anti-inflammatory cytokine Interleukin-1 receptor antagonist (IL-1ra) secretion increased dose-dependently and was significant at 2 mg/mL HA. Interleukin-6 (IL-6), a dual role cytokine, increased in the 0.25 mg/mL HA concentration but then decreased with increasing concentration of HA. Fibroblast growth factor-basic (FGF-β) increased dose-dependently and was significant at 2 mg/mL HA. Vascular endothelial growth factor
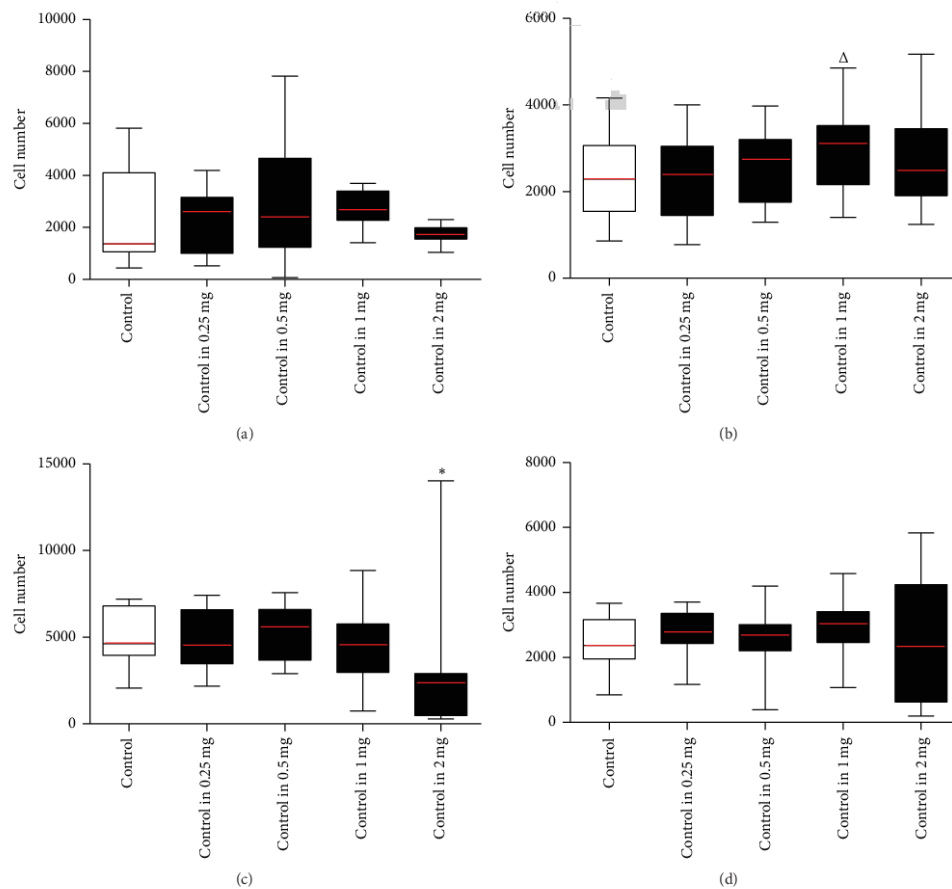
(a)

(b)

(c)

(d)

FIGURE 1: MSCs grown in control media and seeded in HA media. MSCs expanded until passage 2 in control media. Cells from flask (A) were then stripped and cell suspensions were counted using the standard enumeration technique. Cells were seeded into 96-well plates in either control media (control) (− −) or HA media (− +) containing a series of concentrations ranging from 0.25 to 2 mg/mL of HA to make up the five conditions (x-axis). Conditions were tested using five technical replicates per 96-well plate and run in biological triplicate ($n = 3$). Wells were assayed at the endpoint using Cell Counting Kit-8 with standard curves run on every 96-well plate seeded 24 hours prior to endpoint and absorbance read at a wavelength of 450 nm (y-axis). All conditions were compared back to the control using a t-test ($^\Delta p$ value = 0.07, $^*p$ value < 0.05). (a) MSCs seeded in *standard* 96-well plates for 24 hours (adherence), (b) MSCs seeded in *standard* 96-well plates for three days (proliferation), (c) MSCs seeded in *high*-adherence 96-well plates for 24 hours (adherence), and (d) MSCs seeded in *high*-adherence 96-well plates for three days (proliferation) (see Supplementary Table 1).

(VEGF) decreased with increasing concentration of HA and was significant at 2 mg/mL HA.

4. Discussion

Biological therapeutics such as MSC therapy are gaining acceptance for the treatment of inflammatory conditions, including musculoskeletal ailments such as knee osteoarthritis (OA). This is most likely due to the well documented

treatment gap for middle-age sufferers of OA who are not candidates for total knee arthroplasty [5]. The safety of allogeneic MSC therapy will continue to drive the universal donor model because an "off-the-shelf" therapeutic is desirable for scalability and cost. The manufacture of cell therapeutics for the treatment of OA will likely involve the use of agents that can enhance MSC function. The use of HA is attractive as an adjunct, as it is a core component of the extracellular matrix, endogenously abundant in the
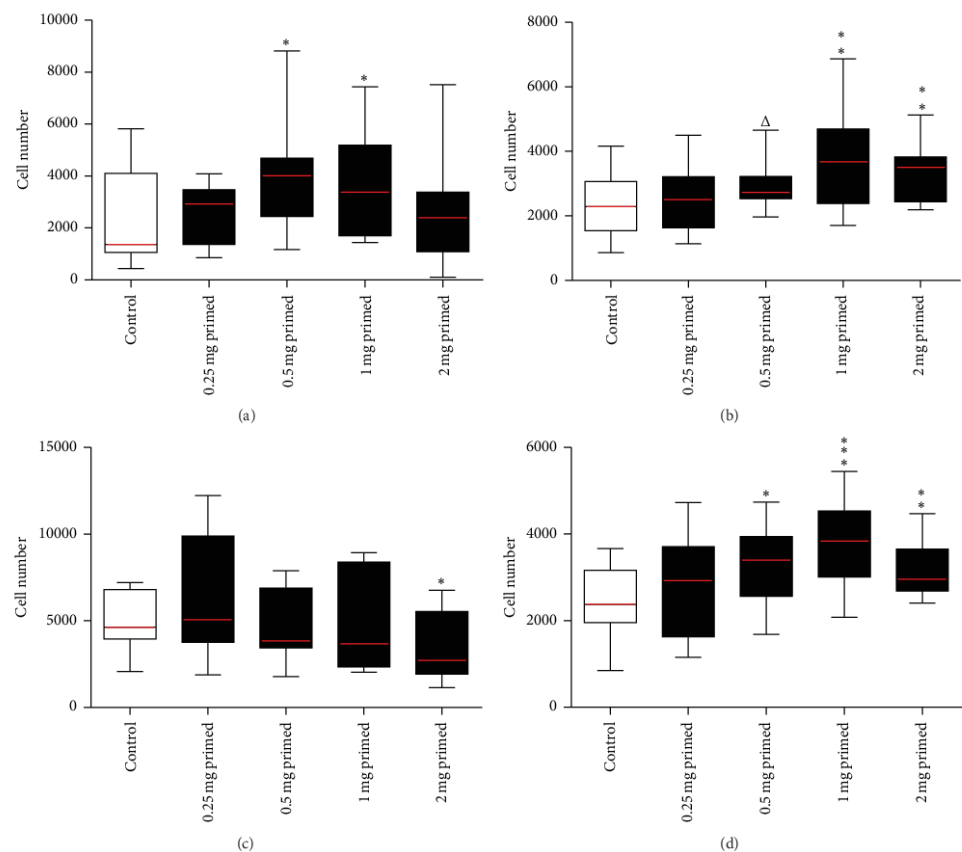
Figure 2: MSCs grown in HA media and seeded in control media (primed). MSCs expanded until passage 2 in control media. Cells were then stripped and cell suspensions were counted using the standard enumeration technique. Cells grown control media, flask (A), were seeded in control media (control) (− −) and MSCs grown in flasks (B–E) HA media were seeded into 96-well plates in control media (HA primed) (+ −), to make up the five conditions (x-axis). Conditions were tested using five technical replicates per 96-well plate and run in biological triplicate ($n = 3$). Wells were assayed at the endpoint using Cell Counting Kit-8 with standard curves run on every 96-well plate seeded 24 hours prior to endpoint and absorbance read at a wavelength of 450 nm (y-axis). All conditions were compared back to the control using a t-test ($^{\Delta}p$ value = 0.1, $^{*}p$ value < 0.05, $^{**}p$ value < 0.01, and $^{***}p$ value < 0.001). (a) MSCs seeded in *standard* 96-well plates for 24 hours (adherence), (b) MSCs seeded in *standard* 96-well plates for three days (proliferation), (c) MSCs seeded in *high*-adherence 96-well plates for 24 hours (adherence), and (d) MSCs seeded in *high*-adherence 96-well plates for three days (proliferation) (see Supplementary Table 1).

knee joint [8, 9] and approved for the treatment of OA. It is important to understand growth kinetics of MSCs with respect to HA. Already there have been case reports [16] and randomised, double-blind, placebo controlled studies [14] where MSCs were suspended in HA either as a vehicle or as a combination for the treatment of OA. These studies did not document any exploration of positive or negative interactions between MSCs and HA [17]. Therefore we tested different concentrations of HA in culture media to assess adherence and early proliferation kinetics of MSCs on both

plastic culture surfaces and equine articular cartilage explants in an *ex vivo* model, as a way to simulate early cell behaviour following an intra-articular injection of this combination. Also, we hypothesised that the high viscosity of HA may interfere with cell binding. To avoid this issue, we explored whether MSCs could be primed with HA to improve growth kinetics or adherence. We employed a novel cartilage disc assay in a time series to determine MSC adherence to cartilage. In the same cartilage assay the physical dispersion of MSCs was assessed using a concentration series of HA media
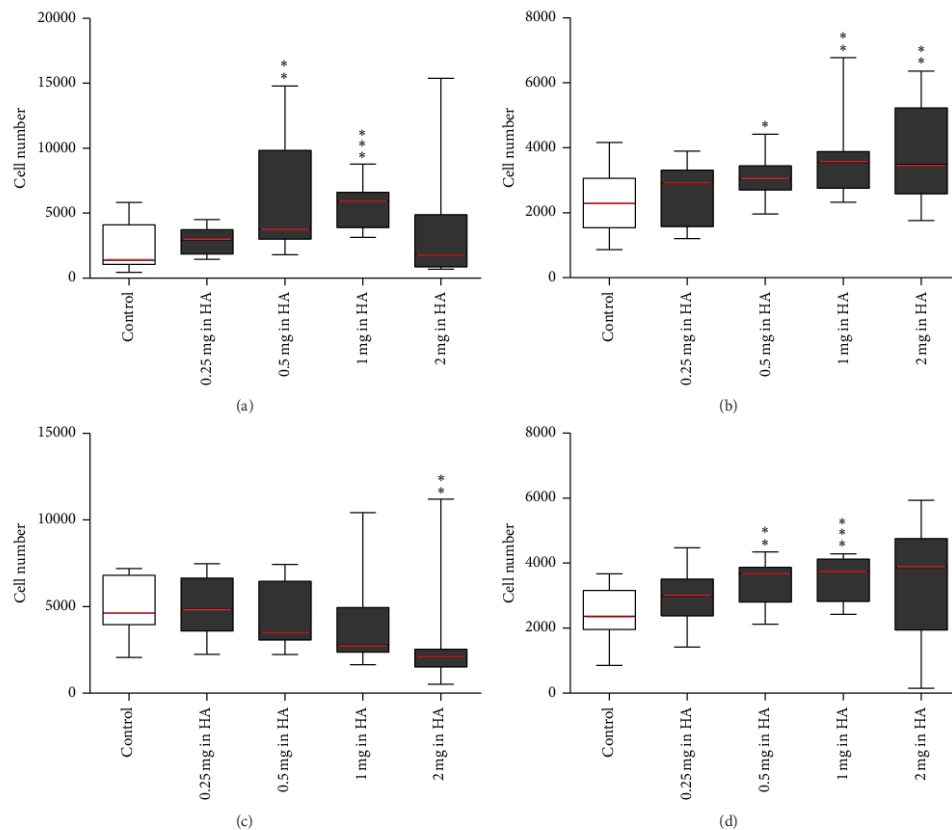
(a)

(b)

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(d)

FIGURE 3: MSCs grown in HA media and seeded HA media. MSCs expanded until passage 2 in control media. Cells were then stripped and cell suspensions were counted using the standard enumeration technique. Cells grown control media, flask (A), were seeded in control media (control) ($-$ $-$) and MSCs grown in flasks (B–E) HA media were seeded into 96-well plates in HA media with the same concentration of HA ($+$ $+$) ranging from 0.25 to 2 mg/mL of HA to make up the five conditions (x-axis). Conditions were tested using five technical replicates per 96-well plate and run in biological triplicate ($n = 3$). Wells were assayed at the endpoint using Cell Counting Kit-8 with standard curves run on every 96-well plate seeded 24 hours prior to endpoint and absorbance read at a wavelength of 450 nm (y-axis). All conditions were compared back to the control using a t-test ($^{*}p$ value < 0.05, $^{**}p$ value < 0.01, and $^{***}p$ value < 0.001). (a) MSCs seeded in *standard* 96-well plates for 24 hours (adherence), (b) MSCs seeded in *standard* 96-well plates for three days (proliferation), (c) MSCs seeded in *high*-adherence 96-well plates for 24 hours (adherence), and (d) MSCs seeded in *high*-adherence 96-well plates for three days (proliferation) (see Supplementary Table 1).

over the cartilage shedding light on the biological relevance of the entanglement point of HA chains.

An allogeneic preparation of MSCs and HA may be developed in a number of ways, with HA only present in the final cellular product, used as a priming agent during culture but not in the final formulation or both. Here we tested three representative conditions and found that cells grown in standard culture and seeded in HA ($-$ $+$) show some increases in adherence and proliferation. However, greater differences were observed in the ($+$ $-$) and ($+$ $+$) conditions,

where HA as a culture supplement exhibited dose-dependent increases in cell number. It is well known that HA molecules behave in solution as highly hydrated randomly kinked coils, which start to entangle at concentrations of less than 1 mg/mL (entanglement point) [18]. The peak seen in the HA dose required for optimal cell attachment is centred on this entanglement point of the HA chains. In this study, for the first time we have attributed biological significance to the physiochemical phenomena of the entanglement point seen in HA chains. At this concentration of HA, all HA molecules
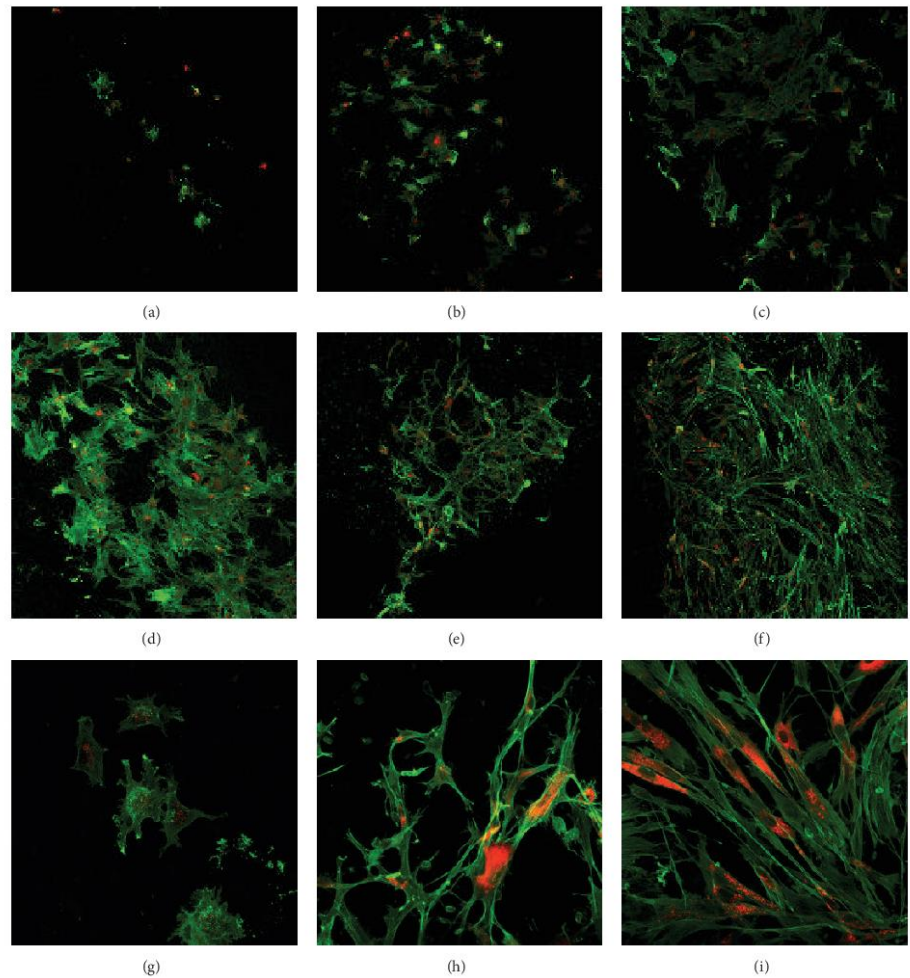
FIGURE 4: Cartilage adherence time course of MSCs. MSCs were cultured until the second passage before use in this experiment. Prior to seeding cells for the time course, the monolayer was washed in PBS and then stained using CM-DiI (orange colour) membrane dye. Cartilage discs were used to plug the bottom of a 96-well plate in the correct orientation. Cells were then stripped using TrypLE, counted using the standard enumeration technique, and then seeded onto the cartilage discs at a density of 5×10^3 cells/disc and the 96-well plate was then incubated at 37°C and 5% CO_2. At each of the time points (1, 2, 3, 4, 8, and 24 hours), the cartilage discs were washed in PBS and then fixed in 4% paraformaldehyde and then washed in PBS. Cartilage discs with attached cells were then permeabilised in 0.1% Triton X-100, washed in PBS, and blocked in 1% Bovine serum albumin in PBS before being stained with F-actin-specific Alexa Fluor 488-phalloidin (green). All images were taken using the OLYMPUS FLUOVIEW FV1000 IX81 inverted confocal microscope. (a) One-hour time point at 10x magnification, (b) two-hour time point at 10x magnification, (c) three-hour time point at 10x magnification, (d) four-hour time point at 10x magnification, (e) eight-hour time point at 10x magnification, (f) 24-hour time point at 10x magnification, (g) one-hour time point at 40x magnification, (h) eight-hour time point at 40x magnification, and (i) 24-hour time point at 40x magnification.

(a)

(b)

(c)

(d)

(e)

(f)
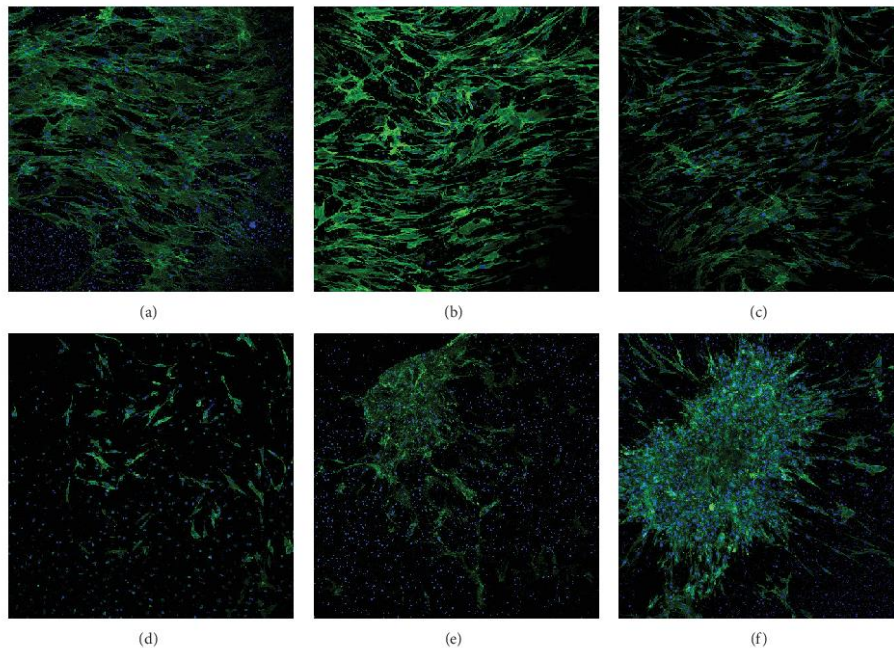
FIGURE 5: MSC dispersion on cartilage with increasing concentration of HA. MSCs were cultured until the second passage before use in this experiment. Cartilage discs were used to plug the bottom of a 96-well place in the correct orientation. Media formulations were then added to each well. The media formulations used were (a) 0.5 mg/mL HA media, (b) 1 mg/mL HA media, (c) 2 mg/mL HA media, (d) 3 mg/mL HA media, (e) 4 mg/mL HA media, and (f) 5 mg/mL HA media. MSCs were then stripped using TrypLE, counted using standard enumeration technique, and then seeded onto the cartilage discs at a density of 5×10^3 cells/disc. The 96-well plate was then incubated for 24 hours at 37°C and 5% CO_2. After 24 hours the cartilage discs were washed in PBS and then fixed in 4% paraformaldehyde. Cartilage discs were washed in PBS and then stained in using Hoechst 33342 (blue) and again washed five times in PBS. Cartilage discs were then permeabilised in 0.1% Triton X-100, washed in PBS, and blocked in 1% Bovine serum albumin in PBS before being stained with F-actin-specific Alexa Fluor 488-phalloidin (green). All images were taken using the OLYMPUS FLUOVIEW FV1000 IX81 inverted confocal microscope at 10x magnification.

are connected via a meshwork and behave like a weak and elastic gel which mimics properties of soft tissue [19].

In contrast to the standard plates, the *high*-adherence plates decreased the number of adherent cells in the presence of HA and for the HA primed cells. According to the manufacturer, the surface is treated (nonbiologically) to improve cell attachment by incorporating more oxygen into the cell culture surface by increasing surface area and rendering it more hydrophilic. Increasing concentrations of HA decreased the number of attached cells within the first 24 hours significantly. HA is a regulator of water homeostasis in the body [8]; therefore it is reasonable to suggest HA interfered with the treated surface, an interference which persisted in the primed conditions.

The constraint of viscosity made it unclear whether increasing HA concentration further would amplify the effects seen in early time points. Viscosity-induced heterogeneity can be seen in the variance of the ranges observed for the 2 mg/mL HA concentration across all the growth kinetics

experiments when cells were seeded in HA. Previous investigations of porcine bone marrow-derived MSCs showed using 4 mg/mL HA (M_W 800 KDa) was optimal at 7 days [20] but no changes in two days. It is counterintuitive to expect optimal growth kinetics at 7 days since HA half-life in the knee joint is reported to be less than 24 hours [21].

Our study was concerned with high molecular weight HA, as it is more applicable as an OA therapeutic [22]. The study of low molecular weight HA found no difference in cell adherence or proliferation between 0.5, 1, and 2 mg/mL concentrations in the early time points. Another adhesion study using the same high molecular weight HA also found a decrease in synovial MSC adhesion with increasing concentrations beyond the entanglement point [23]. We employed a modified falling-ball test to show relative changes in viscosity from control media. The biggest change in viscosity was seen at concentrations above the entanglement point, the flow-rate of which decreased by 31% between 1 and 2 mg/mL concentrations and then a plateau. This further explains
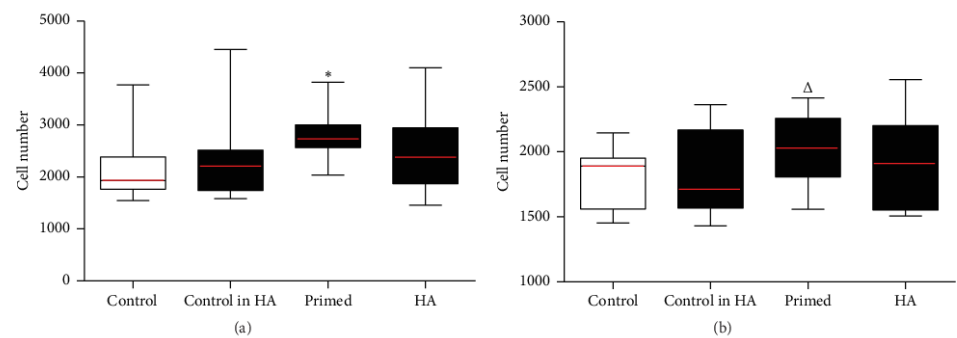
FIGURE 6: Treated MSCs seeded onto articular cartilage explants for 24 hours and three days. MSCs expanded until passage 2 in control media. On the third day flasks were treated with either control media or 1 mg/mL HA media for another three days. Cells were then stripped and cell suspensions were counted using the standard enumeration technique. Cells were seeded into *ultra-low* adherence 96-well plates plugged with *articular cartilage explants* in the correct orientation. Conditions (x-axis) tested were cells grown in the control flask and seeded in control media (control) ($-$ $-$), grown in control media and seeded in 1 mg/mL HA media (control in HA) ($-$ $+$), grown in 1 mg/mL HA media and seeded in control media (primed) ($+$ $-$), and grown in 1 mg/mL HA media and seeded in 1 mg/mL HA media (HA) ($+$ $+$) and articular cartilage explants alone (no cells seeded to serve as a blank absorbance). Conditions were tested using five technical replicates per 96-well plate and run in biological triplicate ($n = 3$). Wells were assayed at the endpoint using Cell Counting Kit-8 and absorbance of the resulting media alone read at a wavelength of 450 nm (y-axis). All conditions were compared back to the control using a t-test ($\Delta = 0.19$, *P value < 0.05). (a) MSCs seeded for 24 hours (adherence) into *ultra-low* adherence 96-well plates plugged with articular cartilage explants and (b) MSCs seeded for three days (proliferation) into *ultra-low* adherence 96-well plates plugged with articular cartilage explants.

decreases seen in cell attachment for concentrations higher than 1 mg/mL as a viscosity constraint in the early phase.

Increased attachment and proliferation on culture surfaces enabled the initial experiments to be performed under controlled conditions but may not reflect cartilage binding. We therefore employed a novel equine-derived cartilage explant culture model *ex vivo* in an attempt to better mimic true architecture of the target tissue in OA, cartilage. Shimaya et al. had shown previously that synovial MSCs will increase adherence to an osteochondral defect with 10 mM magnesium *ex vivo* [24]. However, inconsistencies can be observed in defects created and further limitations of subjectivity can arise when using an image analysis technique to quantify cell adherence. Baboolal et al. utilised a similar technique with osteochondral plugs embedded in agarose to test MSC adherence to cartilage. However, MSCs were labelled with iron-oxide microparticles and semiquantitative image analysis techniques were used to report cell adhesion [23]. Herein we have described a reproducible, consistent, and quantitative method for cell attachment to biologically inactive cartilage in isolation. This enabled the testing of various cell and HA formulations without any modulatory signalling from the cartilage because it was not viable. Some considerations for our technique revolved around the plug integrity in the well; that is, if the cartilage disc curled and the well no longer sealed, the replicate was discarded. For this reason, all conditions were run using five technical replicates. The use of *ultra-low* adherence plates and repeated washing limited nonspecific binding of cells to plastic and therefore gave a true indication of cell adherence to the cartilage.

Growth kinetics on the cartilage surface employed the optimal concentration of 1 mg/mL HA for all the conditions on *ultra-low* adherence plates plugged with the cartilage discs as the new surface. Interestingly, if the cells were seeded in the presence of HA ($-$ $+$ and $+$ $+$), cell adherence did not improve which suggests the HA may interfere with MSCs binding to cartilage. Only the primed MSCs adhered ($p < 0.05$) and proliferated ($p = 0.1$) more than the control. Therefore it is reasonable to expect that the use of HA as a vehicle for MSCs during intra-articular injection for knee OA will interfere with cell attachment to cartilage. Indeed this was consistent with previous investigations, where high molecular weight HA in OA-derived synovial fluid interfered with MSCs binding to cartilage, an effect which could be overcome by hyaluronidase (enzyme used to break down HA) treatment [23]. Furthermore it was shown in a caprine model using a collagen II-induced arthritis that the half-life of HA was an average of 11.5 hours and 20.8 hours in the nonchallenged knees [25].

We showed MSCs substantially adhere after three hours but do not regain fibroblastic morphology until eight hours after seeding. For surgeons injecting allogeneic MSCs into humans, this may indicate the need for an extended unweighted rest period of 8–24 hours after injection to allow MSCs to thoroughly adhere to the cartilage in the joint. Although our model does not take into account the internal pressures of the knee joint, endogenous HA concentration or the catabolic milieu of an OA affected knee. Previous investigations of cell attachment to human arthritic cartilage showed synovial MSCs can adhere within 10 minutes [24];
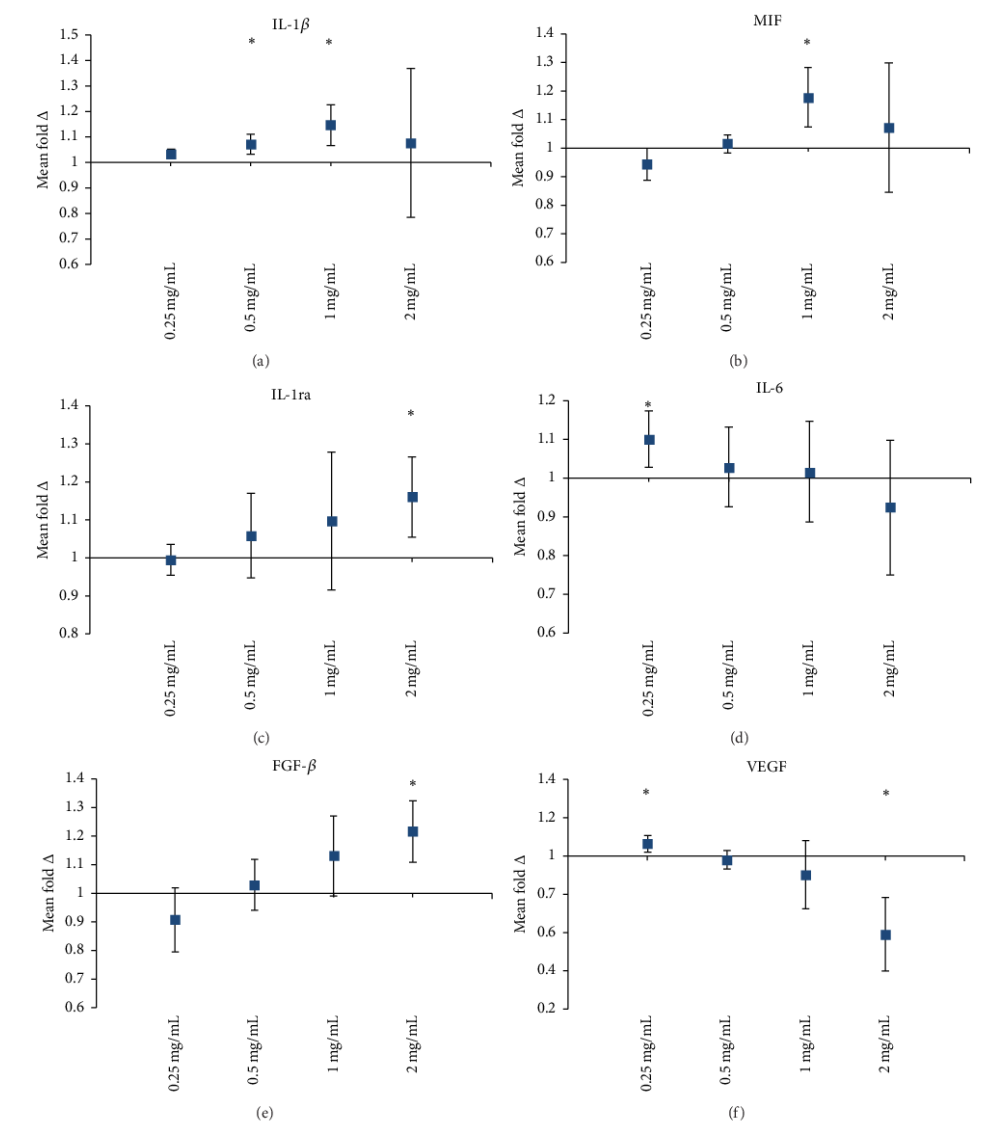
FIGURE 7: Fold change of cytokine secretion by MSCs with HA treatment. Conditions were all cultured in either control media or HA media for three days after the media change. Conditions (*x*-axis) were grown in flasks (B–E) HA media, ranging from 0.25 to 2 mg/mL of HA. These were all compared back to the control condition (cells grown control media, flask (A)). Average fold change in fluorescence (n = 3) ± upper and lower confidence intervals at 95% (*y*-axis). Cytokines with a fold change less than one indicate a decrease with HA media treatment; a fold change greater than one indicates an increase in the cytokine with HA treatment (*cytokines with a fold change clear of the axis at 1 reported as significant). Interleukin-1β (IL-1β), macrophage migration inhibitory factor (MIF), Interleukin-1 receptor antagonist (IL-1ra), Interleukin-6 (IL-6), fibroblast growth factor-basic (FGF-β), and vascular endothelial growth factor (VEGF). See Supplemental Data for complete list of significantly changed cytokines and numerically tabulated summary.

however the cell dose used was more than 200 times greater per square millimetre of cartilage and lacked washing steps to account for loosely bound cells. In light of this, the model cannot take into account internal shearing forces of the joint which may contribute to cell detachment. Also no effort was made to show morphology of cells was fibroblastic and therefore they may have been in the flat bodied and in the initial attachment phase observed in this study. Furthermore, OA-derived synovial fluid was shown to inhibit MSC binding to cartilage [23]; thus an extended rest period beyond 24 hours may be required for thorough adhesion.

Concentration of HA as a vehicle for intra-articular injection of MSCs is important for dispersion and internal cartilage coverage. The dispersion assay with increasing concentration of HA indicated the higher (4 and 5 mg/mL HA) concentrations would form single aggregate colonies. A randomised, double-blind, controlled study which used intra-articular injection of allogeneic bone marrow-derived MSCs with HA as a vehicle at a concentration of 10 mg/mL showed a reduction in pain in patients with OA changes using a visual analogue scale [14]. However, only some patients had increased meniscal volume, which may be *de novo* tissue regeneration. According to our dispersion data, it is likely that the injected cell suspension did not disperse throughout the joint. Furthermore, the endogenous concentration of HA found in postmortem synovial fluid was reported at 1–4 mg/mL of HA [26] and this can decrease with joint pathologies as reported by Dahl et al. to 0.17–1.32 mg/mL [27]. It is unclear if the lack of dispersion and therefore close proximity of MSCs would have an effect on tissue regeneration or migration of MSCs *in vivo*. However, MSCs grown in hanging drop culture as spheroids express higher levels of anti-inflammatory TNF alpha stimulated gene 6 (TSG-6) and stanniocalcin-1 (STC-1) compared to monolayer culture [28] which suggests altered immunomodulatory capacity of MSCs.

Culturing MSCs with HA can alter the cytokine secretion profile. Investigations of the secretome of MSCs when combined with HA have been shown to increase the secretion of macrophage migration inhibitory factor (MIF) [29]. MIF secretion by MSCs in this study peaked significantly only for the 1 mg/mL treated cells. MIF is considered to be a proinflammatory mediator especially in OA and acts in local tissue to increase neutrophil and macrophage migration to regions of inflammation. Our previous investigations also showed increased secretion of MIF by MSCs cultured with HA [29]. Vascular endothelial growth factor (VEGF) decreased with increasing concentration of HA and significantly for the 2 mg/mL concentration. This was consistent with our previous investigations of HA and MSC coculture [29]. VEGF may play an active role in the pathogenesis of OA. Ludin et al. found that if exogenous VEGF was injected into the knees of mice, the joint presented with synovial hyperplasia and cartilage degradation as typically seen in OA disease [30]. Thus a consistent decrease in the secretion of VEGF by MSCs in the presence of HA may suggest therapeutic synergy between the two.

Basic fibroblast growth factor (FGF-β) increased with increasing concentrations of HA. FGF-β is a potent mitogen on MSCs and the increased secretion may help to explain the increase in proliferative capacity of the cells [31, 32]. HA may therefore have an agonistic role in potentiating the secretion of FGF-β by MSCs but this has not been shown. Additionally, an increase in secretion of FGF-β may contribute to increase therapeutic efficacy of MSCs for the treatment of OA. A cartilage defect model in rabbit showed exogenous human FGF-β alone could potentiate articular cartilage resurfacing within 6 weeks of the injury [33].

5. Conclusion

Allogeneic preparations of MSCs will become an increasingly attractive therapeutic in the treatment of knee OA. These preparations are increasingly seen to be tested with HA as a vehicle without consideration for the likely interactions. Here we have shown HA can have profound dose-dependent effects on early growth kinetics of MSC and these effects were founded on the physiochemical phenomena of the HA entanglement point. The use of our *ex vivo* cartilage model further emphasises the limitation of *in vitro* experimentation but also highlighted for the first time that HA primed MSCs can increase cell attachment to cartilage and that the presence of HA did not. Our time course study also suggests patients undergoing MSC therapy for knee OA could benefit from being immobilised and unweighted for 8 hours after injection to allow MSCs to thoroughly adhere in the joint. Additionally, concentration of HA was also shown to greatly affect the dispersion of MSCs. These factors should be considered in future trials with respect to the culture media for MSCs and where HA is being considered as a component of vehicle for the treatment of OA.

Conflict of Interests

Benjamin R. Herbert is an Assistant/Professor of Translational Regenerative Medicine at the University of Sydney. He is also a cofounder, shareholder, and consultant to Regeneus Ltd. Peter Succar is a Ph.D. candidate at Macquarie University and a casual employee at Regeneus Ltd. Edmond J. Breen is the Head of Bioinformatics at the Australian Proteome Analysis Facility (APAF) at Macquarie University and a consultant to Regeneus Ltd. Michael Medynskyj is a full time employee at Regeneus Ltd. Tony Batterham is a consultant for Regeneus Ltd. and director of Quirindi Vet Clinic. The Quirindi Vet Clinic and Regeneus Ltd. contributed funds equally to the publication processing fee.

Authors' Contribution

Peter Succar contributed to concept and design, provision of study material, collection and assembly of data, data analysis and interpretation, paper writing, and final approval of the paper. Michael Medynskyj contributed to concept and design and final approval of paper. Edmond J. Breen contributed to data analysis and interpretation and final approval of paper. Tony Batterham contributed to provision of study material. Mark P. Molloy contributed to administrative support and

final approval of paper. Benjamin R. Herbert contributed to concept and design, financial support, administrative support, paper writing, and final approval of paper.

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A highly selective and sensitive ON–OFF–ON fluorescence chemo-sensor for cysteine detection in endoplasmic reticulum

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ABSTRACT

A new complex between coumarin-based ligand (**CL**) and copper ion has been prepared and applied to be an ON–OFF–ON reversible fluorescence chemosensor for the detection of Cys with high sensitivity and specificity. In HEPES buffer, **CL** displayed high affinity towards Cu^{2+} ion over other physiological and environmental metal ions, accompanied with a 98.4% of fluorescence quenching. In the presence of Cys, the detachment of Cu^{2+} ion of **CL**–Cu^{2+} ensemble led to the liberation of the fluorophore, **CL**, and thus fluorescence was recovered. The results of absorption and emission spectroscopy analyses confirmed that the fluorescence turn ON response of **CL**–Cu^{2+} ensemble was selective towards Cys only with high sensitivity (detection of limit, 7.2×10^{-7} M). Confocal microscopy studies indicated that the lipophilic **CL** targets the endoplasmic reticulum (ER) of live cells, where it could be functioned as a fluorescent chemosensor for visualization of Cys in this organelle. Quantitative fluorescence detection of Cys in ER was successfully realized by flow cytometry analysis. The developed chemosensor was further applied to detect Cys in real urine samples with great recoveries ranges from 95.41% to 107.40%.

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1. Introduction

Biological thiols, including cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) play fundamental roles in living systems (McMahon and Gunnlaugsson, 2012; Lee et al., 2010; Sun et al., 2011; Long et al., 2013; Chen et al., 2015a, 2015b), and are involved in various physiological and pathological processes (Zhang et al., 2010; Lim et al., 2011; Pires and Chmielewski, 2008). As a well-known thiol-containing amino acid in this family, Cys serves as the building block in protein synthesis, detoxification, and metabolism in live organisms (Yang et al., 2011; Chen et al., 2013; Li et al., 2009; Das et al., 2012). Alterations in the level of cellular Cys has been implicated with neurotoxicity, and the deficiency of Cys may lead to many syndromes, such as a hematopoiesis decrease, slow growth, hair depigmentation, leukocyte loss, psoriasis and so on (Shiu et al., 2011; Yin et al., 2013; Shao et al., 2011; Zhou et al., 2013; Wang et al., 2014).

Endoplasmic reticulum (ER), as the first organelle in the secretory pathway (Sevier and Kaiser, 2008), is dedicated to the folding, assembly, and transporting secretory proteins, as well as response to cellular stress (Zhang et al., 2013). When the cells suffering from ER stress, Cys is identified as one of the most important mediator to maintain the redox state of ER environment by trapping free radicals and reactive oxygen species (ROS) (Zhang et al., 2013; Dierks et al., 1997). It is reported that both oxidative and reductive shifts in ER environments are primarily modulated by posttranslational regulation of Ero1 (ER oxidoreductin) activity via changes in the redox state of its two non-catalytic "regulatory" Cys pairs (Gillece et al., 1999; Vishwakarma et al., 2005). Therefore, monitoring Cys levels in the ER environments is highly important for better understanding of its biological functions which could in-turn contribute to early diagnosis of related diseases.

Recently, there has been increasing interesting in the design of analytical methodologies for the detection of Cys levels in vivo (Chen et al., 2010; Liu et al., 2014a, 2014b; Mei et al., 2013; Shang et al., 2009; Park et al., 2013). Among them, instrumental analytical techniques, such as capillary electrophoresis, high performance liquid chromatography (HPLC) (Bakirdere et al., 2010; Bronowicka-Adamska et al., 2011), mass spectra (MS) (Bronowicka-Adamska et al., 2011), electrochemical method are impractical for the high-throughput clinical analysis or research purpose because the intrinsic limitation of high costs and time consuming (Murale et al., 2013; Zhang et al., 2012). Alternatively,

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Fluorescence
"On"
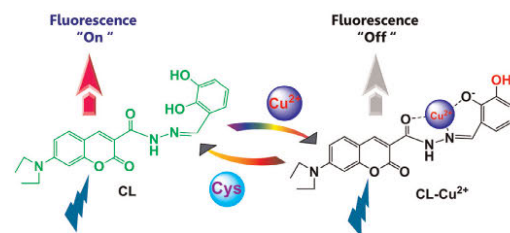
Fluorescence
"Off"

Scheme 1. Schematic illustration of the design and sensing mechanism of **CL** for the reversible detection of Cys.

optical probes using fluorescent chemosensor have been very actively developed in recent years due to the high sensitivity, specificity, simplicity of implementation and fast response time (Kong et al., 2013; Hao et al., 2011; Lee et al., 2008; Yuan et al., 2011). Based on the strong nucleophicity and high binding affinity for metal ions of Cys, a variety of fluorescent chemosensors have been reported for Cys detection (Yang et al., 2013; Wu et al., 2011; Jung et al., 2011; Jiang et al., 2012; Lin et al., 2009; Madhu et al., 2013; Li et al., 2012; Tang et al., 2013). Nevertheless, the probes which can be used to track Cys at subcellular level are scarce (Lim et al., 2011), especially for visualizing Cys level in ER.

In the present work, a new copper complex, **CL**–Cu^{2+} ensemble was designed and facilely synthesized for highly sensitive and selective detection of Cys in live organisms (Scheme 1). The unique ligand, **CL** exhibited high affinity ($K = 1.2 \times 10^5\,M^{-1}$) and specificity to Cu^{2+} ions to form a non-fluorescent 1:1 complex, **CL**–Cu^{2+} ensemble in aqueous solution. In the presence of Cys, the decomplexation of Cu^{2+} ions of the **CL**–Cu^{2+} complex led to significant fluorescence enhancement. The fluorescence switch ON response showed high sensitivity and selectivity to Cys over other naturally amino acids and bioactive species. The proposed displacement mechanism was confirmed by the results of MS study. Interestingly, the results of confocal microscopy imaging of **CL** in live U-343 MGa and MDA-MB-231 cells displayed ER distribution. Thus, quantitative fluorescence detection of Cys at the subcellular level was achieved with confocal microscopy and flow cytometry analysis. The reversibility and the capability of endogenous Cys detection were further demonstrated in the present work.

2. Experiment

2.1. Synthesis and characterization of CL

To a solution of 7-diethylamino-3-hydrazide-coumarin (Munasinghe et al., 2007) (0.275 g, 1.0 mmol) in 30 mL hot methanol at 65 °C, 2,3-dihydroxy-benzaldehyde (0.138, 1.0 mmol) in 10 mL methanol was added. The reaction mixture was refluxed at 65 °C for 7 h. The formed yellow precipitates were filtered, washed with methanol and dried under vacuum to obtain c**L** in 75% yield. ^{1}H NMR (DMSO-d_6, 500 MHz) δ(ppm): 11.80 (s, H), 11.22 (s, H), 9.16 (s, H), 8.75 (s, H), 8.64 (s, H), 7.74 (d, J=10.0 Hz, 1H), 6.93 (d, J=5.0 Hz, 1H), 6.85 (t, J=10.0 Hz, 2H), 6.75 (t, J=5.0 Hz, 1H), 6.66 (s, 1H), 3.50 (q, 4H), 1.16 (t, 6H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ(ppm): 161.77, 159.41, 157.92, 153.37, 150.38, 149.05, 146.61, 146.06, 132.34, 120.90, 119.61, 119.11, 118.00, 111.88, 108.60, 108.37, 96.47, 44.91, 12.80. ESI-MS (m/z): 396.10 [**CL**+H]$^+$. Elemental analysis calcd. for $C_{21}H_{21}N_3O_5$: C 63.79, H 5.35, N 10.63; found: C 63.69, H 5.75, N 10.75.

2.2. Synthesis and characterization of CL–Cu^{2+} ensemble

To a solution of **CL** (39.5 mg, 0.1 mmol) in 1 mL DMSO and 4 mL H_2O, $Cu(NO_3)_2 \cdot 3H_2O$ (26.5 mg, 0.11 mmol) in 1 mL water was added dropwise. After the solution was stirred for 2 h at room temperature, the solvent was evaporated and the product was washed with 2 mL water for three times. **CL**–Cu^{2+} ensemble was then obtained (49.6 mg, 95.6% yield). ESI-MS (m/z): 457.0288 ([**CL**–H+Cu]$^+$), 475.0516 ([**CL**–H+Cu+H_2O]$^+$). Elemental analysis calcd. for $C_{21}H_{20}CuN_4O_8 \cdot H_2O$: C 46.89, H 4.12, N 10.41; found: C 46.52, H 4.45, N 10.18.

3. Results and discussion

3.1. Design, synthesis and sensing mechanism

Our idea that **CL**–Cu^{2+} could have potential for reversible detection of Cys in living biological samples is based on the following considerations: (1) coumarin derivative was chosen as the reporter is due to its excellent photochemical and photophysical properties (Sun et al., 2011; Liu et al., 2014a, 2014b; Yang et al., 2013; Kurishita et al., 2010), such as long-wavelength absorption and emission, high fluorescence quantum yield and high stability against light; (2) (2-hydroxybenzylidene) acetohydrazide moiety could capture Cu^{2+} ions with appropriate affinity (Shi et al., 2011); (3) the complexation of Cu^{2+} ions with **CL** would lead to fluorescence quenching due to the notorious paramagnetic nature of Cu^{2+} ions (Meng et al., 2015a, 2015b; Zhang et al., 2011; Lim et al., 2006); (4) the fluorescence could be recovered in the presence of Cys because of the decomplexation of Cu^{2+} ions from **CL**–Cu^{2+} ensemble (Jung et al., 2011; Shi et al., 2011); (5) both **CL** and **CL** –Cu^{2+} could be cell membrane penetrable due to its lipophilicity, which enables it to be used for intracellular Cys detection (Huang et al., 2009).

Comprehensive consideration of this hypothesis, we synthesized the ligand, **CL**, and its Cu^{2+} ions complex, **CL**–Cu^{2+} (Fig. S1). The structure of **CL** and **CL**–Cu^{2+} were well characterized by NMR, MS, and elemental analysis (Figs. S2–5). The proposed displacement mechanism of **CL**–Cu^{2+} towards Cys was further confirmed by the results of MS study. As shown in Fig. S6, upon the addition of Cu^{2+} ions into the solution of **CL** in HEPES buffer, peak at m/z=457.1 could be assigned to [**CL**–H+Cu]$^+$. This result revealed that 1:1 stoichiometry complex between **CL** and Cu^{2+} ions. Upon the addition of Cys, ESI-MS analysis (Fig. S7) displayed the peaks at 396.3, 418.2 which could be attributed to [**CL**+H]$^+$, and [**CL**+Na]$^+$; 457.2, 535.2 to be assigned to [**CL**–H+Cu]$^+$, and [**CL**–H+Cu+DMSO]$^+$; and 201.2 to be assigned [Cys–H+Cu+H_2O]$^+$. All of these evidences firmly supported that the decomplexation of Cu^{2+} by Cys led to the liberation of fluorophore, **CL**.

The complexation of **CL** with Cu^{2+} ions was then investigated by the evaluation of the changes in fluorescence spectra in HEPES buffer. **CL** displayed strong green fluorescence maximum at 489 nm in HEPES buffer (Φ_1=0.72). As expected, the addition of Cu^{2+} ions (1.0 equiv.) to **CL** led to more than 98.4% of the fluorescence quenching at 489 nm (Φ_2=0.06), which could be ascribed to the paramagnetic quenching effect of the Cu^{2+} ions (Fig. 1a) (Meng et al., 2015a, 2015b; Zhang et al., 2011; Chen et al., 2015a, 2015b). In addition, the fluorescence quenching displayed high sensitivity toward Cu^{2+} ions. As shown in Fig. S9, the dose-dependent fluorescence quenching of **CL** towards Cu^{2+} exhibited a good linearity. The detection limit for Cu^{2+}, calculated as the concentration corresponding to three standard deviations of the background signal, is $2.5 \times 10^{-7}\,M$ (Meng et al., 2015a, 2015b; Zhang et al., 2011). Negligible changes of fluorescence intensities
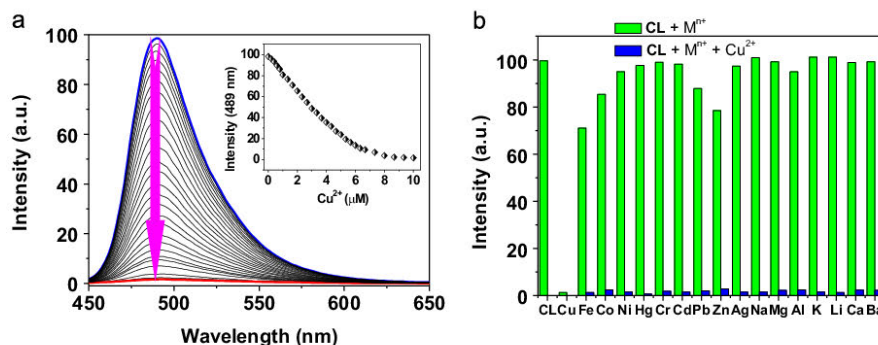
Fig. 1. (a) Fluorescence emission spectra of **CL** (10 μM) in the presence of different concentration of Cu^{2+} ions (0–10 μM) in HEPES aqueous buffer (DMSO: H_2O=5:5, 20 mM, pH=7.4). Inset: plot of emission intensity at 489 nm vs. [Cu^{2+}]. (b) Fluorescence responses of **CL** (10 μM) to various cations. The green bars represent the emission changes of **CL** in the presence of cations of interest (10 μM). The blue bars represent the changes of the emission that occurs upon the subsequent addition of 10 μM of Cu^{2+} ions to the above solution. λ_{em}=489 nm, λ_{ex}=430 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were observed when the **CL** was treated with others physiological and environmental important metal ions, such as Fe^{3+}, Hg^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+}, Ag^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Li^+, K^+, and Na^+ (Fig. 1b). Based on fluorescence titration data, the association constant (K) which was evaluated by the Benesi–Hildebrand method was found to be $1.2 \times 10^5\ M^{-1}$ (Fig. S10) (Meng et al., 2015a, 2015b). The changes of absorption spectra of **CL** towards Cu^{2+} ions were also investigated in this work. **CL**

displays maximum absorption band centered at 445 nm. Upon increasing concentration of Cu^{2+} ions (0–1.0 equiv.), the maximum absorption at 445 nm was gradually reduced with a continuous hypochromatic shift of about 6 nm (Fig. S11). Other competitive metal ions did not induce obvious UV–vis absorption responses (Fig. S12).
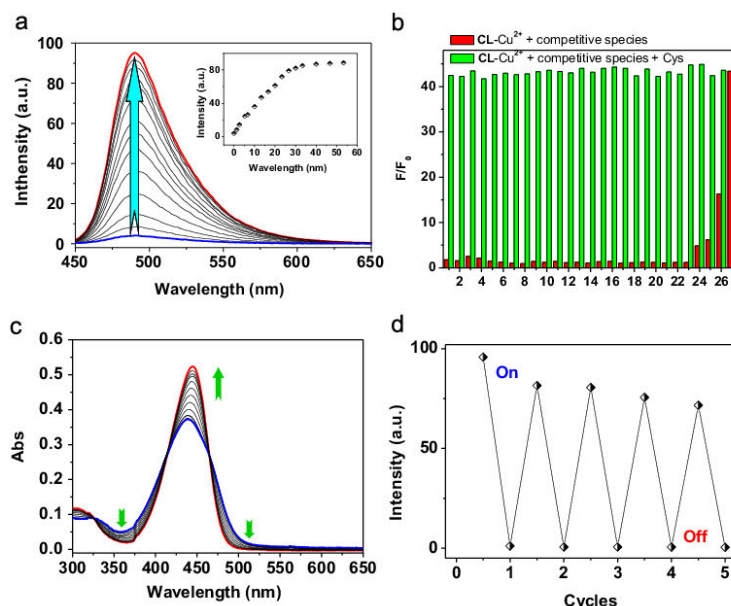

Fig. 2. (a) Fluorescence spectra of **CL**–Cu^{2+} (10 μM) in the presence of different amounts of Cys (0–60 μM). Inset: plot of emission intensity of **CL**–Cu^{2+} (10 μM) at 489 nm as a function of Cys (0–60 μM). (b) Normalized fluorescence changes of **CL**–Cu^{2+} to 60 μM various competitive species (red bar) and to the mixture 60 μM tested species with 60 μM Cys (green bar). F_0 and F is the fluorescence emission intensity at 489 nm of **CL**–Cu^{2+} without (F_0) and with (F) tested species: 1. Ala, 2. Asp, 3. Asn, 4. Arg, 5. Gln, 6. Glu, 7. Gly, 8. Ile, 9. Lys, 10. Leu, 11. Phe, 12. Pro, 13. Ser, 14. Thr, 15. Tyr, 16. Typ, 17. Val, 18. Met, 19. Cl^-, HCO_3^-, 20. NO_2^-, 21. NO_3^-, 22. HSO_4^-, 23. PPi, 24. GSH, 25. His, 26. Hcys, 27. Cys. (c) Changes of UV–vis absorption spectra of **CL**–Cu^{2+} (10 μM) in the presence of different amounts of Cys (0–60 μM). (d) Fluorescent intensity of **CL** (10 μM) at 489 nm upon the alternate addition of Cu^{2+}–Cys with several concentrations ratio (0: 0, 10: 0, 10: 30, 40: 30, 40: 120, 130: 120, 130: 390 and 400: 390 μM, respectively). All the spectra measurements were performed in HEPES aqueous buffer (DMSO: H_2O=5:5, 20 mM, pH=7.4). Excitation was performed at 430 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Fluorescence response of CL–Cu^{2+} towards Cys

It is well-known that Cys is one of the basic component of copper-dependent metalloenzymes, such as cytochrome c oxidase (Liu et al., 2014a, 2014b). Thus, based on the high affinity of Cys with copper, we reasoned that Cys can snatch Cu^{2+} ions of the **CL**–Cu^{2+} ensemble, accompanied with fluorescence switch ON. Towards this hypothesis, we measured the fluorescence response of the **CL**–Cu^{2+} ensemble towards Cys in HEPES buffer. As shown in Fig. 2a, upon addition of 60 μM Cys, both the intensity and overall pattern of the emission spectrum closely match those of native **CL** state ($\Phi_3 = 0.65$), indicating the release of the flouorephore, **CL** by the addition of Cys. The fluorescence turn ON response of **CL**–Cu^{2+} towards Cys was obtained within a few seconds (Fig. S13). An excellent linear correlation was observed by plotting the fluorescence intensity against the concentrations of Cys (Fig. S14). The detection limit, calculated according to the reported method, is 7.2×10^{-7} M (Meng et al., 2015a, 2015b; Zhang et al., 2011).

Subsequently, the experiments on specificity of **CL**–Cu^{2+} towards Cys was conducted by the treatment of **CL**–Cu^{2+} with biothiols (Cys, Hcys, GSH and Met), non-S-containing amino acids (Ala, Asp, Asn, Arg, Gln, Glu, Gly, His, Ile, Lys, Leu, Phe, Pro, Ser, Thr, Tyr, Typ, Val) and biologically important anions such as Cl^-, NO_2^-, NO_3^-, HCO_3^-, HSO_4^-, PPi, ADP, AMP and ATP, respectively. As shown in Fig. 2b, significantly fluorescent enhancement (43.4-fold) was observed with the addition of 60 μM Cys. Meanwhile, only 4.8-fold, 6.2-fold and 16.3-fold enhancements were observed in the presence of equal amounts of GSH, His and Hcys, which was profound enough to discriminate Cys from His and other biothiols. No obvious changes in fluorescence intensities were observed when other amino acids and anions were added. However, further addition of 1 equiv. of Cys to the above solution led to significant fluorescence enhancement. The fluorescence intensities were comparable to the solution of **CL**–Cu^{2+} with 1 equiv. of Cys only, demonstrating that the specific fluorescence response of **CL**–Cu^{2+} towards Cys were not disturbed by competitive biomolecules and anions. These results clearly confirm that Cu^{2+} mainly interact through the co-operative effect of the thiol-amino-carboxylic acid function of Cys, the single functional group such as $-NH_2$, $-SH$ and $-S-S-$ do not bind to Cu^{2+} effectively (Jung et al., 2011; Liu et al., 2014a, 2014b).

The changes of UV–vis spectra of **CL**–Cu^{2+} ensemble with various amino acids, Hcy, GSH and anions were examined in HEPES buffer. As shown in Fig. 2c, the absorption band centered at 445 nm increased gradually with the addition of increasing concentrations of Cys. The addition of 60 μM Cys led to a completely recovery of the absorption spectrum. Meanwhile, a mild decrease of the absorbance of **CL**–Cu^{2+} was observed when the solution was treated with Hcy. Additionally, other competitive species did not induce obvious changes of UV–vis absorption (Fig. S15).

Reversibility is one of the most important factors that should be considered for the development of chemosensors for the detection of analytes in practical applications. As shown in Fig. 2d, a reversible change in the fluorescence intensity of **CL**–Cu^{2+} at 489 nm can be repeated more than 5 times by the addition of Cu^{2+}/Cys, indicating that **CL** can be developed as a reversible fluorescence ON–OFF–ON chemosensor for Cys detection.

3.3. Fluorescence detection of Cys in live cells

Prior to the confocal microscopy imaging in live cells, the long-term cellular toxicity of **CL** were evaluated by means of a MTT assay (Meng et al., 2015a, 2015b; Li et al., 2011). In this work, the cytotoxicity of **CL** towards two cell lines (human neuronal glioblastoma cell line, U-343 MGa cells and breast carcinoma cell line,

MDA-MB-231) was investigated. Fig. S17 represents the results of both U-343 MGa and MDA-MB-231 cells were incubated with different concentration of **CL** (0, 0.2, 2, 4, 10, and 50 μM) for 24 h. U-343 MGa cell viabilities remained approximately 86%, and MDA-MB-231 cell viabilities remained more than 90% even at the high concentration of 50 μM. The results demonstrated that **CL** is low cytotoxicity both at low and high concentrations and will not kill the U-343 MGa and MDA-MB-231 cells being probed.

Having demonstrated the high biocompatibility of **CL**, we next examined the ability of **CL** to track Cu^{2+} ions, and its **CL**–Cu^{2+} ensemble to detect Cys level in live U-343 MGa and MDA-MB-231 cells. It is well-known that the lipophilicity of the fluorescence imaging probe is critical for their cellular uptake (Komatsu et al., 2013). In the present work, the lipophilicity of **CL** and **CL**–Cu^{2+} ensemble was examined by measuring the partition coefficient ($\log P_{o/w}$) between 1-octanol and water. We confirmed that the $\log P$ values of **CL** and **CL**–Cu^{2+} were 1.06 and 1.38, respectively. Given that the $\log P$ values of fluorescence chemosensors that are known to have good cellular membrane permeability are within the range of 0–5, we reasoned that **CL** and **CL**–Cu^{2+} are all able to penetrate the cells through a passive diffusion pathway (Komatsu et al., 2013).

With this in mind, we then assessed the intracellular uptake mechanism of **CL** into U-343 MGa and MDA-MB-231 cells. The cells were incubated with 1 μM **CL** under reduced temperature condition (4 °C) for 30 min, washed with PBS for three times and then subjected to confocal microscopy imaging analysis. Figs. S18 and S19 represent the results of confocal fluorescence images of U-343 MGa cells stained with 1 μM **CL** at 4 °C, and 37 °C, respectively. As expected, bright green intracellular fluorescence was observed when U-343 MGa cells incubated at both 4 °C, and 37 °C, indicating that **CL** was taken up by the cells through an energy-independent pathway, like passive diffusion. This result was further supported by the fluorescence imaging of cellular uptake into MDA-MB-231 cell line. As shown in Figs. S20 and S21 strong green fluorescence signals were clearly observed even when the cells stained with 1 μM **CL** at 4 °C.

Interestingly, cellular localization of **CL** seemed to be limited to the cytoplasm. As shown in Figs. S19d and S21d, quantitative analysis by line plots of imaging both U-343 MGa and MDA-MB-231 cells revealed that the signal ratio between the cytoplasm and nucleus reached 19.8 and 15.4, respectively, implying weak nuclear uptake of **CL**. These results attracted our attention to investigate the subcellular localization of **CL**. Considering the lipophilicity of **CL** ($\log P_{o/w}$, 1.06), it would be highly likely that **CL** has potential to target cellular membrane structures, like the endoplasmic reticulum (ER) (Gill et al., 2013). To address this hypothesis, intracellular co-localization experiments of **CL** with organelle-specific fluorescent molecule ER-Tracker™ Red, a commercial ER tracker were conducted in U-343 MGa and MDA-MB-231 cells. Cells were incubated with 1 μM **CL** for 30 min, and then stained with 1 μM ER-Tracker™ Red for another 30 min. As shown in Figs. 3, S22, and S23, **CL** displayed bright-green fluorescence in channel 1 (Ch1, $\lambda_{ex} = 473$ nm, $\lambda_{em} = 490–525$ nm), and ER-Tracker™ Red showed red fluorescence in channel 2 (Ch2, $\lambda_{ex} = 559$ nm, $\lambda_{em} = 560–620$ nm). The merged images of Ch1 and Ch2 overlapped perfectly (Figs. 3d, S22d and S23d), suggesting that **CL** can specifically localize in the ER of live U-343 MGa and MDA-MB-231 cells. The intensity profiles of the linear regions of interest across U-343 MGa and MDA-MB-231 cells stained with **CL** and ER-Tracker™ Red also vary in close synchrony (Fig. 3f). The Pearson's correlation coefficients are 0.96 for U-343 MGa cells, 0.98 for MDA-MB-231 cells, and the Mander's overlap coefficients are 0.98 for U-343 MGa cells, 0.93 for MDA-MB-231 cells. All of the coefficients are close to 1 in our experiments, which represent the high-colocalization of **CL** and ER-Tracker™ Red in both U-343 MGa
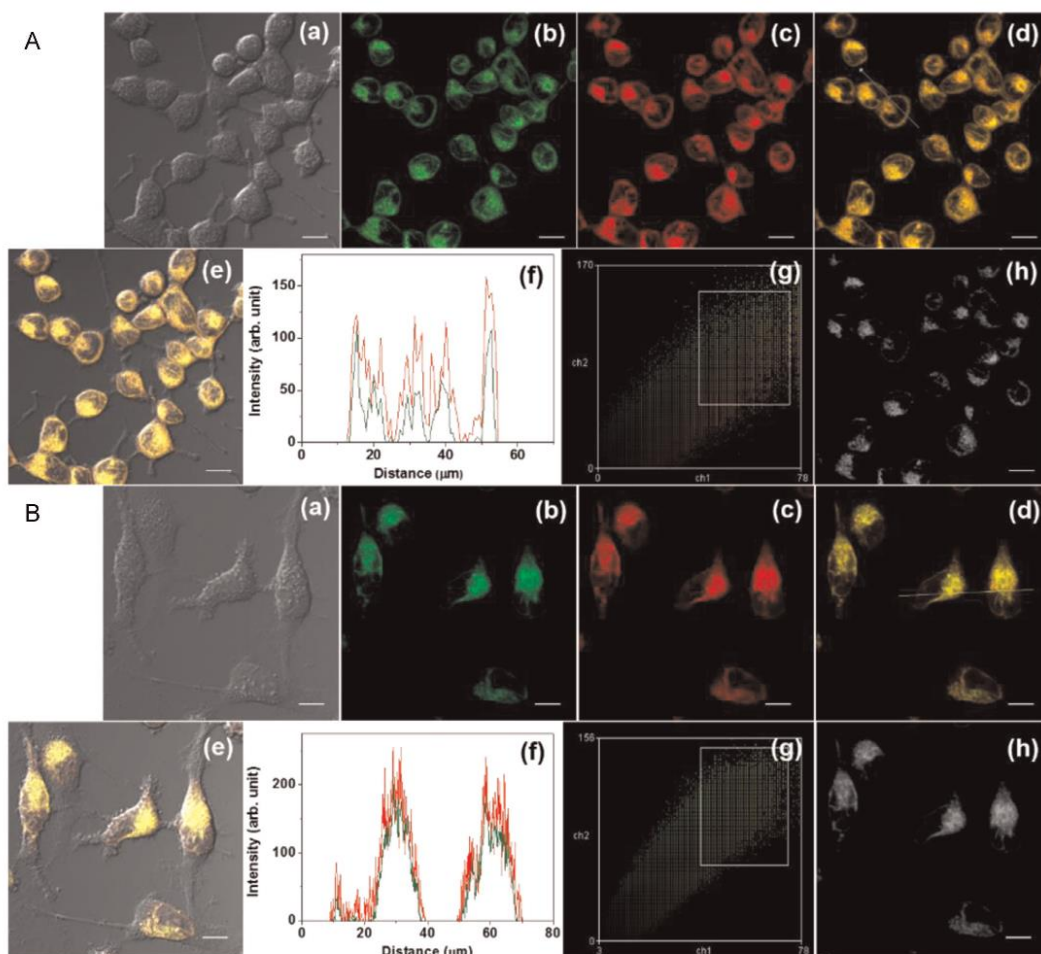
Fig. 3. Co-localization analysis of **CL** and ER-Tracker™ Red in U-343 MGa (A) and MDA-MB-231 (B) cells. DIC (a), and images of cells stained with **CL** (b, channel 1: λ_{ex} =473 nm, λ_{em}=490–525 nm), ER-Tracker™ Red (c, channel 2: λ_{ex}=559 nm, λ_{em}=560–620 nm), and overlay of channel 1 and channel 2 (d). (e) Merged images of (d) and their DIC. (f) Fluorescence intensity profiles of regions of interest (ROI) across U-343 MGa (A) and MDA-MB-231 cells, respectively. (g) Intensity correlation plot of U-343 MGa and MDA-MB-231 cells stained with **CL** and ER-Tracker™ Red. (h) Simulated images (the white pixels represented by the intensity correlation plot (g) was highlighted by selecting the points with a white rectangular selection).

and MDA-MB-231 cells (Li et al., 2004). In addition, a high correlationship was found in the intensity scatter plot of Ch1 and Ch2 (Fig. 3g), indicating **CL** accumulates predominantly in ER. Selecting a rectangular area on the intensity scatter plot, the corresponding white pixels on stimulated images were obtained in accordance with the overlay image, corroborated that **CL** is ER localized.

Further exploration of **CL** for targeting ER was conducted by 3D imaging analysis of live U-343 MGa (Fig. S24) and MDA-MB-231 (Fig. S25) cells. The images were captured by serially scanning at increasing depths along z-axis of both U-343 MGa and MDA-MB-231 cells. Similar with Figs. 3, S22, and S23, as shown in an xy images of the cells obtained at certain depth, the merged yellow color ER were clearly observed as the co-stained with **CL** and ER-Tracker™ Red (Figs. S24a–d and S25a–d). By virtue of 3D reconstruction of serial xy sections, the yellow color ER in U-343

MGa (Fig. S24e) and MDA-MB-231 (Fig. S25e) were perfectly visualized in the xz and yz cross-sectional images. Taken together, these results demonstrated that **CL** is an ER specific molecule, and fluorescence detection of Cys in this organelle is achievable by using **CL** as a chemosensor.

Next, the capability of **CL** to detect Cys in live cells was evaluated by confocal microscopic and flow cytometric analysis. To demonstrate the reversible fluorescence response towards Cys, the U-343 MGa and MDA-MB-231 cells were incubated with **CL** for 30 min at 37 °C in a humidified, 5% CO_2/95% air (v/v) incubator, and were sequentially treated with Cu^{2+} ions and Cys. Fig. 4 represents the results of fluorescence imaging and flow cytometric analysis of Cu^{2+} ions and Cys in live U-343 MGa cells. A strong green fluorescence could be observed when the cells stained with **CL** for 30 min. The **CL**-deposited cells were then incubated with
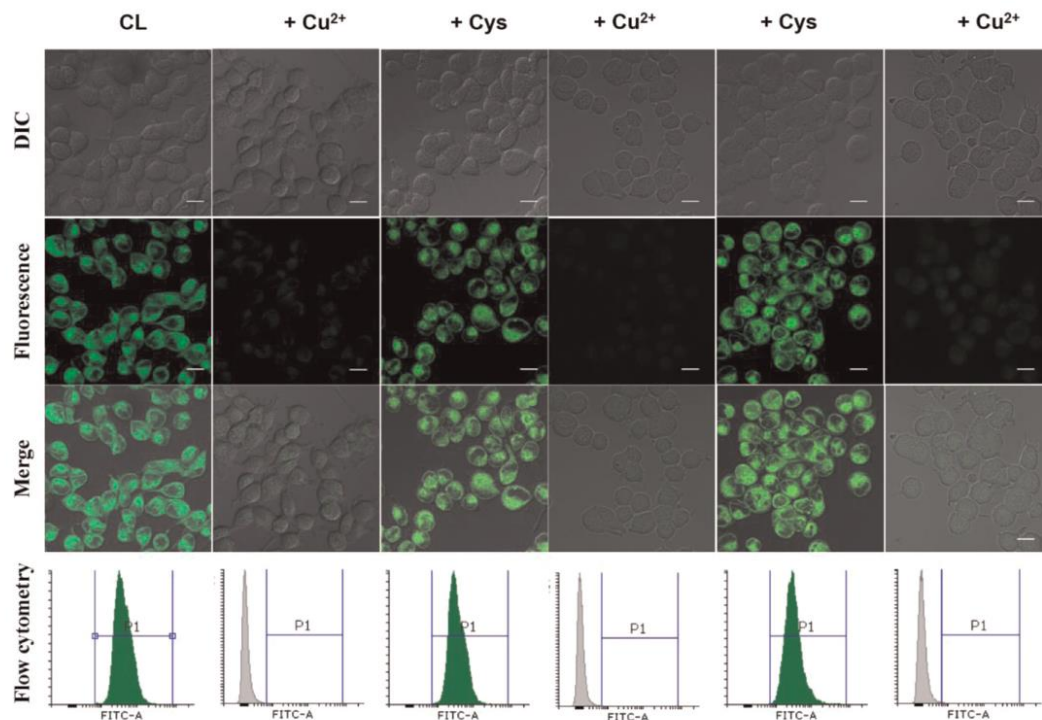
Fig. 4. Confocal microscopy and flow cytometry analysis of reversible cycles in U-343 MGa cells. U-343 MGa cells were stained with **CL** (1 μM) for 30 min at 37 °C, and then sequentially treated with Cu^{2+} ions (10 μM) for 15 min, Cys (10 μM) for 30 min. The intracellular "ON–OFF–ON" cycles were determined by the shifts of histograms of flow cytometric analysis with excitation at 488 nm. Scale bar, 20 μm.

Cu^{2+} ions for 15 min. Their fluorescence images became dim, implying that the nonfluorescent **CL**–Cu^{2+} ensemble was formed in the live cells through complexation of Cu^{2+} ions with **CL**. The intense green fluorescence of cells recovered upon incubation these cells with exogenous Cys for 30 min, suggesting that the dissociation of **CL**–Cu^{2+} ensemble by Cys. To test the reversibility of **CL** in live cells, the cells were subsequently treated with Cu^{2+} ions and Cys. The fluorescence became faint in the presence of Cu^{2+} ions, but was significantly enhanced again when the cells were further incubated with Cys. Similar behaviors were observed under the microscopic imaging analysis of Cys in live MDA-MB-231 cells (Fig. S26). By the intracellular complexation/decomplexation interaction modulated by the incubation of Cu^{2+}–Cys, the "ON–OFF–ON" visualization of Cys levels in live cells was achieved by using **CL** as a chemosensor. More importantly, the analysis of cell viability using the PI exclusion stain showed that almost all cells remain alive after sequential treatment with Cu^{2+}–Cys (Figs. S27 and S28).

The "ON–OFF–ON" fluorescence response in live cells was further quantitatively analyzed by the flow cytometry through measuring the fluorescence of 10,000 cells from each population (Meng et al., 2015a, 2015b). Figs. 4 and S26 represent the shift of fluorescence signal measured of live U-343 MGa and MDA-MB-231 cells stained with **CL**, and then sequentially treated with Cu^{2+} ions and Cys. Based on the measurement of mean fluorescence intensity (MFI) of the cell population, the reversible fluorescence response of intracellular Cys level was investigated in the present work (Fig. S29). As the control groups, U-343 MGa and MDA-MB-

231 cells only showed negligible background fluorescence. Significant fluorescence enhancement was observed when the cells incubated with **CL** for 30 min at 37 °C (MFI: 4948.96 for U-343 MGa cells and 5530.46 for MDA-MB-231 cells). However, when these cells treated with Cu^{2+} ions, fluorescence intensity of 99% cells were switched OFF (70.45-fold, and 65.29-fold fluorescence quenching for U-343 MGa and MDA-MB-231 cells, respectively). In the presence of Cys, the intracellular fluorescence increased significantly again (MFI: 4168.60 for U-343 MGa cells and 4588.21 for MDA-MB-231 cells). Consistent with the results of confocal fluorescence images, reversible fluorescence quantity of Cys in live cells was achieved by the sequential treatment of Cu^{2+} ions and Cys.

Following the results from the above confocal fluorescence imaging, we then examined the detection of endogenous Cys level in live U-343 MGa and MDA-MB-231 cells by using **CL**–Cu^{2+} ensemble as the chemosensor. The cells were pretreated with different concentrations of N-ethylmaleimide (NEM, a thiol blocking reagent) to reduce the concentration of intracellular Cys prior to incubation with **CL**–Cu^{2+}. As shown in Figs. 5 and S27, a bright fluorescence was definitely observed when the cells were incubated with **CL**–Cu^{2+} for 30 min, while the fluorescence decreased for the cells pretreated with NEM (Jung et al., 2011), indicating that intracellular Cys was involved in the fluorescence switch ON imaging. The results of fluorescence images were further supported by the quantitative flow cytometric analysis by the test of the shift of fluorescence signal and MFI (Figs. 5, S30 and S31). As shown in Fig. S30, the MFI of U-343 MGa and MDA-MB-231 cells was decreased with increasing concentrations of NEM.
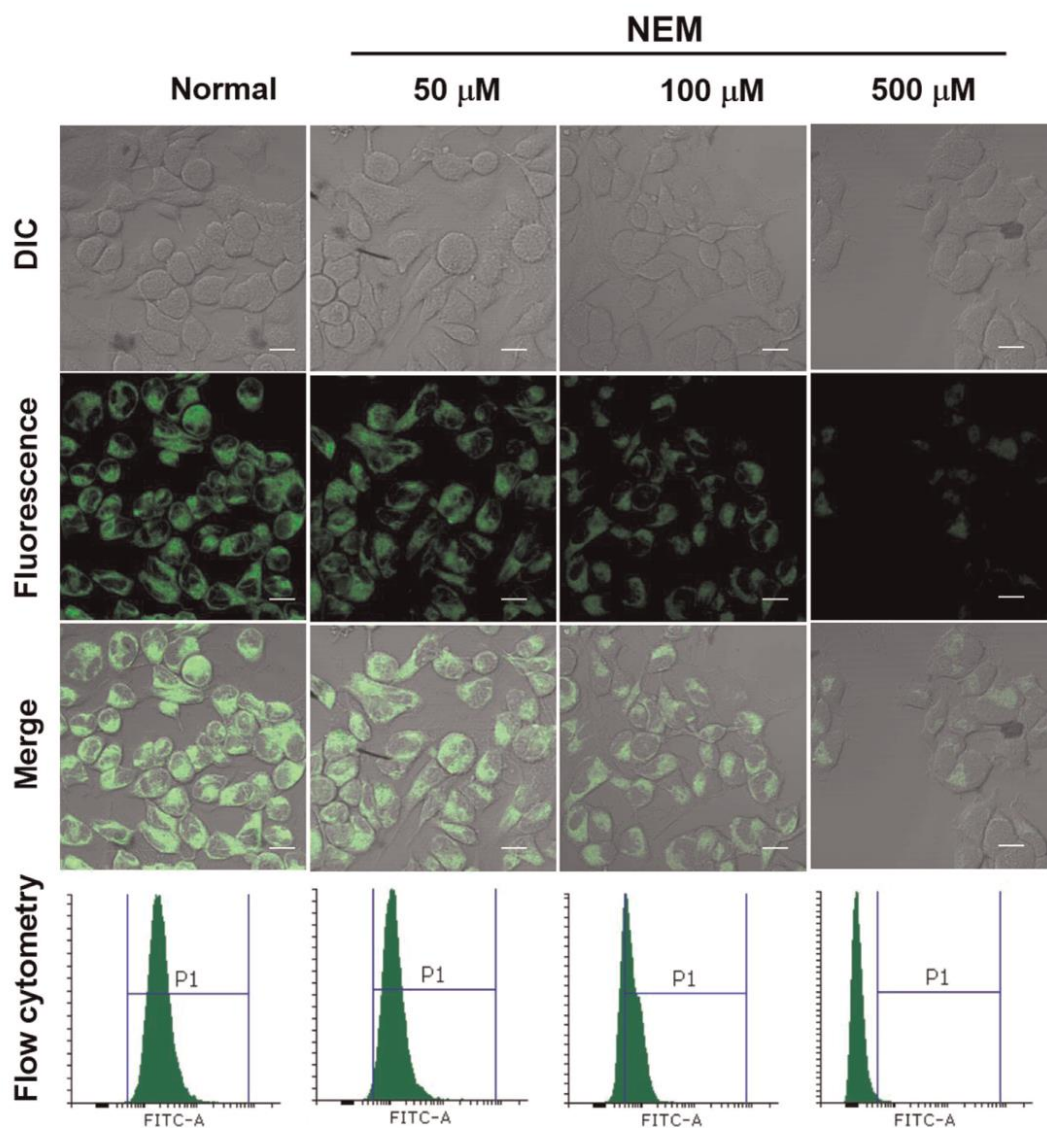
Fig. 5. DIC, fluorescence and merged images of U-343 MGa cells treated with **CL**–Cu^{2+} (1 μM). The cells were treated with NEM at variable concentrations for 0.5 h at 37 °C before incubation with **CL**–Cu^{2+}. Bottom panels represent the shifts of histograms of flow cytometric analysis with excitation at 488 nm. Scale bar, 20 μm.

Comparing with the control group, only approximately 4-fold fluorescent enhanced could be recorded when the cells treated with 500 μM NEM prior incubation with **CL**–Cu^{2+}. The results demonstrate that **CL**–Cu^{2+} could be used as chemosensor for the quantification of endogenous Cys level in live cells.

To evaluate the reliability of the proposed method, the chemosensor, **CL**–Cu^{2+} was further applied to the detection of Cys in three urine samples. As shown in Table S1, The recoveries of spiked Cys are in the range of 95.41–107.40%, suggesting that the present chemosensor has promise for the detection of Cys in real samples.

4. Conclusions

In conclusion, highly selective and sensitive detection of Cys in aqueous solution and even in single cell level was successfully achieved by utilizing the ensemble chemosensor **CL**–Cu^{2+} derived

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from the complex of the coumarin-based fluorescent chemosensor, **CL** and copper ion. The unique ligand, **CL** exhibited strong fluorescence, which was effectively quenched by the complexation with Cu^{2+} ions. In the presence of Cys, fluorescence recovery was observed due to the decomplexation of **CL**–Cu^{2+} ensemble. This displacement approach has been confirmed by the results of MS studies. The results of confocal microscopy imaging studies demonstrated that **CL** could be taken up into live cells through a passive diffusion pathway and then localized in the lipid-dense endoplasmic reticulum. To the best of our knowledge, this is the first chemosensor for visualization of Cys level at ER in live cells. Thus, the experiments on reversible fluorescence imaging of Cys and imaging of endogenous Cys level in this organelle were conducted. In addition, the quantitative fluorescence detection of Cys at subcellular level was also confirmed by the flow cytometry analysis in a rapid, sensitive, and quantitative fashion. We expect that the success of the present findings will not only provide a new approach for the design of fluorescent chemosensors for Cys detection, but also pave the way for the development of new chemosensor for the quantitative detection of other bioactive species at subcellular level.

Acknowledgments

Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.06.077.

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7.2 Appendix of Ethics approvals

10 July 2014

Associate Professor Benjamin Herbert
Department of CBMS
Faculty of Science
Macquarie University NSW 2109

Dear Associate Professor Herbert

RE: *Investigation of osteoarthritis-derived joint material cultured with adipose-derived cells*

Thank you for submitting the above application for ethical and scientific review. Your application was first considered at the HREC (Medical Sciences) meeting held on 28 November 2013 and by the Macquarie University Scientific Sub-Committee (SSC) out of session. At this meeting the Committee requested further information and your responses were reviewed at the HREC (Medical Sciences) meeting held on 27 February 2014 and out of session. Additional information was then reviewed by the HREC (Medical Sciences) Executive and Ethics Secretariat.

The requested information was received with correspondence on 20 January, 18 June and 8 July 2014.

I am pleased to advise that ethical and scientific approval has been granted for this project to be conducted at:

- Macquarie University

This research meets the requirements set out in the *National Statement on Ethical Conduct in Human Research* (2007 – Updated March 2014) (the *National Statement*).

Details of this approval are as follows:

Reference No: 5201300753

Approval Date: 10 July 2014

The following documentation has been reviewed and approved by the HREC (Medical Sciences):

Documents reviewed	Version no.	Date
Macquarie University Ethics Application Form	2.3	July 2013
Correspondence from Dr Herbert responding to the issues raised by the HREC (Medical Sciences)		Received 20/01/2014, 18/06/2014 & 8/07/2014
MQ Participant Information and Consent Form (PICF) entitled *Investigation of osteoarthritis-derived*	4	8/07/2014

1

This letter constitutes ethical and scientific approval only.

Standard Conditions of Approval:

1. Continuing compliance with the requirements of the *National Statement,* which is available at the following website:

http://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research

2. This approval is valid for five (5) years, subject to the submission of annual reports. Please submit your reports on the anniversary of the approval for this protocol.

3. All adverse events, including events which might affect the continued ethical and scientific acceptability of the project, must be reported to the HREC within 72 hours.

4. Proposed changes to the protocol must be submitted to the Committee for approval before implementation.

It is the responsibility of the Chief investigator to retain a copy of all documentation related to this project and to forward a copy of this approval letter to all personnel listed on the project.

Should you have any queries regarding your project, please contact the Ethics Secretariat on 9850 4194 or by email ethics.secretariat@mq.edu.au

The HREC (Medical Sciences) Terms of Reference and Standard Operating Procedures are available from the Research Office website at:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics

The HREC (Medical Sciences) wishes you every success in your research.

Yours sincerely

Professor Tony Eyers
Chair, Macquarie University Human Research Ethics Committee (Medical Sciences)

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research* (2007) and the *CPMP/ICH Note for Guidance on Good Clinical Practice.*

2

MACQUARIE UNIVERSITY

Human Research Ethics Committee

REQUEST FOR AMENDMENT FORM

This form is to be completed and forwarded to the Ethics Secretariat for **ALL** amendments/modifications including extensions to approved ethics protocols.

This form must be signed by the Chief Investigator (if you are an Honours, postgraduate or HDR student, your supervisor must be nominated as the Chief Investigator) and emailed to ethics.secretariat@mq.edu.au or forwarded to the Research Ethics Officer, Research Office, Level 3, Research HUB, Building C5C. Electronic submission of this form is equivalent to the signature of the Chief Investigator/Supervisor.

Handwritten forms will not be accepted.

1. **Chief Investigator/Supervisor:** Benjamin Herbert

   Faculty: Science

   Department: CBMS

   Email: benjamin.herbert@mq.edu.au

(Note: If the project is to be undertaken by an Honours/postgraduate/HDR student, the supervisor will be considered the Chief Investigator. The student may be named as a co-investigator.)

2. **Project Title:** Investigation of the properties and secretions of adipose tissue and cells isolated from adipose tissue

3. **Human Research Ethics Committee Reference No:** 5201100385

4. **Name/s of Student/s (if applicable):**

5. **Names of Co-Investigators/Associate Supervisors/Research Assistants:** Mr Cameron Hill, Mr Michael Medynskyj, Mr Sinead Blaber, Ms Katherine Wongtrakul-kish & Ms Rebecca Webster

6. Reason for amendment/s:
Please provide details of the changes you propose and explain why these are necessary (eg. recruitment, methodology, substantial change in sample size)

Multiple persons are being recruited to work on the project. They represent significant new resources and expertise.

7. Potential inconveniences or risks to participants: ☐ Yes ☒ No
If YES, please outline these and include any changes to confidentiality provisions, psychological or physical risks, increased time commitments, etc,. Please explain how you will reduce inconveniences and/or risks to participants.

8. Adding/removing personnel: ☒ Adding ☐ Removing
If **Adding**, provide the following (If more than one, please copy this page).
If **Removing,** please complete name and title sections only.

Name:	PETER SUCCAR
Title:	MR
Personnel type:	☒ Staff ← OR → ☐ Student
Staff / Student no.:	MQX803448
Qualifications:	MASTER OF PHILOSOPHY
Positions held: (if student, specify Faculty, Department, degree and course in which enrolled)	RESEARCH ASSISTANT
Has the new personnel received a copy of the approved application?	YES
What specific skills will the new personnel bring to the project?	Cell culture, differentiation into neuronal lineage using adipose lipoaspirate
Full mailing address:	60 BALMORAL AVENUE, CROYDON PARK

	peter.succar@gmail.com
Tel No. (W):	
Tel No: (H):	9745 2948
Mobile No:	0412855572
Fax number:	

Working with children and young people (please mark one with an X)	N/A	☒
	Working with children check – details attached	☐
	Prohibited Employment Dec aration Form attached	☐
	Currently employed as a teacher in Australia	☐
	Other evidence attached	☐

Name:	JERRAN NAIDOO
Title:	MR
Personnel type:	☐ Staff ← OR → ☒ Student
Staff / Student no.:	
Qualifications:	student
Positions held: (if student, specify Faculty, Department, degree and course in which enrolled)	STUDENT, FACULTY OF SCIENCE - CBMS
Has the new personnel received a copy of the approved application?	YES
What specific skills will the new personnel bring to the project?	Cell culture, differentiation into neuronal lineage using adipose lipoaspirate
Full mailing address:	95 Stoney Creek Road Beverly Hills 2209 jerranx@gmail.com jerran.naidoo@students.mq.edu.au
Tel No. (W):	0403301353
Tel No: (H):	0403301353
Mobile No:	0403301353
Fax number:	

Working with children and young people (please mark one with an X)	N/A	☒
	Working with children check – details ttached	☐

Full mailing address:	
Tel No. (W):	
Tel No: (H):	
Mobile No:	
Fax number:	

Working with children and young people (please mark one with an X)	N/A	☐
	Working with children check – details attached	☐
	Pro ibited Employment Declaration Form attached	☐
	Currently employed as a teacher in Australia	☐
	Other evidence attached	☐

9. **Expected date of implementation of amendments to research:**

   Date: [March 2012] (dd/mm/yyyy)

10. **Will the amendment/s impact on the research funding arrangements?** ☐ Yes ☒ No ☐ N/A
    If YES, please provide details.

11. **Does the amendment comply with legislative requirements?:** ☐ Yes ☒ No ☐ N/A
    (for example, Privacy Act 1998 (Section 95), and Children and Young Persons Act 1989.) If YES, please attach appropriate Appendix. http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/ human_research_ethics/forms

12. **Attach copies of amended information statement and consent form, advertisement**. ☐ Yes ☐ No ☒ N/A

13. **Attach copies of amended questionnaires,** ☐ Yes ☐ No ☒ N/A

surveys, interview questions.

14. Any other information (permission/approvals) ☐ Yes ☐ No ☒ N/A
If YES, please provide details and attach these.

CHECK LIST

Copies of amended material (refer to 12-15): ☐ Yes ☐ No ☒ N/A

Compliance with legislative requirements: ☐ Yes ☐ No ☒ N/A

IMPORTANT NOTICE: ELECTRONIC SUBMISSION OF THIS FORM IS EQUIVALENT TO THE SIGNATURE OF THE CHIEF INVESTIGATOR.